

Institut für Tierwissenschaften, Abt. Tierzucht und Tierhaltung
der Rheinischen Friedrich – Wilhelms – Universität Bonn

**Porcine Alveolar Macrophage Responses to Gram-positive and Gram-negative Bacterial
Components in Innate Immunity**

I n a u g u r a l – D i s s e r t a t i o n

zur

Erlangung des Grades

Doktor der Agrarwissenschaft

(Dr. agr.)

der

Landwirtschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

Vorgelegt im September 2013

von

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aus

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Diese Dissertation ist auf dem Hochschulschriftenserver der ULB Bonn

http://hss.ulb.uni-bonn.de/diss_online elektronisch publiziert

E-mail: diss-online@ulb.uni-bonn.de

Universitäts- und Landesbibliothek Bonn

Landwirtschaftliche Fakultät – Jahrgang 2013

Zugl.: ITW; Bonn, Univ., Diss., 2013

D98

Referent :	Prof. Dr. Karl Schellander
Koreferent :	Prof. Dr. Dr. Helga Sauerwein
Tag der mündlichen Prüfung :	25. November 2013
Erscheinungsjahr:	2013

Dedicated to

My parents

For everything they have done for me

My wife Shamsun Nahar

For her tremendous support and encouragement

My son Arwarh Sabahat Nibir

For his love and naughtiness that make me smile all the time

Porcine alveolar macrophage responses to Gram-positive and Gram-negative bacterial components in innate immunity

Alveolar macrophages (AMs) are important in the regulation of innate immune responses in the lung through their phagocytosis and the production of a variety of compounds, like cytokines and nitric oxide (NO). Newborn animals are known to manifest increased susceptibility to pulmonary infection, and thus we hypothesize that this may be due in part to a deficiency in the function of AMs. To dissect the age-related immune responses, AMs were isolated from 5 (newborn), 40 (post-weaned) and 120 (young) day old pigs, and stimulated with lipopolysaccharide (LPS). Cytokine mRNA and protein were assayed by quantitative real-time PCR and ELISA, respectively. We report here that AM cells were relatively less abundant in broncho-alveolar lavage fluid in newborn piglets and their phagocytic capacity to *Escherichia coli* was lower than those of young pigs ($P < 0.05$). LPS inducible mRNA for TLR-4, LBP, CD-14 and MyD-88 had higher expression in newborn piglets compared with those of young pigs ($P < 0.05$) in a time-dependent manner. The kinetics results showed that AMs from newborn piglets were significantly less capable of producing IL-1 β , IL-6, IL-12 β , TNF- α , IL-8 and GM-CSF than post-weaned piglets or young pigs. In contrast, IL-10 was significantly higher in newborn piglets. The reasons for differences in cytokine production may reflect maturation of immune cells which indicate functional differences of cell population and may underlay the enhanced susceptibility of early born animals to pulmonary infections. Only very low levels of NO were detected in young pigs. Future investigations in more animals and across a wider age range are recommended.

Furthermore, in another study, AMs were incubated for 24 h with various concentrations of LPS, lipoteichoic acid (LTA), LPS plus LTA or control. Overall, compared to LPS, LTA was a relatively weak inducer in releasing cytokines in a dose-dependent manner. Co-stimulation augmented TLR-2, CD-14 and MyD-88 mRNA, and subsequently produced elevated levels of IL-6, TNF- α and IL-8 when compared to LPS and LTA alone. Additionally, phagocytosis of macrophages was significantly increased following low concentration of LPS treatment. Collectively, our data suggest that during pulmonary infections, an exacerbated inflammatory response may occur inducing the enhanced release of cytokines. The mechanism underlying the synergistic up-regulation of TLRs-associated genes and inflammatory cytokines by combined stimulation is of concern.

Angeborenen Immunität: Reaktion von porcinen Alveolarmakrophagen auf Gram-positive und Gram-negative bakteriellen Komponenten

In der Lunge sind Alveolarmakrophagen (AM) für die Regulation der angeborenen Immunantwort durch Phagozytose und die Herstellung einer Vielzahl von Verbindungen, wie Zytokine und Stickstoffmonoxid (NO) von entscheidender Bedeutung. Da neugeborene Tiere eine erhöhte Anfälligkeit für pulmonale Infektion zeigen, wird vermutet, dass dies zum Teil auf einen Mangel in der Funktion der AMs zurückzuführen ist. Um die altersbedingte Immunantwort zu untersuchen, wurden AMs von 5 (Neugeborenen), 40 (Abgesetzten) und 120 (Jungtieren) Tage alten Schweinen isoliert und mit einem Lipopolysaccharid (LPS) stimuliert. Die Expression von Zytokinen -mRNAs und -Proteinen wurde durch quantitative Echtzeit-PCR und ELISA getestet. Die Ergebnisse zeigten, dass in der broncho-alveolären Lavage-Flüssigkeit von neugeborenen Ferkeln relativ wenige AM Zellen nachzuweisen waren, ihre Phagozytosekapazität von *Escherichia coli* war geringer als bei jungen Schweinen ($P < 0,05$). Die Expression von TLR-4, LBP, CD-14 und MyD-88 der LPS induzierten Zellen war in neugeborenen Ferkeln höher als im Vergleich zu denen von jungen Schweinen ($P < 0,05$). Weiterhin zeigten die kinetischen Ergebnisse, dass AMs von neugeborenen Ferkeln deutlich weniger in der Lage sind IL-1 β , IL-6, IL-12 β , TNF- α , IL-8 und GM-CSF zu produzieren als abgesetzte Ferkel oder junge Schweine. Im Gegensatz dazu war IL-10 signifikant höher bei neugeborenen Ferkeln. Gründe für Unterschiede in der Produktion von Zytokinen könnten auf die Reifung von Immunzellen zurückzuführen sein, welche auf funktionale Unterschiede der Zellpopulation hinweisen und unterlegen damit die verstärkte Anfälligkeit der neu geborenen Tiere auf pulmonale Infektionen. NOs konnten nur in sehr geringe Mengen in jungen Schweinen nachgewiesen werden. Daher sollten in zukünftigen Untersuchungen mehr Tiere und einen größerer Altersbereich berücksichtigt werden.

In einer weiteren Studie wurden AMs 24 h mit unterschiedlichen Konzentrationen von LPS, Lipoteichonsäure (LTA), LPS plus LTA oder Kontrolle inkubiert. Insgesamt gesehen war LPS gegenüber LTA ein relativ schwacher Induktor für die Freisetzung von Zytokinen. Die Co-Stimulation erhöht die Expression von TLR-2, CD-14 und MyD-88 und führt anschließend zu einer erhöhten Produktion von IL-6, TNF- α und IL-8, im Vergleich zu LPS und LTA allein. Weiterhin war die Phagozytose von Makrophagen deutlich bei niedriger LPS Konzentration erhöht. Insgesamt deuten unsere Daten darauf hin, dass bei Infektionen der Lunge, eine verschärfte Entzündungsreaktion eine erhöhte Freisetzung von Zytokinen induziert, die zu septischen Schock und Tod führen können. Dabei scheint der Mechanismus der synergistischen Hochregulation von TLR-assozierten Genen und inflammatorischen Zytokinen durch kombinierte Stimulation von Bedeutung zu sein.

Contents	Page
Abstract	III
Zusammenfassung	IV
List of abbreviations	VII-VIII
List of tables	IX
List of figures	X-XIII
1.1 Introduction	1-7
1.2 Materials and Methods	8-10
1.3 Results and Discussion	11-15
1.4 Summary and conclusion	16-17
1.5 References	18-27

2 Annex	Page
2.1 Chapter 1	
Expression of Toll-like receptors and downstream genes in lipopolysaccharide-induced porcine alveolar macrophages	28-54
Published in: <i>Veterinary Immunology and Immunopathology</i> , 2012. 15; 146: 62-73. (DOI: 10.1016/j.vetimm.2012.02.001)	
2.2 Chapter 2	
Age-related changes in phagocytic activity and production of pro-inflammatory cytokines by lipopolysaccharide stimulated porcine alveolar macrophages	55-86
Published in: <i>Cytokine</i> , 2012. 60: 707-717. (DOI: 10.1016/j.cyto.2012.08.011)	
2.3 Chapter 3	
Age-associated differential production of IFN- γ , IL-10 and GM-CSF by porcine alveolar macrophages in response to lipopolysaccharide	87-110
Published in: <i>The Veterinary Journal</i> , 2013, 198: 245-251. (DOI: 10.1016/j.tvjl.2013.07.026)	
2.4 Chapter 4	
Alveolar macrophage phagocytic activity is enhanced with LPS priming and combined stimulation of LPS and LTA synergistically induce pro-inflammatory cytokines in pigs	111-139
Published in: <i>Innate Immunity</i> , 2013, 19: 631-643. (DOI: 10.1177/1753425913477166)	

List of abbreviations

ANOVA	:	Analysis of variance
AM	:	Alveolar macrophage
BAL	:	Bronchoalveolar lavage
B2M	:	Beta-2-microglobulin
Bp	:	Base pairs
cDNA	:	Complementary DNA
Ct	:	Cycle threshold
CD14	:	Cluster differentiation-14
DNA	:	Deoxynucleic acid
dNTP	:	Deoxyribonucleoside triphosphate
DPBS	:	Dulbecco,s phosphate-buffered saline
<i>E. coli</i>	:	<i>Escherichia coli</i>
ELISA	:	Enzyme-linked immunosorbent assay
FITC	:	Fluorescein isothiocyanate
HKGs	:	Housekeeping genes
IL	:	Interleukin
IRAK-4	:	Interleukin-1 receptor-associated kinase-4
IFN γ	:	Interferon gamma
LPS	:	Lipopolysaccharide
LTA	:	Lipoteichoic acid
LBP	:	Lipopolysaccharide binding protein
MD2	:	Myeloid differentiation-2
MgCl ₂	:	Magnesium chloride
MyD88	:	Myeloid differentiation primary response gene-88
MCP1	:	Monocyte chemotactic protein-1
MIP2	:	Macrophage inflammatory protein-2
min	:	Minute

List of abbreviations (Continued)

miRNA	:	Micro ribonucleic acid
mRNA	:	Messenger RNA
NaCl	:	Sodium chloride
NO	:	Nitric oxide
NTC	:	No template control
NF- κ B	:	Nuclear factor kappa beta
OP	:	Optical density
PA	:	Phagocytic assay
PAMP	:	Pathogen associated molecular patterns
PBMC	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffer saline
PBST	:	Permiabilisation solution-Tritonx-100
PGN	:	Peptidoglycan
pH	:	pH value
PPIA	:	Peptidylprolyl isomerase A (cyclophilin A)
PRR	:	Pattern recognition receptors
PMNs	:	Polymorphonuclear cells
qRT-PCR	:	Quantitative real-time reverse transcriptase polymerase chain reaction
RBC	:	Red blood cell
S.E.	:	Standard error
TRAF6	:	TNF-receptor associated factor-6
TLR	:	Toll-like receptors
TNF α	:	Tumor necrosis factor alpha
UV	:	Ultra-violet light

List of tables	Page
2. Chapter 1	
Table 1: Product sizes and nucleotide sequence details of primers used for real time quantitative PCR analysis of mRNA expression of porcine TLRs, associated molecules and two house keeping genes	48
3. Chapter 2	
Table 1: Oligonucleotide sequences designed for the quantitative real-time PCR analysis of mRNA expression of porcine cytokines and two house keeping genes, with the respective annealing temperature, amplicon products (bp) and GenBank accession number	74
4. Chapter 3	
Table 1: Oligonucleotide sequences designed for the quantitative real-time PCR analysis of mRNA expression of porcine cytokines and two house keeping genes, with the respective annealing temperature, amplicon products (bp), GenBank accession number and references	103
5. Chapter 4	
Table 1: Product sizes and nucleotide sequence details of primers used for real time quantitative PCR analysis of mRNA expression of porcine TLRs, associated molecules and two reference genes	133

List of figures

2. Chapter 1		Page
Figure 1:	A dose-dependant relative expression ratios of TLRs mRNA were quantified by qRT-PCR in LPS-induced alveolar macrophage cells of 5-day-old newborn and 120-day-old young pigs	49
Figure 2:	Age-wise differential expression of TLRs (2, 4, 5 and 9) mRNA. The data shown using the same values presented in Fig. 1	50
Figure 3:	A time-dependant relative expression pattern of TLRs mRNA were quantified by qRT-PCR in LPS-induced alveolar macrophage cells of 5-day-old, newborn and 120-day-old, young pigs	51
Figure 4:	Kinetics relative expression of LPS-signaling TLR4 associated molecules were performed using the same samples presented in Fig. 3	52
Figure 5:	A time-course relative expression analysis of TLR-downstream genes was performed using the same samples presented in Fig. 3	53
Figure 6:	Concentrations level of pro-inflammatory cytokines protein by LPS-stimulated alveolar macrophage cells relative to that of un-treated cells	54
2. Chapter 2		
Figure 1:	Differential cell count percentages from BALF of 5-day-old newborn piglets, 40-day-old post-weaned piglets and 120-day-old young pig lungs	75
Figure 2:	Phagocytic ability of alveolar macrophages from pigs of different ages	76

Figure 3:	Expression levels of proinflammatory cytokines mRNA by LPS-stimulated alveolar macrophages relative to that of untreated cells	77
Figure 4:	Expression levels of IL12 β and TNF α were quantified using the same samples presented in Fig. 3	78
Figure 5:	Expression levels of IL18 and IL8 were quantified using the same samples presented in Fig. 3	79
Figure 6:	Expression levels of MIP2 and MCP1 were quantified using the same samples presented in Fig. 3	80
Figure 4:	Concentration levels of pro-inflammatory cytokines protein by LPS-stimulated alveolar macrophages relative to that of untreated cells	81
Figure 5:	Concentrations of cytokines protein IL12 β and TNF α was assayed using the same samples presented in Fig. 7	82
Figure 6:	Concentration of chemokine IL8 was assayed using the same samples presented in Fig. 7	83
Figure 7:	Kinetics profiles of the curves for IL1 β and IL6 mRNA expression in comparison with secreted IL1 β and IL6 mRNA, respectively in different age groups (Supplementary figure 1)	84
Figure 8:	Kinetics profiles of the curves for IL12 β and TNF α mRNA expression in comparison with secreted IL12 β and TNF α mRNA, respectively in different age groups (Supplementary figure 2)	85
Figure 9:	Kinetics profiles of the curves for chemokine IL8 expression in comparison with secreted IL8 mRNA in different age groups (Supplementary figure 3)	86

2. Chapter 3

Figure 1:	Effects of LPS stimulation on alveolar macrophage cell viability	104
Figure 2:	Dose-dependent cytokine mRNA expression was quantified by qRT-PCR in LPS-induced alveolar macrophage cells of 5-day-old newborn piglets, 40-day-old post-weaned piglets, and 120-day-old young pigs	105
Figure 3:	Age-related production of IFN- γ (mRNA and protein) by LPS-stimulated alveolar macrophages in a time-dependent manner	106
Figure 4:	Production level of IL-10 mRNA and protein was quantified using the same samples presented in Fig. 3	107
Figure 5:	Expression level of GM-CSF mRNA was quantified using the same samples presented in Fig. 3	108
Figure 6:	Kinetics comparison of IFN- γ mRNA and protein release by alveolar macrophages were assessment by qRT-PCR and ELISA, respectively in different age groups (Supplementary figure 1)	109
Figure 7:	Kinetics comparison of IL-10 mRNA and protein production by alveolar macrophages were assessment by qRT-PCR and ELISA, respectively in different age groups (Supplementary figure 2)	110

2. Chapter 4

Figure 1:	Effect of LPS-induced phagocytosis on porcine alveolar macrophages <i>in vitro</i>	134
Figure 2:	Effects of LPS, LTA and their combined stimulation on alveolar macrophage cell viability	135
Figure 3:	Comparative dose-dependent mRNA expression of TLRs and their associated pathway molecules in PAMP-induced porcine alveolar macrophages	136
Figure 4:	Expression levels of TLRs and their associated signalling molecules by PAMP-stimulated porcine alveolar macrophages	137
Figure 5:	Comparative cytokine production by PAMP-induced alveolar macrophages in a dose-dependent manner	138
Figure 6:	Synergistic effect of LTA with LPS on induction of cytokines in alveolar macrophages	139

1.1 Introduction

The immune system is a system of biological structures and is a very complex system working synergistically to protect the host from any infections or insults and to maintain homeostasis (Cooper and Herrin 2010). It comprises two functional types of responses, innate or cellular or non-specific and adaptive or humoral or acquired responses. The innate immune response plays a critical role in the host defense system from any pathogenic infectious agents in a non-specific manner. It does not confer long lasting or life-long protection to the host. This non-specific immune system provides immediate defense against infection, and can be found in all living organisms, animals and humans (Beutler 2004). In the respiratory tract, the lung, such a vulnerable organ is protected from microbial infections by highly efficient innate immune system that acts as the body's first line of defense. As the first line of defense against inhaled substances, alveolar macrophages (AMs) are important in the regulation of immune responses in the lung (Hauschildt and Kleine 1995). As the primary inflammatory cells found within the airways, bronchoalveolar lavage (BAL) fluids containing AMs play a central role in the constant recognition of potentially pathogenic organisms entering the lung (Monick and Hunninghake 2003; Twigg, 2004). These cells are equipped with different evolutionarily conserved receptors, known as the Toll-like receptors (TLRs), which have been shown to play a role in the innate immune response (Droemann et al. 2003; Droemann et al. 2005).

Toll-like receptors (TLRs) are a family of cell-surface molecules involved in the recognition of structurally conserved pathogen-associated molecular patterns (PAMPs) (Kawai and Akira 2005; Takeda and Akira 2005). After recognizing the PAMPs by TLRs, there is induction of opsonization, reactive oxygen and nitrogen mediators, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways and cytokines production and induction of apoptosis (Akira 2009; Medzhitov 2007). Among PAMPs, LPS is the major component of the outer membrane of Gram-negative bacteria, acts as endotoxin and elicits strong immune responses by producing a range of inflammatory mediators by many cell types, but especially in macrophages, monocytes and B cells. Before being recognized by TLR-4, LPS needs to be bound to lipopolysaccharide binding protein (LBP) and then with the help of cluster differentiation factor-14 (CD-14) (membrane bound and

soluble) and myeloid differentiation factor-2 (MD-2), TLR-4 signaling is initiated (Gioannini and Weiss 2007; Miyake 2007). Subsequently, adapter proteins MyD-88, TIR domaincontaining adapter protein (TIRAP) as well as IL-1 receptor-associated kinase (IRAK) are recruited into the signaling complex. Following autophosphorylation, IRAK dissociates itself from MyD-88 to activate TNF-R-associated factor-6 (TRAF-6). Then TRAF-6 detaches I κ B to assist in the nuclear translocation of NF- κ B and subsequent production of inflammatory cytokines and antimicrobial effector molecules (Takeuchi and Akira 2010).

The immune responsiveness to infection varies according to the age of the individual which is thought to be associated with TLR expression (van Duin et al. 2007). Immune cells recognize pathogens via TLRs, the basic signaling receptor of innate immunity. Induction of the expression of TLRs and the associated signalling molecules by bacterial products has been reported in monocytes, PBMC, splenic macrophages, alveolar macrophages and dendritic cells (Juarez et al. 2010; Maris et al. 2006; Uddin et al. 2012). Expression of swine TLRs has been identified in immune cells, gut associated tissue, lymphoid and adipose tissues (Burkey et al. 2007; Gabler and Spurlock, 2008; Tohno et al. 2005) but not in porcine AM cells. Studies in neonates regarding TLR and CD-14 expression on peripheral blood monocytes have generated discrepancy data, compared to adult animals (Forster-Waldl et al. 2005; Henneke et al. 2003; Levy et al. 2004; Yan et al. 2004). In addition, studies in neonatal immune cells showed increased TLR, LBP and CD-14 expression upon LPS stimulation in neonates compared to adults (Lee et al. 2000; Levy et al. 2009; Yerkovich et al. 2007). Despite the growing interest in the investigation without the involvement of TLRs in the host's defense against microbial infection of pig diseases, nothing on age-related TLR expression and effector molecule production in LPS-induced BAL cells has been reported as so far. Therefore, it is very important to evaluate the age-related kinetic changes of TLRs and downstream genes expression and their regulation in the pulmonary immune cells in response to bacterial products.

The innate immune response plays a critical role in the host defense system. There is a great deal of variation in disease severity associated with bacterial infection in early born animals, which is likely a result of differences in regulation of the host response to the pathogens. In general, neonates have an immature immune system compared to that of adults. It is unknown whether neonates are more vulnerable to get pulmonary

infections compared to older animals. Newborn and early postnatal piglets are highly susceptible to a wide range of infectious disease, especially respiratory disease complex (Cutler 2006). In the respiratory tract, AMs have important roles in conducting the first line of defense against invading pathogens by two important and effective means. First, AMs directly bind, phagocytose, and kill pathogens, and whereas some earlier studies have demonstrated deficient phagocytic capacity of AM in the neonates compared with adults (Kurland et al. 1988; Sherman et al. 1977). In contrast, other contradictory reports suggest that neonatal and adult AMs are equivalent in their capacity to phagocytose bacteria (Conly and Speert 1991; Speer et al. 1988). Second, AMs are able to secrete a large range of inflammatory mediators including cytokine, chemokine and NO (Dempsey et al. 2003). Cytokines are acting directly on the pathogens while others such as chemokines exert their effects indirectly by recruiting other components of the immune system. Functional maturity and production of NO by AMs is beneficial in killing microorganisms entering the lung. This response is necessary for mounting an effective immune response, although it must be tightly regulated so as not to obstruct alveolar air exchange (Rubins 2003). The functional deficiency of neonatal AMs includes impaired phagocytosis, chemotaxis, and production of cytokine and chemokine (Cheung et al. 1986; Mills 1983; Sherman et al. 1977). During early postnatal life there are relative deficiencies in the number and differentiation of AMs. Thus, AMs may contribute to diminish the host's defense and increase neonatal susceptibility to respiratory infection (Coonrod et al. 1987). During neonatal infections, an exacerbated inflammatory response often occurs inducing the enhanced expression of TLRs and release of inflammatory mediators, which may lead to septic shock and death of the individual (Tatad et al. 2008).

Alveolar macrophages express various inflammatory cytokine genes in response to respiratory diseases (Murtaugh et al. 1996). Cytokines are important mediators of immune and inflammatory responses in humans and animals (McWilliam et al. 1996). Recently, increasing attention has been paid to the involvement of the cytokine and chemokine response in neonatal immune defense mechanism. The ability of newborn children to produce cytokine and chemokine is different from adults (Marodi 2006). Understanding ontogeny of inflammatory mediator's production in pigs is not detailed as in humans. The altered ability of newborns to produce inflammatory cytokines could be partly responsible for the higher sensitivity to newborns to infectious diseases.

Through *in vitro* studies the expression of interleukin (IL)-1 β , IL-6 and TNF- α mRNA in un-stimulated and LPS-stimulated porcine AM cells was investigated (Lee et al. 2004; Scamurra et al. 1996; Vezina et al. 1995). Age-dependent variations of cytokine expression levels in blood plasma, peripheral blood phagocytes and lymphatic organs were observed in pigs (Mikami et al. 2002; Moya 2007; Zelnickova et al. 2008a) but data on cytokine protein secretion by AMs are not available. Moreover, it has been reported that different cytokines can be expressed by immune cells at different time points after initial stimulation with mitogens, such as LPS and phytohemagglutinin (PHA) (Choi et al. 2002a; Choi et al. 2002b; Reddy and Wilkie 2000; Yoo et al. 1995). Another study using AMs from a rat model reported similar findings, where LPS-stimulated AMs exhibited decreased capacity to produce NO and IL-1 β , as well as impaired phagocytic ability (Bakker et al. 1998). Neonatal AMs isolated from premature infants with chronic lung disease had increased IL-1 β and IL-6 secretion compared to healthy controls, indicating these cells had the capacity to produce these cytokines under the right conditions (Kotecha et al. 1996). In a lamb model, neonatal viral infection was shown to induce a mixed cytokine response in AMs, as evidenced by increased IL-4 and IL-10 gene transcripts in addition to significantly increased IL-1 β and IL-8 gene expression (Fach et al. 2006). The altered ability of newborns to produce inflammatory cytokines and chemokines can be in part responsible for the higher sensitivity of newborns to infectious diseases. However, the specific role of the age-related AM in initiation and modulation of the immune response to LPS in terms of active cytokine secretion remains unknown. Determination of cytokine profiles during the time course stimulation would give important information on immunostimulatory and immunosuppressive mechanisms. To the author's knowledge, understanding the age-associated variability expression and the kinetics pattern of pro-inflammatory cytokine, Th-type cytokines and chemokine production by AMs has not been well reported in pigs.

Taking into account that, on one hand AMs express TLRs and, on the other hand these cells play a crucial role in the inflammatory process in the pulmonary system; it is necessary to know the effects of different bacterial components and whether the co-stimulation has the synergistic action on the expression of TLRs and down-stream signalling genes and cytokine production from porcine AMs. AM cells exhibit a TLR pattern, which serve their need in defense against pathogens. TLRs act as pivotal

signalling proteins of the inflammatory response during sepsis. Gram-negative bacteria release the TLR-4 ligand LPS whereas the cell wall component lipoteichoic acid (LTA from Gram-positive bacteria) is primarily bound by TLR-2. Both LTA and LPS are antigenic and able to activate monocytes/macrophages to secrete various inflammatory mediators, which are highly relevant for the pathogenesis of pulmonary inflammation and injury (Baumgarten et al. 2006; Knuefermann et al. 2007). Hence it may be speculated that the inflammatory responses depend on specific TLR stimulation; and thus, various virulence factors might induce different inflammatory responses. To induce cytokine release by macrophages in response to extracellular LPS via TLR-4, another molecule MD-2 is required (Schroder et al. 2003) which binds with high affinity to the ectodomain of the TLR-4 and affects subcellular distribution of TLR-4 (Nagai et al. 2002). Although LTA is much less reported than LPS, LTA is already regarded as the Gram-positive equivalent to LPS in some aspects (Akira et al., 2006; Beutler et al. 2006). Lipoteichoic acid share pathophysiological properties and recognition mechanisms such as CD-14 and TLRs, with LPS (Ginsburg, 2002). Activated TLRs transmit signals through MyD88-dependent or independent pathways which in turn activate intra-cellular signaling cascades and ultimately result in the induction of innate immune inflammatory mediators (Akira 2009; Krishnan et al. 2007). However, synergistic interactions between bacterial components and their activation of TLR signalling have been suggested to contribute to the pathophysiology of sepsis (Takada and Galanos 1987; Wray et al. 2001).

Sepsis is the systemic inflammatory response to the infection of Gram-negative and Gram-positive bacteria. It is associated with a generalized activation and systemic expression of the host's inflammatory pathways (Matot and Sprung 2001). Infections of the respiratory tract are frequently caused by co-infections of Gram-positive and Gram-negative bacteria. Specially, bacterial pneumonia, often caused by Gram-positive pathogens, is the most frequent cause of sepsis (Laterre et al. 2005). Macrophages are involved in the innate immune response through phagocytosis and the production of a variety of compounds, like nitric oxide (NO) (Dempsey et al. 2003). Nitric oxide is a very important molecule involved in a wide range of physiologic and pathologic processes in mammalian systems and its production by macrophages is fundamental for immune defense (MacMicking et al. 1997). Recent studies have shown that LPS stimulation of murine peritoneal macrophages may be important in mediating functions

that are important for bacterial killing (Victor et al. 1998), whereas other studies have shown a decrease in phagocytic activity when macrophages are treated with LPS (Smith et al. 1998). A more complete understanding of the effects of LPS on phagocytosis and a thorough investigation of the molecular mechanisms that are involved are therefore clearly needed.

PAMP-stimulated immune cells secrete pro-inflammatory cytokines, such as TNF- α , IL1- β and IL-6, initiating both innate and adaptive defense responses, and also release the anti-inflammatory cytokine, IL-10 (O'Garra and Murphy 2009; Pulendran and Ahmed 2006). Over-production of these cytokines and NO might cause inflammatory diseases (Bogdan 2001). In this thesis, we have reported that different cytokines can be produced by porcine AM cells after initial stimulation with LPS (Chapter 2 and 3). In addition, some investigations have been published on the effects of LPS and other PAMPs in porcine immune cells (Ramjeet et al. 2005; Sorensen et al. 2011; Yancy et al. 2001) but the AMs responses to LTA and co-stimulation with LPS has been never studied in pig. It has been reported that combinations of TLR ligands synergistically augment various cytokine production by macrophages (Bagchi et al. 2007) which not only achieve the antibacterial action but also concurrently trigger severe inflammatory response. If this phenomenon is not controlled, the systemic inflammation may progress to septic shock and result in multi-organ failure. We, therefore, hypothesized that LPS and LTA would induce differential inflammatory mediators from porcine AMs and their combination would have a synergistic effect. This notion promoted us to investigate the effects responsible for macrophage cytokine induction by LPS and LTA and their co-stimulation. Therefore, an accurate characterization of TLRs expression and signalling as well as the investigation of consecutively induced inflammatory mediators (Ishii et al. 2006) is essential for understanding a different inflammatory responses to varying stimuli. It might be the fundament for developing efficient strategies for diagnosis and treatment of sepsis.

Objectives

Taking together this background, several experiments were conducted in this thesis to achieve the following aims:

1. The overall first aim described in the following chapter was to address the role of freshly isolated AMs in terms of their innate immune responses during stimulation time with Gram-negative bacterial cell wall component in pigs at different ages. Therefore, to evaluate the age-related porcine AMs innate immune competence, we performed several study with the following objectives:

- To examine the cellular components in BAL fluids.
- To determine NO production and the phagocytic activity of AM cells for the selected fluorescein-labeled Gram-negative bacterium.
- To quantify the kinetics of TLRs and down stream pathway gene expression.
- To investigate the kinetics of inflammatory cytokine and chemokine production.

2. The general second aim defined in the current thesis was to investigate the differential innate immune responses by porcine AMs with the stimulation of Gram-negative and Gram-positive bacterial components. Hence, to analyze the influence of different TLR ligands in AMs immune modulation, we performed this experiment with the following objectives:

- To compare the induction of TLRs, the associated pathway genes and inflammatory mediators production in porcine AMs following stimulation with LPS and LTA in a dose-dependent manner.
- To evaluate whether co-stimulation of LPS and LTA would result in augmented induction of TLRs and down-stream pathway genes and inflammatory molecules by porcine AM cells.

In addition, we first determined the phagocytic ability of AMs with different concentrations of LPS for the selected fluorescein-labeled Gram-negative bacteria.

1.2 Materials and methods

To achieve the objectives of this research several materials and methods were used. The particular materials and methods are described in details in the different chapters in this thesis. The experimental animals and cells, and the value of some methods and their descriptions are briefly described here.

1.2.1 Animals and cells

Clinically healthy German Landrace pigs (male) were used for isolation of AM cells (Chapter 1, 2 and 3). The pigs were conventionally housed and euthanised at the following ages for cell isolation: 5 days (newborn piglets, $n = 3$), 40 days (post-weaned piglets, $n = 3$) and 120 days (young, $n = 3$). In addition, post-weaned Pietrain piglets ($n = 3$) were used for isolation of cells (Chapter 4). All animals were obtained from the teaching and research station of Frankenforst, University of Bonn, Germany. The experiments were done according to the institutional guidelines and animal husbandry regulations of Germany (ZDS 2003).

Alveolar macrophages from lungs were obtained by bronchoalveolar lavage (BAL) using ice-cold sterile calcium-magnesium free Dulbecco's phosphate-buffered saline (D-PBS, pH 7.4; Sigma Aldrich). In all experiments, the collected cells were re-suspended in 2mM L-glutamine-containing complete RPMI-1640 medium (Sigma-Aldrich). For each experiment, AMs were cultured separately at 2×10^6 cells/mL/well into 24-well cell culture plates (Cell-Star). Adherent cells were treated with various concentrations of LPS from *Escherichia coli* 026:B6 (Sigma-Aldrich, Chapter 1, 2 and 3). In addition, various doses of LTA from *Staphylococcus aureus* and LPS from *Escherichia coli* 026:B6 were used (Sigma-Aldrich, Chapter 4). Medium in triplicate wells were added in the same culture plate as control. Cell pellets and supernatants were collected at different time points for mRNA quantification and protein measurement, respectively.

1.2.2 Quantitative real-time polymerase chain reaction (qRT-PCR)

In recent years, qRT-PCR has emerged as a robust and extensively used methodology for biological investigation because it can detect and quantify very small amounts of specific nucleic acid sequences. As a research tool, a major application of this

technology is the rapid and accurate assessment of gene expression as a result of physiology, pathophysiology, or development (Valasek and Repa 2005). In this thesis, mRNA transcription for TLRs, downstream genes and cytokines was quantified by qRT-PCR. For this purpose, total RNA was extracted from harvested cells using PicoPure RNA isolation kit following the manufacturer's manual (Arcturus, Invitrogen). Total RNA was then reversed transcribed into cDNA and purified for qRT-PCR. Briefly, nine fold serial dilution of plasmids DNA were prepared and used as template for the generation of the standard curve. All experiments were performed by the StepOnePlus qRT-PCR System (Applied Biosystems). Primer sequences were designed using FASTA product of the GenBank mRNA sequences for *Sus scrofa* using Primer3 program (Rozen and Skaletsky 2000). Two housekeeping genes (*PPIA* and *B2M*) were selected from previously published data (Cinar et al. 2012). Normalization of data was performed according to the Vandesompele's procedure (Vandesompele et al. 2002)

1.2.3 Enzyme-linked immunosorbent assay (ELISA)

Immune competence of pigs can be monitored by measuring the immune response induced by infection or antigens. The immune responsiveness of animals can also be detected by measuring the serum concentration of immune response components like cytokines and TLRs (Buduneli et al. 2011; Uddin et al. 2012). For measuring the serum cytokines ELISA is the mostly practiced, easy and cost effective immunological assay for a large number of animals (Andreotti et al. 2003). The uses of ELISA has increased dramatically in the immunological as well as in diagnostic research (Lequin 2005). In the present thesis, concentrations of cytokine proteins were measured using commercially available specific porcine enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D System). Results are reported as the mean values of duplicate wells of protein concentrations (pg/ml) in the cell culture supernatant. The concentrations were detected according to the standard using microplate data compliance software SoftMax Pro (Molecular Devices GmbH).

1.2.4 Phagocytosis assay

Phagocytosis is a very important physiological process which is characterized by the ingestion of foreign particles and killing of microorganisms by phagocytic leukocytes (granulocytes, monocytes, and macrophages). It provides a first line of host defense

against potential pathogens. Phagocytosis is used as a model of microbe-innate immune interactions, resulting in increased understanding of the consequences of these interactions. (Choi et al. 2002). In this thesis, *in vitro* phagocytosis assay was performed with Vybrant Phagocytosis Assay Kit (Molecular Probes Inc.) according to manufacturer instructions (Chapter 2 and 4). Using this method, the process of phagocytosis can be quantified by following the internalization of fluorescein-labeled *Escherichia coli* (K-12 strain) bioparticles. The amount of fluorescent bacteria that has been engulfed by cells was measured as mean fluorescence intensity. The number of cells that were significantly phagocytic is expressed as a percentage of the whole population according to the calculation formula of assay kit.

1.2.5 Nitric oxide (NO) production assay

In mammals including humans, NO is an important cellular signaling molecule involved in many physiological and pathological processes (Choi et al. 2002). NO plays an important role in neurotransmission, vascular regulation, immune response and apoptosis. This molecule is rapidly oxidized to nitrite and nitrate which are used to quantitate NO production. In chapter 3 and 4 for this thesis, the Griess Reagent assay system (Promega) was used to measure nitrite formed by the spontaneous oxidation of NO using a standard sodium nitrite curve (Breathnach et al. 2006). Concentrations were calculated from a standard sodium nitrite curve.

1.2.6 Data analysis

In this thesis, the data were analyzed using the SAS software package v.9.2 (SAS Institute). The one-way factorial analysis of variance with Tukey's test was used to detect any possible effect of LPS and or LTA concentration and incubation time on the mRNA expression and production of cytokines within the same age group. Differences between age groups and PAMP treated groups were determined using student's *t* test. All data are reported as the mean of three animals \pm SEM. All results were deemed significant for $P < 0.05$.

1.3 General Results and Discussion

The main focus of our research was to investigate the age-associated innate immune responses of alveolar macrophages following stimulation with Gram-negative bacterial cell wall components. Experiments aimed at dissecting the roles of inflammatory cells such as alveolar macrophages, role of endotoxin such as LPS and TLR-4 signaling, examining the lung responses to *E. coli* LPS stimulation in pigs at different ages. The studies described here explore the role of AMs during LPS stimulation in newborn piglets compared to post-weaned piglets and young pigs in terms of cytokine expression and secretion. However, based on these current experiments, there appear to be functional differences in the ability of the newborn piglets AMs to secrete inflammatory cytokine proteins compared with older pigs.

It is becoming increasingly clear that many factors contribute to the age-related susceptibility of neonates to bacterial infections of the respiratory system. In particular, the innate immune response plays a critical role in the host's defense. In the present thesis, first we describe the age-related changes in BAL fluid cellular content and phagocytic capacity of AMs for selected Gram-negative bacteria (fluorescein-labeled *E. coli*, K-12) in newborn, post-weaned piglets and young pigs. In chapter 2, we have shown that AMs were relatively less abundant in structurally immature lungs in newborn piglets and cells are not completely efficient in function when compared with young animals. As AM cell density in the lung increased with advancement of age, however, phagocytic activity also increased with maturational changes of AMs in young pigs. In general, the phagocytic activity results suggest that AMs from newborn piglets had a significantly lower phagocytic capacity than their post-weaned and young counterparts. Consistent with earlier investigations (Zeidler and Kim 1985; Dickie et al. 2009), we observed that the phagocytosis percentage of opsonised particles was significantly lower in 5-day-old piglets than in older pigs. In the present study, production of NO by AMs was not detected in any age groups, except for very low levels in young pigs. Our results confirmed previous studies, which also failed to induce NO production in porcine AMs, Kupffer cells (KC) and PBMC following stimulation with LPS and IFN- γ (Akunda et al. 2001; Zelnickova et al. 2008b).

In chapter 1, we showed the mRNA level of TLR-4 was the highest expressed gene in a dose-dependant manner of LPS in newborn piglets and young pigs. On AM cells, TLR (2, 4, 5 and 9) mRNA expressions were significantly increased after LPS stimulation in both ages. Our data reveal that LPS-induced AMs resulted in an increase in the levels of mRNA transcript for TLR-2 and TLR-4, and TLR-4 was significantly greater enhanced in neonate AMs than in those of young animals. Higher TLR-4 levels on peripheral blood monocytes were reported after LPS stimulation in neonates, compared with infants and adults (Yerkovich et al. 2007). Presumably, this increases the responsiveness of immune cells to subsequent microbial insults. From the results it may be speculated that BAL containing AM cells of newborn piglets are more sensitive to LPS than those of older animals which may due to less pre-exposure to nosocomial challenges.

Our findings demonstrated that neonatal piglet AMs are markedly more capable of expressing LBP, CD-14 and MyD-88 compared with those of young pigs. Experimental data is not available to explain why AMs produced these molecules in neonates. LBP has been shown to opsonize LPS-bearing particles for recognition and uptake by macrophages (Wright et al. 1989) and enhance the effects of LPS on CD-14 bearing cells by accelerating the transfer of LPS to CD-14 (Hailman et al. 1994). Modulation of immune response by LPS and the role of LBP and CD-14 as bacterial pattern recognition receptors are important in the case of bacterial infections. Hence, it could be speculated that LPS-induced AMs are more host sensitive via the MyD-88 dependent pathway in newborns than in young animals in the respiratory system against microbial infections. Collectively our data suggest that the increased LBP and CD-14 mRNA expression on LPS stimulation in neonatal AM cells may participate in the exacerbated inflammatory response often occurring during neonatal infection. Moreover, the effects of LPS on the up-regulation of the IRAK-4 and TRAF-6 gene expression on AMs may play a role in further augmentation of innate immune responses. Upon LPS stimulation, MyD-88 recruits and activates a death domain-containing kinase, IL-1 receptor-associated kinase-4 (IRAK-4) to TLR-4 and is activated by phosphorylation and then associates with downstream gene TRAF-6. This cascade leads to the activation of signaling transduction pathways of NF- κ B to produce inflammatory cytokines, and in addition to other immune related genes (Takeda and Akira 2005; O'Neill et al. 2009).

Little is known so far about the age-related kinetic production of inflammatory mediators by porcine AMs in response to LPS. AMs production of pro-inflammatory cytokines may contribute to the differences of disease susceptibility in early born animals compared to older animals. Therefore, in order to investigate the ability of AMs in producing pro-inflammatory cytokines, we assessed IL-1 β , IL-12 β , IL-6 and TNF- α production (both mRNA and protein) in newborn piglets, post-weaned and young pigs in a time-dependent manner. Surprisingly, our data reveal that LPS-induced AMs of newborn piglets showed higher expression of mRNA transcripts for TLRs and associated signaling molecules, and on the contrary, lower production of pro-inflammatory cytokine than those of older pigs. In addition, the ability of AMs to produce the chemokine IL-8 increased with advancement of age in a time-course manner. Interestingly following exposure to LPS, the production of IL-8 significantly increased as very early as 1 h in all ages. Based on our observations, we hypothesized that LPS exerts striking time-dependent modulatory effects with age on the cytokine secretion, and it may be plausible that pro-inflammatory cytokines are playing an important part of the host's defense response in the lung and in the pathogenesis of pulmonary infections.

In contrast, AMs from newborn piglets were more capable in producing a critical anti-inflammatory mediator, IL-10 than those of post-weaned piglets and young pigs (Chapter 3). The IL-10 has been shown to inhibit Th-1 cytokine production as well as antigen-presenting cell functions (de Waal Malefyt et al. 1991; Fiorentino et al. 1991). In lung, IL-10 is produced by AMs in response to LPS stimulation (Chanteux et al. 2007). Hence, increases in IL-10 secretion by LPS-treated AMs could suppress Th-1 responses and contribute to the overall Th-2 cytokines bias in the lung. However, IL-10 is a pleiotropic cytokine with a broad spectrum of immunosuppressive and anti-inflammatory effects, which is produced predominantly by macrophages (Moore et al. 2001). In this study, AMs from newborn piglets did produce higher amount of IL-10 than those of post-weaned and young pigs in response to LPS stimulation. Hence, it can be speculated that elevated levels of IL-10 could suppress the production of pro-inflammatory mediators, particularly in early born piglets which may have an impact on the cellular pulmonary immune system and may contribute to the increase susceptibility of early born animals to bacterial infection.

In the present thesis, baseline IFN- γ mRNA levels were very low and the IFN- γ protein was undetected in all age groups (Chapter 3). Following stimulation, IFN- γ mRNA levels were low in newborn piglets and only slightly higher in post-weaned and young pigs. Our results suggest that LPS-stimulated porcine AMs are only producing low levels of IFN- γ . IL-2 and IL-4 mRNA expression was not detected in our cell samples. The absence or low level of IL-4 mRNA expression in these cells may be due to the low stability of mRNA in AMs as demonstrated in Th1 cells compared with Th2 cells (Butler et al. 2002). The kinetics mRNA level of MIP-2, MCP-1 and GM-CSF were significantly higher in older pigs than in newborn piglets (Chapter 2 and 3). In general, chemokines are involved in the onset of inflammation; they mediate mainly the chemotaxis of macrophages and monocytes by controlling leukocyte recruitment and clearance of pathogens (Dessing et al. 2007; Melgarejo et al. 2009). The GM-CSF gene regulates diverse functions of AMs critical to surfactant homeostasis and the innate pulmonary immune response in mice (Chen et al. 1988; Shibata et al. 2001). Differential expression of these pro-inflammatory genes could help to explain the age-related susceptibility of pulmonary infection. Although these *in vitro* data may not fully reflect the *in vivo* situation, they suggest that differences in cytokine production may play a role for the increased susceptibility of neonatal animals. With these data (Chapter 1, 2 and 3), we could not explain in detail how cytokine protein release is regulated by inflammation of AMs with age. The reasons for differences in inflammatory cytokine production in pigs may reflect maturation of immune cells which indicate functional differences of cell population.

Furthermore, we focused on the role of a various concentrations of LPS in regulating phagocytosis of fluorescein-labeled *Escherichia coli* (K-12 strain) bioparticles by porcine AMs (chapter 4). Interestingly we noticed that macrophages exhibited a significant enhancement in their ability to phagocytise bacteria when exposed to as little as 0.1 $\mu\text{g}/\text{mL}$ of LPS and inhibited the phagocytic ability with higher doses of LPS. Based on this finding, we may speculate that the lack of significant phagocytosis enhancement after stimulation with higher LPS concentrations can be attributed to LPS-mediated early induced cytokines, such as TNF- α and IL-8.

In chapter 4, our results demonstrated that LTA was unable to stimulate TLR-4 and MD-2, whereas LPS induced an increased expression of TLR-4, TLR-2 and MD-2

mRNA, regardless of the concentrations used. We also reported that AMs responded to the presence of both LPS and LTA by up-regulating CD-14 and MyD-88 in a dose-dependent manner. In accordance with our results, it has been shown that LTA activates immune cells via TLR-2, whereas TLR-4 and MD-2 are not involved (Schroder et al. 2003). Based on the present study, we could only state that both LPS and LTA represent immuno-stimulatory compounds, however, the LPS receptor is TLR4-MD2, and that of LTA is still unclear. Moreover, the effects of LPS and LTA on the up-regulation of the IRAK4 and TRAF6 gene expression on cells may play a role in further augmentation of innate immune responses. In my experiment, we show differences in cytokine release *in vitro*, in response to LPS compared with LTA, regardless of the concentrations used. Overall, LTA appears to be a less potent cytokine-inducer than LPS and the production of inflammatory cytokines is reported to be affected by the concentration of PAMP in swine (Sorensen et al. 2011). In contrast, the lack of detectable IL-1 β , IL-6 and IFN- γ in response to LTA may be relatively less sensitive to porcine AM cells. This finding may speculate the low sensitivity of lung and pulmonary cells to Gram-positive bacterial stimuli via the MyD88-dependent pathway.

In addition, we examined the potential synergistic production of inflammatory cytokines provoked by combination of LPS and LTA. Our results showed that co-stimulation of LTA with LPS led to the synergistic activation of TLR-2, CD-14 and MyD-88 genes. Additionally, co-stimulation produced elevated levels of IL-6, TNF- α and IL-8 when compared to LPS and LTA alone at 24 h post-stimulation. Although co-stimulation resulted in greater IL-6 production, there was no significant production when stimulated with LTA alone. Simultaneous stimulation with LPS and LTA results in amplified cytokine signaling during polymicrobial infections in humans and leads to poor outcomes and an increased risk of septic shock and mortality (Sato et al. 2000; Lin et al. 2009). Considering our results, we suggest that during pulmonary infections, an exacerbated inflammatory response may occur inducing the enhanced release of cytokines. Still there is need for additional experiments to understand the molecular mechanism underlying the synergistic up-regulation of TLRs-associated genes and inflammatory cytokines by PAMP combinations.

1.4 General Summary and Conclusions

The present study describes the age-related changes in the expression of TLRs and downstream genes, and the production of inflammatory mediators in response to LPS in a dose- and-time dependent manner by porcine AMs. In this thesis, we also study the age-related changes in BAL fluid cellular content and phagocytic capacity of AMs for selected Gram-negative bacteria (fluorescein-labeled *E. coli*, K-12) in newborn, post-weaned piglets and young pigs. We reported that AMs were relatively less abundant in structurally immature lungs in newborn piglets. As AM cell density in the lung increased with advancement of age, however, phagocytic activity also increased with maturational changes of AMs in young pigs in a time-dependent manner. We also demonstrated that AMs of newborn piglets are more sensitive to LPS in regards to the expression of TLR-4 and associated genes, and also produced more anti-inflammatory cytokine IL-10 than those of young pigs. In contrast, the ability of AMs from piglets to produce pro-inflammatory cytokines was reduced compared to the young pigs. The reasons for these age-associated differences in cytokine production could be due to functional differences of AMs. These may reflect the development of the relatively immature immune system and influence the pathogenesis of pulmonary infection in pigs. Taken together, based on our findings we may conclude that differential age-related changes in the expression of TLRs and downstream genes, and pro-inflammatory cytokine could contribute to a different age-related innate immune response during pulmonary infection. Ultimately this study provides insight into the complex immune system changes associated with age in pigs and the findings may explain the increased susceptibility of newborn piglets to respiratory pathogens. The understanding of the response of piglet AMs to LPS will provide important insights into some effect of LPS and Gram-negative bacteria on the lung. This may help to formulate therapy to prevent the over expression of TLRs and associated genes by newborn piglets AMs that can contribute to pathological tissue damage.

In another study for this thesis, we used the porcine AMs model to investigate the *in vitro* immuno-modulating responses of LPS, LTA and their co-stimulation. For characterization of inflammatory cascade we monitored a variety of endpoints like TLRs and downstream pathway molecules expression, production of inflammatory molecules, and phagocytic activity as well as NO production by porcine AMs. After stimulation with LPS we observed a strong inflammatory reaction with up-regulation of TLRs and associated signaling molecules,

enhanced cytokines and chemokine release in a dose-dependent manner. On the other hand, stimulation of cells with an equipoint dose of LTA had a markedly lesser impact on the induction of TLR4-CD14-MyD88 pathways genes, and cytokines and chemokine production. Although co-stimulation augmented TLR-2, CD-14 and MyD-88 mRNA expression, and subsequently produced elevated levels of IL-6, TNF- α and IL-8 when compared to LPS and LTA alone. Additionally, phagocytosis of macrophages was significantly increased following low concentration of LPS treatment used in this study, and we may contemplate that the lack of significant augmentation of phagocytosis after stimulation with higher doses of LPS can be attributed to LPS-induced early induction cytokine. According to our study we could say that LTA is a potent mediator of innate immune response, but compared to LPS, it was a relatively weak inducer in immuno-stimulating response and induces less cytokine secretion. Simultaneous stimulation of LTA and LPS results in amplified inflammatory mediators during co-infections of pulmonary immune cells with Gram-negative bacteria and Gram-positive bacterial components, and that may lead to poor outcomes of pulmonary infections. We hope that our findings could expand the understanding of molecular mechanisms involved in the pathogenesis of bacterial infections and may potentially help in developing the therapeutic possibilities for interference with inflammatory responses of sepsis in a more efficient and specific way.

Recommendations for future works

The findings reported in this thesis only scratch the descriptive age-related and pathogen associated molecular patterns-related innate immune response of AMs in pigs. Further investigations to determine the precise effects of LPS on porcine AMs *in vivo* through functional study across a wider age range are necessary. Another interesting direction to take would be to further explore the mechanisms underlying the enhanced responsiveness of AM cells upon co-stimulation of Gram-negative and Gram-positive bacterial components. The reasons for genetic differences and influences in cytokine and chemokine production in pigs are unknown with respect to the present state of our knowledge. It will be of concern to learn to what extent genetic and age-related factors contribute to the variation in cytokine and chemokine production in pigs. Such knowledge will unwrap an era in obtaining better immunological resistance through breeding programmes.

1.5 References

- Akira S (2009): Pathogen recognition by innate immunity and its signaling: Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 85, 216-216.
- Akira S, Uematsu S, Takeuchi O (2006): Pathogen recognition and innate immunity. Cell 124, 783-801.
- Akunda JK, Ahrens FA, Kramer TT (2001): Evaluation of phagocytosis, bactericidal activity, and production of superoxide anion, nitric oxide, and tumor necrosis factor-alpha in Kupffer cells of neonatal pigs. Am. J. Vet. Res. 62, 1040-1045.
- Andreotti PE, Ludwig GV, Peruski AH, Tuite JJ, Morse SS, Peruski LF (2003): Immunoassay of infections agents. Biotechniques 35, 1212-1212.
- Bagchi A, Herrup EA, Warren HS, Trigilio J, Shin HS, Valentine C, Hellman J (2007): MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. J. Immunol. 178, 1164-1171.
- Bakker JM, Broug-Holub E, Kroes H, van Rees EP, Kraal G, van Iwaarden JF (1998): Functional immaturity of rat alveolar macrophages during postnatal development. Immunol. 94, 304-309.
- Baumgarten G, Knuefermann P, Wrigge H, Putensen C, Stapel H, Fink K, Meyer R, Hoeft A, Grohe C (2006): Role of Toll-like receptor 4 for the pathogenesis of acute lung injury in Gram-negative sepsis. Eur. J. Anaesth. 23, 1041-1048.
- Beutler B (2004): Innate immunity: an overview. Mol Immunol. 40, 845-859.
- Beutler B, Jiang ZF, Georgel P, Crozat K, Croker B, Rutschmann S, Du X, Hoebe K (2006): Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. Annu. Rev. Immunol. 24, 353-389.
- Bogdan C (2001): Nitric oxide and the immune response. Nature Immunol. 2, 907-916.
- Breathnach CC, Sturgill-Wright T, Stiltner JL, Adams AA, Lunn DP, Horohov DW (2006): Foals are interferon gamma-deficient at birth. Vet. Immunol. Immunopathol. 112, 199-209.
- Buduneli N, Ozcaka O, Nalbantsoy A (2011): Salivary and plasma levels of Toll-Like Receptor 2 and Toll-Like Receptor 4 in Chronic Periodontitis. J. Periodontol. 82, 878-884.

- Burkey TE, Skjolaas KA, Dritz SS, Minton JE (2007): Expression of Toll-like receptors, interleukin 8, macrophage migration inhibitory factor, and osteopontin in tissues from pigs challenged with *Salmonella enterica* serovar Typhimurium or serovar Choleraesuis. *Vet. Immunol. Immunopathol.* 115, 309-319.
- Butler NS, Monick MM, Yarovinsky TO, Powers LS, Hunninghake GW (2002): Altered IL-4 mRNA stability correlates with Th1 and Th2 bias and susceptibility to hypersensitivity pneumonitis in two inbred strains of mice. *J. Immunol.* 169, 3700-3709
- Chanteux H, Guisset AC, Pilette C, Sibille Y (2007): LPS induces IL-10 production by human alveolar macrophages via MAPKinases- and Sp1-dependent mechanisms. *Respir. Res.* 8: 71.
- Chen BD, Mueller M, Chou TH (1988): Role of granulocyte/macrophage colony-stimulating factor in the regulation of murine alveolar macrophage proliferation and differentiation. *J. Immunol.* 141, 139-144.
- Cheung AT, Kurland G, Miller ME, Ford EW, Ayin SA, Walsh EM (1986): Host defense deficiency in newborn nonhuman primate lungs. *J. Med. Primatol.* 15, 37-47.
- Choi IS, Collisson EW, Maheswaran SK, Yoo HS, (2002a): Evaluation of cytokine gene expression in porcine spleen cells, peripheral blood mononuclear cells, and alveolar macrophages by competitive RT-PCR. *FEMS Immunol. Med. Microbiol.* 34, 119-126.
- Choi IS, Shin NR, Shin SJ, Lee DY, Cho YW, Yoo HS (2002b): Time course study of cytokine mRNA expression in LPS-stimulated porcine alveolar macrophages. *J. Vet. Sci.* 3, 97-102.
- Cinar MU, Islam MA, Uddin MJ, Tholen E, Tesfaye D, Looft C, Schellander K (2012): Evaluation of suitable reference genes for gene expression studies in porcine alveolar macrophages in response to LPS and LTA. *BMC Res. Notes.* 5, 107.
- Conly ME, Speert DP (1991): Human neonatal monocyte-derived macrophages and neutrophils exhibit normal nonopsonic and opsonic receptor-mediated phagocytosis and superoxide anion production. *Biol. Neonate.* 60, 361-366.
- Coonrod JD, Jarrells MC, Bridges RB (1987): Impaired pulmonary clearance of pneumococci in neonatal rats. *Pediatr. Res.* 22, 736-742.

- Cooper MD, Herrin BR (2010): How did our complex immune system evolve? *Nat. Rev. Immunol.* 10, 2-U11.
- Cutler RS, Fahy VA, Cronin GM, Soicer EM (2006): Prewaning mortality. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.), *Diseases of swine*. Blackwell publishing, IA, USA, pp. 993-1011.
- de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE (1991): Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* 174, 1209-1220.
- Dempsey PW, Vaidya, SA, Cheng G (2003): The art of war: Innate and adaptive immune responses. *Cell Mol. Life Sci.* 60, 2604-2621.
- Dempsey RJ, Sailor KA, Bowen KK, Tureyen K, Vemuganti R (2003): Stroke-induced progenitor cell proliferation in adult spontaneously hypertensive rat brain: effect of exogenous IGF-1 and GDNF. *J. Neurochem.* 87, 586-597.
- Dessing MC, van der Sluijs KF, Florquin S, van der Poll T (2007): Monocyte chemoattractant protein 1 contributes to an adequate immune response in influenza pneumonia. *Clin. Immunol.* 125, 328-336.
- Dickie R, Tasat DR, Alanis EF, Delfosse V, Tsuda A (2009): Age-dependent changes in porcine alveolar macrophage function during the postnatal period of alveolarization. *Dev. Comp Immunol.* 33, 145-151.
- Droemann D, Goldmann T, Branscheid D, Clark, R, Dalhoff K, Zabel P, Vollmer E (2003): Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung. *Histochem. Cell Biol.* 119, 103-108.
- Droemann D, Goldmann T, Tiedje T, Zabel P, Dalhoff K, Schaaf B (2005): Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients. *Respir. Res.* 6: 68.
- Fach SJ, Brockmeier SL, Hobbs LA, Lehmkuhl HD, Sacco RE (2006): Pulmonary dendritic cells isolated from neonatal and adult ovine lung tissue. *Vet. Immunol. Immunopathol.* 112, 171-182.
- Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A (1991): IL-10 inhibits cytokine production by activated macrophages. *J Immunol.* 147, 3815-3822.

- Forster-Waldl E, Sadeghi K, Tamandl D, Gerhold B, Hallwirth U, Rohrmeister K, Hayde M, Prusa AR, Herkner K, Boltz-Nitulescu G, Pollak A, Spittler A (2005): Monocyte toll-like receptor 4 expression and LPS-induced cytokine production increase during gestational aging. *Pediatr. Res.* 58, 121-124.
- Gabler NK, Spurlock ME (2008): Integrating the immune system with the regulation of growth and efficiency. *J. Anim. Sci.* 86, E64-74.
- Ginsburg I (2002): Role of lipoteichoic acid in infection and inflammation. *Lancet Infect. Dis.* 2, 171-179.
- Gioannini TL and Weiss JP (2007): Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells. *Immunol. Res.* 39, 249-260.
- Hailman E, Lichenstein HS, Wurfel MM, Miller DS, Johnson DA, Kelley M, Busse LA, Zukowski MM, Wright SD (1994): Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J. Exp. Med.* 179, 269-277.
- Hauschildt S, Kleine B (1995): Bacterial stimulators of macrophages. *Int. Rev. Cytol.* 161, 263-331.
- Henneke P, Osmers I, Bauer K, Lamping N, Versmold HT, Schumann RR (2003): Impaired CD14-dependent and independent response of polymorphonuclear leukocytes in preterm infants. *J. Perinat. Med.* 31, 176-183.
- Ishii KJ, Uematsu S, Akira S (2006). 'Toll' gates for future immunotherapy. *Curr. Pharm. Design.* 12, 4135-4142.
- Juarez E, Nunez C, Sada E, Ellner JJ, Schwander SK, Torres M (2010): Differential expression of Toll-like receptors on human alveolar macrophages and autologous peripheral monocytes. *Respir. Res.* 11: 2.
- Kawai T, Akira S (2005): Pathogen recognition with toll-like receptors. *Curr. Opin. Immunol.* 17, 338-344.
- Knuefermann P, Baumgarten G, Koch A, Schwederski M, Velten M, Ehrentraut H, Mersmann J, Meyer R, Hoefl A, Zacharowski K, Grohe C (2007): CpG oligonucleotide activates toll-like receptor 9 and causes lung inflammation in vivo. *Respir Res.* 8: 72.
- Kotecha S, Wilson L, Wangoo A, Silverman M, Shaw RJ (1996): Increase in interleukin (IL)-1 beta and IL-6 in bronchoalveolar lavage fluid obtained from infants with chronic lung disease of prematurity. *Pediatr. Res.* 40, 250-256.

- Krishnan J, Selvarajoo K, Tsuchiya M, Lee G, Choi S (2007): Toll-like receptor signal transduction. *Exp. Mol. Med.* 39, 421-438.
- Kurland G, Cheung AT, Miller ME, Ayin SA, Cho MM, Ford EW (1988): The ontogeny of pulmonary defenses: alveolar macrophage function in neonatal and juvenile rhesus monkeys. *Pediatr. Res.* 23, 293-297.
- Laterre PF, Garber G, Levy H, Wunderink R, Kinasewitz GT, Sollet JP, Maki DG, Bates B, Yan SCB, Dhainaut JF, Comm PCE (2005): Severe community-acquired pneumonia as a cause of severe sepsis: Data from the PROWESS study. *Crit. Care Med.* 33, 952-961.
- Lee DY, Cho YW, Kang SG, Shin NR, Choi IS, Shin SJ, Yoo HS (2004): Quantitative analysis of interleukin-6 expression in porcine spleen cells and alveolar macrophages using real-time PCR. *Vet. Res. Commun.* 28, 503-513.
- Lee PT, Holt PG, McWilliam AS (2000): Role of alveolar macrophages in innate immunity in neonates: evidence for selective lipopolysaccharide binding protein production by rat neonatal alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.* 23, 652-661.
- Lequin RM (2005): Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA). *Clin. Chem.* 51, 2415-2418.
- Levy E, Xanthou G, Petrakou E, Zacharioudaki V, Tsatsanis C, Fotopoulos S, Xanthou M (2009): Distinct roles of TLR4 and CD14 in LPS-induced inflammatory responses of neonates. *Pediatr. Res.* 66, 179-184.
- Levy O, Zarembek KA, Roy RM, Cywes C, Godowski PJ, Wessels MR (2004): Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. *J. Immunol.* 173, 4627-4634.
- Lin JN, Tsai YS, Lai CH, Chen YH, Tsai SS, Lin HL, Huang CK, Lin HH (2009): Risk factors for mortality of bacteremic patients in the emergency department. *Acad. Emerg. Med.* 16, 749-755.
- MacMicking J, Xie QW, Nathan C (1997): Nitric oxide and macrophage function. *Annu. Rev. Immunol.* 15, 323-350.

- Maris NA, Dessing MC, de Vos AF, Bresser P, van der Zee JS, Jansen HM, Spek CA, van der Poll T (2006): Toll-like receptor mRNA levels in alveolar macrophages after inhalation of endotoxin. *Eur. Respir. J.* 28, 622-626.
- Marodi L (2006): Neonatal innate immunity to infectious agents. *Infect Immun.* 74, 1999-2006.
- Matot I, Sprung CL (2001): Definition of sepsis. *Intens. Care Med.* 27, S3-S9.
- McWilliam AS, Napoli S, Marsh AM, Pemper FL, Nelson DJ, Pimm CL, Stumbles PA, Wells TN, Holt PG (1996): Dendritic cells are recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli. *J. Exp. Med.* 184, 2429-2432.
- Medzhitov R (2007): Recognition of microorganisms and activation of the immune response. *Nature* 449, 819-826.
- Melgarejo E, Medina MA, Sanchez-Jimenez F, Urdiales JL (2009): Monocyte chemoattractant protein-1: a key mediator in inflammatory processes. *Int. J. Biochem. Cell Biol.* 41, 998-1001.
- Mikami O, Muneta Y, Mori Y, Yokomizo Y, Nakajima Y (2002): Expression of proinflammatory cytokine mRNA in the lymphatic organs of adult and neonatal pigs. *Vet. Immunol. Immunopathol.* 90, 203-207.
- Mills EL (1983) Mononuclear phagocytes in the newborn: their relation to the state of relative immunodeficiency. *Am. J. Pediatr. Hematol. Oncol.* 5, 189-198.
- Miyake K (2007): Innate immune sensing of pathogens and danger signals by cell surface toll-like receptors. *Semin. Immunol.* 19, 3-10.
- Monick MM and Hunninghake GW (2003): Second messenger pathways in pulmonary host defense. *Annu. Rev. Physiol.* 65, 643-667.
- Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A (2001): Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 19: 683-765.
- Moya SL, Boyle LA, Lynch PB, Arkins S (2007): Age-related changes in proinflammatory cytokines, acute phase proteins and cortisol concentrations in neonatal piglets. *Biol. Neonate. Neonatol.* 91: 44-48.
- Murtaugh MP, Baarsch MJ, Zhou Y, Scamurra RW, Lin G (1996): Inflammatory cytokines in animal health and disease. *Vet. Immunol. Immunopathol.* 54, 45-55.

- Nagai Y, Akashi S, Nagafuku M, Ogata M, Iwakura Y, Akira S, Kitamura T, Kosugi A, Kimoto M, Miyake K (2002): Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat. Immunol.* 3, 667-672.
- O'Garra A and K M Murphy (2009): From IL-10 to IL-12: how pathogens and their products stimulate APCs to induce T(H)1 development. *Nat Immunol.* 10, 929-932.
- O'Neill LA, Bryant CE, Doyle SL (2009): Therapeutic targeting of toll-like receptors for infectious and inflammatory diseases and cancer. *Pharmacol. Rev.* 61, 177-197.
- Pulendran B and Ahmed R (2006): Translating innate immunity into immunological memory: Implications for vaccine development. *Cell.* 124, 849-863.
- Ramjeet M, Deslandes V, St Michael F, Cox AD, Kobisch MN, Gottschalk M, Jacques M (2005): Truncation of the lipopolysaccharide outer core affects susceptibility to antimicrobial peptides and virulence of *Actinobacillus pleuropneumoniae* serotype 1. *J. Biol. Chem.* 280, 39104-39114.
- Reddy NR and Wilkie BN (2000): Quantitation of porcine cytokine and beta 2-microglobulin mRNA expression by reverse transcription polymerase chain reaction. *J. Immunol. Methods.* 233, 83-93.
- Rozen S and Skaletsky H (2000): Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* 132, 365-386.
- Rubins JB (2003): Alveolar macrophages: wielding the double-edged sword of inflammation. *Am. J. Respir. Crit. Care Med.* 167, 103-104.
- Sato S, Nomura F, Kawai T, Takeuchi O, Muhlradt PF, Takeda K, Akira S (2000): Synergy and cross-tolerance between toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways. *J. Immunol.* 165, 7096-7101.
- Scamurra R, Arriaga C, Sprunger L, Baarsch MJ, Murtaugh MP (1996): Regulation of interleukin-6 expression in porcine immune cells. *J. Interferon Cytokine Res.* 16, 289-296.
- Schroder NWJ, Morath S, Alexander C, Hamann L, Hartung T, Zahringer U, Gobel UB, Weber JR, Schumann RR (2003): Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J. Biol. Chem.* 278, 15587-15594.

- Sherman M, Goldstein E, Lippert W, Wennberg R (1977): Neonatal lung defense mechanisms: a study of the alveolar macrophage system in neonatal rabbits. *Am. Rev. Respir. Dis.* 116, 433-440.
- Shibata Y, Berclaz PY, Chronos ZC, Yoshida M, Whitsett JA, Trapnell BC (2001): GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1. *Immunity.* 15, 557-567.
- Smith ME, van der Maesen K, Somera FP (1998): Macrophage and microglial responses to cytokines in vitro: Phagocytic activity, proteolytic enzyme release, and free radical production. *J. Neurosci. Res.* 54, 68-78.
- Sorensen NS, Skovgaard K, Heegaard PM (2011): Porcine blood mononuclear cell cytokine responses to PAMP molecules: comparison of mRNA and protein production. *Vet. Immunol. Immunopathol.* 139, 296-302.
- Speer CP, Gahr M, Wieland M, Eber S (1988): Phagocytosis-associated functions in neonatal monocyte-derived macrophages. *Pediatr. Res.* 24, 213-216.
- Takada H and Galanos C (1987): Enhancement of endotoxin lethality and generation of anaphylactoid reactions by lipopolysaccharides in muramyl-dipeptide-treated mice. *Infect. Immun.* 55, 409-413.
- Takeda K and Akira S (2005): Toll-like receptors in innate immunity. *Int Immunol.* 17, 1-14.
- Takeuchi O and Akira S (2010): Pattern recognition receptors and inflammation. *Cell* 140, 805-820.
- Tatad AM, Nesin M, Peoples J, Cheung S, Lin H, Sison C, Perlman J, Cunningham-Rundles S (2008): Cytokine expression in response to bacterial antigens in preterm and term infant cord blood monocytes. *Neonatology* 94, 8-15.
- Tohno M, Shimosato T, Kitazawa H, Katoh S, Iliev ID, Kimura T, Kawai Y, Watanabe K, Aso H, Yamaguchi T, Saito T (2005): Toll-like receptor 2 is expressed on the intestinal M cells in swine. *Biochem. Biophys. Res. Commun.* 330, 547-554.
- Twigg H L 3rd (2004): Macrophages in innate and acquired immunity. *Semin. Respir. Crit. Care Med.* 25, 21-31.
- Uddin MJ, Nuro-Gyina PK, Islam MA, Tesfaye D, Tholen E, Looft C, Schellander K, Cinar MU (2012): Expression dynamics of toll-like receptors mRNA and cytokines in porcine peripheral blood mononuclear cells stimulated by bacterial lipopolysaccharide. *Vet. Immunol. Immunopathol.* 147, 211-222.

- Valasek MA, Repa JJ (2005): The power of real-time PCR. *Adv Physiol Ed.* 29, 151-159.
- van Duin D, Mohanty S, Thomas V, Ginter S, Montgomery RR, Fikrig E, Allore HG, Medzhitov R, Shaw AC (2007): Age-associated defect in human TLR-1/2 function. *J. Immunol.* 178, 970-975.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002): Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3, RESEARCH0034.
- Vezina SA, Roberge D, Fournier M, Dea S, Oth D, Archambault D (1995): Cloning of porcine cytokine-specific cDNAs and detection of porcine tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-1 beta gene expression by reverse transcription PCR and chemiluminescence hybridization. *Clin. Diagn. Lab. Immunol.* 2, 665-671.
- Victor VM, Minano M, Guayerbas N, Del Rio M, Medina S, De la Fuente M (1998): Effects of endotoxic shock in several functions of murine peritoneal macrophages. *Mol. Cell Biochem.* 189, 25-31.
- Wray GM, Foster SJ, Hinds CJ, Thiemermann C (2001): A cell wall component from pathogenic and non-pathogenic gram-positive bacteria (peptidoglycan) synergises with endotoxin to cause the release of tumour necrosis factor-alpha, nitric oxide production, shock, and multiple organ injury/dysfunction in the rat. *Shock* 15, 135-142.
- Wright SD, Tobias PS, Ulevitch RJ, Ramos RA (1989): Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages. *J. Exp. Med.* 170, 1231-1241.
- Yan SR, Qing G, Byers DM, Stadnyk AW, Al-Hertani W, Bortolussi R (2004): Role of MyD88 in diminished tumor necrosis factor alpha production by newborn mononuclear cells in response to lipopolysaccharide. *Infect. Immun.* 72, 1223-1229.
- Yancy H, Ayers SL, Farrell DE, Day A, Myers M (2001): Differential cytokine mRNA expression in swine whole blood and peripheral blood mononuclear cell cultures. *Vet. Immunol. Immunopathol.* 79, 41-52.

- Yerkovich ST, Wikstrom ME, Suriyaarachchi D, Prescott SL, Upham JW, Holt PG (2007): Postnatal development of monocyte cytokine responses to bacterial lipopolysaccharide. *Pediatr. Res.* 62, 547-552.
- Yoo HS, Maheswaran SK, Lin G, Townsend EL, Ames TR (1995): Induction of inflammatory cytokines in bovine alveolar macrophages following stimulation with *Pasteurella haemolytica* lipopolysaccharide. *Infect. Immun.* 63, 381-388.
- ZDS (2003): Richtlinie für die Stationsprüfung auf Mastleistung, Schlachtkörperwert und Fleischbeschaffenheit beim Schwein, 10.12.2003.
- Zeidler RB and Kim HD (1985): Phagocytosis, chemiluminescence, and cell volume of alveolar macrophages from neonatal and adult pigs. *J. Leukoc. Biol.* 37, 29-43.
- Zelnickova P, Leva L, Stepanova H, Kovaru F, Faldyna M (2008a): Age-dependent changes of proinflammatory cytokine production by porcine peripheral blood phagocytes. *Vet. Immunol. Immunopathol.* 124, 367-378.
- Zelnickova P, Matiasovic J, Pavlova B, Kudlackova H, Kovaru F, Faldyna M (2008b): Quantitative nitric oxide production by rat, bovine and porcine macrophages. *Nitric Oxide.* 19, 36-41.

2. Chapter 1 (Published: *Veterinary Immunology and Immunopathology*, 2012, 146: 62-73)

Expression of Toll-like receptors and downstream genes in lipopolysaccharide-induced porcine alveolar macrophages

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Article type and status: research and new

Short title: Expression of TLRs and cytokine production in swine alveolar macrophages

Key words: Toll-like receptors; lipopolysaccharide; mRNA; alveolar macrophages; cytokine protein; newborn piglets; young pigs

Abstract

The aim of the present study was to determine the age-related kinetic changes of Toll-like receptors (TLRs) and downstream genes expression, and secretion of cytokine in lipopolysaccharide (LPS) stimulated porcine alveolar macrophages (AM). For this purpose, AMs were isolated from 5-day-old newborn piglets and 120-day-old young pigs. mRNA expression and cytokine measurement was determined by quantitative real-time PCR and ELISA, respectively. First, AMs were incubated for 24 h in the absence or presence of increasing concentrations of LPS. Results showed the up-regulation of TLRs 2, 4, 5 and 9 mRNA from all concentrations of LPS used, as compared to non-stimulated cells, and TLR-4 was the highest expression in both ages ($P<0.05$). Furthermore, quantitative analysis demonstrated increased expression of mRNAs encoding TLRs 2, 4, 5 and 9, LBP, CD-14, MD-2, MyD-88, IRAK-4 and TRAF-6 in both ages in a time-dependant manner ($P<0.05$). Overall, LPS inducible mRNA for TLR-4, LBP, CD-14 and MyD-88 had higher expression in newborn piglets compared with those of young pigs ($P<0.05$). The level of cytokine protein IL-6 and TNF- α in supernatant fluid significantly varied with time of incubation and age of animals. Their concentration increased immediately at 1 h after LPS stimulation and remained significantly higher up to 48 h in both ages. Production of pro-inflammatory cytokine protein IL-6 and TNF α in supernatant was significantly higher in young pigs than those of piglets. This study suggests that differential age-related changes in the expression of TLRs and downstream genes, and pro-inflammatory cytokine could contribute to a different age-related innate immune response during pulmonary infection. Further investigation is warranted to determine the precise effects of LPS on porcine AMs by means of a functional study across a wider age range.

Introduction

Toll-like receptors (TLRs) are a family of cell-surface molecules involved in the recognition of structurally conserved pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) (Kawai and Akira 2005; Takeda and Akira 2005). As the primary inflammatory cells found within the airways, bronchoalveolar lavage (BAL) fluids containing alveolar macrophages (AMs) play a central role in the constant recognition of potentially pathogenic organisms entering the lung (Pabst 1996; Twigg 2004). Alveolar macrophages are equipped with different TLRs, which have been shown to play a role in the innate immune

response (Droemann et al. 2003; Droemann et al. 2005). However, it has been well accepted that recognition of LPS by TLR-4 is mainly through a series of interactions with several proteins including the LPS binding protein (LBP), cluster differentiation factor-14 (CD-14) and myeloid differentiation factor-2 (MD-2) (Gioannini and Weiss 2007; Miyake 2007). Upon LPS recognition, TLR-4 undergoes oligomerization and recruits its downstream adaptors through interactions with the toll-interleukin-1 receptor (TIR); activating the IL-1 receptor associated kinase (IRAK) through the myeloid differentiation factor-88 (MyD-88) adaptor proteins and mediating signal transduction through TNF-receptor associated factor-6 (TRAF-6) protein. Thereafter, this signaling cascade leads to the activation of the transcription nuclear factor (NF)- κ B, which subsequently results in production of inflammatory cytokines and antimicrobial effector molecules (Takeuchi and Akira 2010).

The immune responsiveness to infection varies according to the age of the individual which is thought to be associated with TLR expression (van Duin et al. 2007). Immune cells recognize pathogens via TLRs, the basic signaling receptor of innate immunity. Expression of swine TLRs has been identified in immune cells, gut associated tissue, lymphoid and adipose tissues (Burkey et al. 2007; Gabler et al. 2008; Tohno et al. 2006). Studies in neonates regarding TLR and CD-14 expression on peripheral blood monocytes have generated discrepancy data, compared to adult animals (Forster-Waldl et al. 2005; Henneke et al. 2003; Levy et al. 2004; Yan et al. 2004). In addition, studies in neonatal immune cells showed increased TLR, LBP and CD-14 expression upon LPS stimulation in neonates (Lee et al. 2000; Levy et al. 2009; Yerkovich et al. 2007). But, the differential age-associated expression of TLRs and downstream genes in LPS-induced inflammatory responses by porcine BAL cells remain unclearly defined.

During neonatal infections, an exacerbated inflammatory response often occurs inducing the enhanced expression of TLRs and release of inflammatory mediators, which may lead to septic shock and death of the individual (Tatad et al. 2008). Inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) are quickly produced after induced inflammation and play an important role in pathogenesis of bacterial infectious diseases and are directly responsible for driving the development of the inflammation (Choi et al. 2006). The altered ability of newborns to produce inflammatory cytokines could be partly responsible for the higher sensitivity to newborns to infectious diseases. Through *in vitro* studies the expression of interleukin-1 beta (IL-1 β), IL-6 and TNF- α mRNA in un-stimulated and LPS-stimulated porcine AM cells was studied (Lee et al. 2004; Scamurra et al. 1996; Vezina et al. 1995). The age-related changes in cytokine mRNA expression were observed in

plasma, lymphatic organs and peripheral blood phagocytes of pigs (Feng et al. 2003; Mikami et al. 2002; Moya 2007), but data on cytokine protein secretion by AMs is not available. Despite the growing interest in the investigation into the involvement of TLRs in the host's defense against microbial infection of pig diseases, nothing on age-related TLR expression and effector molecule production in LPS-induced BAL cells has been reported as of yet. Therefore, the objective of the present study was to investigate the age-related kinetic effects of the expression patterns of TLRs, associated downstream genes, and secretion of effector molecules of LPS stimulated cultured porcine AMs. In addition, the response patterns of TLRs (2, 4, 5 and 9) mRNA in AMs, following exposure to different concentration of LPS were determined.

Materials and methods

Animals

Groups of three German Landrace, 5-day-old newborn piglets and 120-day-old young pigs were euthanized for sampling. All animals were clinically healthy and housed under conventional environmental conditions at the teaching and research station of Frankenforst, University of Bonn, Germany with institutional guidelines (ZDS 2003). Animals were handled according to the animal protection laws of Germany.

Preparation of alveolar macrophage cells

Alveolar macrophage cells were obtained from BAL fluid of animals. In newborn piglets, lungs were lavaged with 100 ml ice-cold sterile calcium-magnesium free Dulbecco's phosphate-buffered saline (D-PBS, pH 7.4; Sigma Aldrich) that was instilled gently in 25 ml aliquots into each of the two adjacent lung sub-segments and withdrawn immediately. In young animals, 200 ml of PBS was instilled in the lungs in a similar fashion. BAL fluid from three animals of the two age groups were collected in separate tubes and filtered through sterile gauze. BAL fluids were mixed with RBC lysis buffer to lyse any residual erythrocytes. BAL cells were then re-suspended with D-PBS and centrifuged at 4°C for 10 min at 400×g. Pellets of BAL cells were washed twice with sterile 1×D-PBS at 500×g for 10 min and re-suspended in 2 mM L-glutamine-containing complete RPMI-1640 media (Sigma Aldrich) supplemented with 10% fetal calf serum (Invitrogen) and containing antibiotic (penicillin and streptomycin) and antimycotic (amphotericin).

Cell counts and viability test

The harvested BAL cells were used for determination of AMs cell counts, purity and viability. Assays were completed within 2 h of BAL fluid collection. The differential cell count was performed on cytopspins stained with a modified commercially available Giemsa stain (Reastain Quick-Diff kit, REAGENA). Alveolar macrophages purity was 93 and 85% in young pigs and piglets, respectively. Other cells were mostly polymorphonuclear cells (PMNs) and lymphocytes. The total BAL cell count was obtained for each BAL fluid using a haemocytometer. Cell viability in culture was assessed by the Trypan blue dye exclusion method for culture period (>98% in all cases).

Stimulation of alveolar macrophage cells with LPS

First, AMs (2×10^6 cells/well) were treated with 0.0, 0.01, 1.0, 5.0 and 10.0 μg of LPS per ml of media, to determine their response to different concentrations. Treated AMs were incubated in an atmosphere of 95% humidified air and 5% CO_2 at 37°C for 24 h. For further studies, 2×10^6 cells were cultured in a final volume of 1 ml medium in each well of an ultra-low attachment polystyrene 24-well plate (CellStar). All plates were incubated at 37°C with 5% CO_2 and 95% air atmosphere for 72 h. After 1 h incubation, non-adhering cells were removed by gentle washing with media, and 1 ml of fresh complete medium was added for further cell culture. The remaining attached cells were >95% macrophages (as tested in the control well by 0.1% neutral dye, 10 min.). Adherent cells (AMs) were stimulated with LPS of *Escherichia coli* 055:B5 (Sigma) (10.0 $\mu\text{g}/\text{ml}$). Cell pellets and supernatants were collected at 1, 8, 24, 48 and 72 h after incubation. After each time point of incubation the cells were washed with cold sterile D-PBS, scraped from the plates in RNA cell lysis solution, and stored at -80°C until use.

RNA extraction and cDNA synthesis

Harvested AMs were washed in ice cold RPMI-1640 media and the total RNA was extracted using Pico-Pure RNA isolation kit following the manufacturer's manual (Arcturus, Invitrogen). The extracted total RNA was treated with RNase-Free DNase Set (Qiagen) for 15 min at 37°C . Total RNA concentration was measured by absorption at 260 nm and purity and concentration were checked by determining the OD ratio 260/280 nm using a NanoDrop-8000 spectrophotometer (Thermo Scientific). Total RNA was then reverse transcribed into cDNA with SuperScript-II RT kit for qRT-PCR (Invitrogen). All samples were reverse transcribed

under the same conditions. The synthesized cDNA was stored at -20°C and used in quantitative real-time (qRT) PCR reactions as a template.

Analysis of gene expression by qRT-PCR

The oligonucleotides were selected for mRNA of porcine TLRs, associated molecules and two house keeping genes based on the information described in table 1. Two endogenous control transcripts of house keeping genes, coding for cyclophilin A (PPIA) and beta-2-microglobulin (B2M), were used. PPIA and B2M were initially tested for their stability in expression with LPS stimulation and were both found to be stable in AMs. Primers were designed by using FASTA product of the GenBank mRNA sequences for *Sus scrofa* using Primer3 program (Rozen and Skaletsky 2000). Each of the primers was used in the PCR reaction to amplify their corresponding gene and the products were confirmed using agarose gel electrophoresis. The PCR products were then purified using the QIAquick PCR purification kit (Qiagen) according to manufacturer's protocol. For the qRT-PCR, a nine-fold serial dilution of the plasmid was used. For this purpose, the purified PCR fragments were ligated into a plasmid pGEM®-T vector (Promega) and the ligated products were inserted into competent JM109 cells (*E. coli*) following routinely used protocols. The competent cells were cultured in ampicillin treated LB-agar plates including 20 μl of X-Gal. White or blue colonies were picked based on the β -galactosidase activity to check the presence or absence of inserted DNA fragments, following M13 PCR. The best insert, based on the M13 PCR results, was further incubated in ampicillin added LB-broth medium in a shaking incubator at 37°C overnight. Finally the plasmid DNA was isolated by using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) as described by the manufacturer. To determine plasmid size and quality, 5 μl of plasmid was run together with 2 μl of loading buffer using agarose gel electrophoresis. An aliquot of DNA plasmid was subjected to sequence check for product confirmation by sequencing (Beckman Coulter) and the rest was stored at -20°C to be used as a template for setting up the standard curve in real-time PCR.

Experiments and subsequent data analysis were performed using the StepOnePlus™ qRT-PCR System (Applied Biosystems). Plasmid DNA of 9-fold serial dilution was used as the template for the generation of the standard curve. In each run, the 96-well microtiter plate contained each cDNA sample, plasmid standards for the standard curves and a no-template control. The no-template control (NTC) was included in each run for each gene to check for contamination. The qRT-PCR was set up using 2 μl first-strand cDNA template, 7.4 μl deionized H_2O , 0.3 μM of forward and reverse gene specific primers and 10 μl 1xPower

SYBR Green I (BioRad) master mix with ROX as reference dye. The thermal cycling conditions were 3 min at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. The mRNA copy numbers of the target gene and housekeeping gene was calculated according to the standard curve method. A standard curve was derived from the serial dilutions of plasmid DNA. Plasmid DNA was produced by cloning the amplicons of interest into the plasmids. After plasmid DNA isolation, a nine fold serial dilution was adjusted from 10^9 copies to 10 copies. The PCR amplification efficiency of each primer pair was calculated from the slope of the standard curve. Melting curve analysis was constructed to verify the presence of gene-specific amplification and for the absence of primer dimer. Agarose gel electrophoresis was performed to test for the specificity of the amplicons. The expression level of transcript (target gene) was normalised relatively to the average transcript of porcine housekeeping genes (HKGs) B2M and PPIA (table 1), where the expression (copy number) of the target gene was divided by the geometric mean of the expression (copy number) of two HKGs (Vandesompele et al. 2002). Final results were reported as the relative expression level after normalization using the HKGs.

ELISA assays of cytokine concentration in the supernatant

The concentration of pro-inflammatory cytokines (IL-6 and TNF- α) was measured from LPS-stimulated AM cultured supernatant using commercially available specific porcine ELISA Kits such as Quantikine® porcine IL-6 Immunoassay (Cat. P6000B) and Quantikine® porcine TNF- α immunoassay (Cat. PTA00) (R&D System, Minneapolis), following the manufacturer's instructions. The optical density (OD) value was detected using ELISA plate reader (Molecular Devices GmbH) using 450 nm wavelengths. To ensure the repeatability of the experiment, each sample was measured twice and the average concentration was used as protein level (pg/ml) in cell culture supernatant. The concentrations were detected according to the standard using microplate data compliance software SoftMax Pro (Molecular Devices GmbH).

Statistical analysis

The PROC GLM (ver9.2; SAS, SAS Institute Inc., Cary, NC, USA) analysis was performed to detect the effect of dose, age and incubation time on the expression of TLRs and associated molecules. Generalized linear models (Proc GLM with Tukey) were used to determine any possible effect of time and concentration on TLRs and time effects on the relative expression of TLR-associated molecules. Differences in gene expression and cytokine production levels

between age groups were determined using the t-test in SAS. All data are reported as the mean of three animals \pm SEM. Values of $P < 0.05$ were considered to be statistically significant.

Results

Effect of LPS on TLRs in AMs

The dose-dependent responses of LPS to TLRs 2, 4, 5 and 9 in AMs of newborn and young pigs were initially observed. In general, low concentrations of LPS induced low levels of TLR expression, and high concentrations induced high levels of expression. The expression levels of TLR-4 and 2 mRNA was increased significantly by the stimulation of all the applied concentrations of LPS when compared to the control in both age groups (Fig. 1 (a) and (b), $P < 0.05$). Increasing the concentration of LPS from 1.0 to 10.0 $\mu\text{g/ml}$ did not result in any change in TLR-4 and TLR-2 mRNA abundance, except a slight but not significant increase at 10.0 $\mu\text{g/ml}$. Next, we observed that the expression of TLR5 and 9 was highest with the higher dosage of LPS (5.0 and 10.0 $\mu\text{g/mL}$) (Fig. 1 (c) and (d), $P < 0.05$). On the contrary, lower LPS concentration (0.1 and 1.0 $\mu\text{g/ml}$) did not influenced the TLR-5 and TLR-9 expression in both age groups. There was no difference in expression between age groups in any concentration of LPS treatment. The dose-dependant general expression of TLRs 2, 4, 5 and 9 in newborn piglets and young pigs are illustrated in Figure 2. In both age groups, the order of expression always showed TLR-4 as the highest, followed by TLR-2 at each concentration (Fig. 2(a) and (b), $P < 0.05$). Overall, TLR-5 and TLR-9 were moderately expressed with higher dosages of LPS in newborn piglets and young pigs, respectively and vice versa.

For the time-dependent response of mRNA expression from TLRs 2, 4, 5 and 9 in the AMs of newborn 5-day-old piglets and 120-day-old young pigs, a concentration of 10.0 $\mu\text{g/mL}$ of LPS was used. The overall results showed that both TLR-4 and TLR-2 mRNA baseline levels were significantly enhanced in newborn and young animals, as compared to non-stimulated cells (time 0 h) (Fig. 3(a) and (b)). However, newborns tended to produce higher TLRs, especially TLR-4, than the young pigs. Stimulation of AMs with LPS increased the mRNA of TLR-4 as early as 24 h and 8 h in the newborn and young groups, respectively (Fig. 2(a), $P < 0.05$). In contrast, up-regulation of TLR-2 was observed as early as 8 h after LPS exposure in both age groups (Fig. 3(b), $P < 0.05$). The mRNA levels of both TLR-4 and TLR-2 increased substantially in neonates and adults (compared with non-stimulated cells) up to the 48 h and decreased thereafter at 72 h (Fig. 2(a) and (b)). The expression level of TLR-4

remained higher in newborn piglets from 24 h to 48 h compared with those of young pigs (Fig. 3(a), $P<0.01$). For TLR-2 there was no statistical difference between the age groups. The overall up-and-down regulation of TLR-4 and TLR-2 was observed in a similar fashion in newborn piglets and young pigs, and TLR-4 expression was higher in neonatal AMs in comparison to those from young pigs.

TLR-5 was significantly influenced by post-stimulation time. The expression increased as early as 1 h post-treatment and maintained the higher level until 48 h in both ages (Fig. 3(c), $P<0.05$). The expression was at peak level at 8 h and 24 h in piglets and young pigs respectively. The TLR-5 expression was at a higher level from 1 to 48 h in young pigs, but was not significant between age groups. TLR-9 was significantly influenced by post-stimulation time in piglets and young pigs. In both ages, the TLR-9 expression peaked at 24 h and maintained the levels thereafter until 48 h (Fig. 3(d), $P<0.05$). Though, there was a non-significant trend of increasing expression in AMs of young pigs in comparison to piglets.

Expression patterns of TLR associated molecules LBP, CD-14 and MD-2 in LPS-stimulated AMs

In order to examine the effects of LPS in the expression of LBP, CD-14 and MD-2 molecules, AMs were incubated for 1, 8, 24, 48 and 72 h. Overall, AMs from neonates tended to produce higher levels of LBP and CD-14 molecules. LBP showed sensitivity to LPS as early as 8 h, as compared to untreated cells (time 0 h) and peaked at 24 h post induction and was down-regulated thereafter at 48 h in newborn and young animals (Fig. 4(a), $P<0.05$). LPS initiated the up-regulation of the LBP molecule in the AMs of newborn piglets as early as 1 h and up to 72 h compared with those of young pigs (Fig. 4(a), $P<0.05$, $P<0.01$).

CD-14 mRNA expression increased in response to LPS treatment in both newborn and young animals. Up-regulation of CD-14 mRNA levels commenced at 8 h and 24 h in neonates and young pigs respectively, as compared to untreated control cells (Fig. 4(b), $P<0.05$). The expression was steadily enhanced and peaked in both age groups at 48 h post induction with LPS and decreased thereafter at 72 h of incubation. After stimulation, CD-14 mRNA levels remained higher from 24 h to 72 h in newborn piglets compared with those of young pigs (Fig. 4(b), $P<0.05$). The LPS-induced MD-2 molecule in AMs was first evident at 8 h, with the highest increase achieved at 24 h, and decreased steadily thereafter in newborn and young animals (Fig. 4(c), $P<0.05$). Significant difference of MD-2 molecule expression was not observed between age groups (Fig. 4(c)).

Expression pattern of downstream TLR-signaling molecules MyD-88, IRAK-4 and TRAF-6 in LPS-induced AMs

Significant up-regulation of the adaptor molecule MyD-88 was observed as early as 24 h and 8 h in newborn and young pigs respectively, as compared to non-stimulated control cells (Fig. 5(a)). The mRNA levels were up-regulated gradually with time up to 72 h after LPS exposure in both ages (Fig. 5(a), $P < 0.05$). After 8 h of post LPS exposure, MyD-88 mRNA expression from AMs of newborn piglets was higher than those of young pigs up to 72 h incubation.

The signaling molecule IRAK-4 was increased as early as 1 h, with the highest increase achieved at 8 h and decreased thereafter at 24 h in both ages (Fig. 5(b), $P < 0.05$). LPS-induced AM cells also showed up-regulation of TRAF-6 expression level with the peaked at 24 h (Fig. 5(c), $P < 0.05$). Overall, up-regulation and down-regulation expression manner of IRAK-4 and TRAF6 genes in both age groups was almost the same. No significant differences were observed between newborn and young animals in the production of IRAK-4 and TRAF-6 mRNA from AMs after LPS stimulation.

Kinetics of cytokine protein secretion

The overall kinetics of the cytokine production results showed that protein concentration of IL-6 and TNF- α in culture supernatant was significantly affected by the time of incubation and age of the animals (Fig. 6). As expected, LPS stimulation increased IL-6 secretion early at 1 h and after the early period of induction, it was dramatically increased at 8 h, and maintained higher levels up to 48 h, as compared to un-stimulated cells (time 0 h) in piglets and young animals (Fig. 6 (a)). The maximal production level was achieved at 24 h and 8 h after stimulation of culture in piglets and young pigs respectively. However, the secretion of IL-6 in both ages then sharply decreased (48 h) and returned to almost basal level by 72 h post-stimulation. The IL-6 protein level remained significantly higher from 8 h to 48 h in young pigs compared to those of neonatal piglets (Fig. 6 (a)).

The pro-inflammatory cytokine TNF- α secretion significantly increased in response to LPS treatment in both ages as early as 1 h, as compared to un-treated cells. The highest production levels of TNF- α was detected at 8 h post-stimulation with LPS and sustained significantly higher levels up to 48 h in both age groups (Fig. 6 (b)). TNF α secretion in both age groups was declined after the peak and reached the control level at 72 h post-stimulation. Immediately after 1 h post LPS exposure to 8 h, TNF- α secretion by AM cells of young pigs remained significantly higher than those of newborn piglets (Fig. 6 (b)).

Discussion

Expression analysis of TLRs and downstream genes in LPS-induced porcine AM cells

Little is known so far about the age-related kinetic expression of TLRs and downstream genes in porcine AMs. In the present study, we examined and compared the effects of LPS stimulation on TLRs and associated signaling molecule expression levels from AMs of 5-day-old newborn piglets and 120-day-old young pigs. The results from the first experiment showed the mRNA level of TLR-2 and TLR-4 was up-regulated by all concentrations of LPS used in both age groups, as compared to non-stimulated cells (Fig. 1, $P < 0.05$). TLR-5 and TLR-9 expression was also significantly increased with higher dosage of LPS. In a dose-dependant manner, TLR-4 mRNA was the highest expressed gene and others (TLR-2, 5 and 9) were also significantly up-regulated in both age groups after 24 h incubation (Fig. 2). However, increasing the concentration of LPS did not result in any change in TLR-2 and TLR-4 mRNA abundance, except for a slight but non-significant increase at 10.0 $\mu\text{g}/\text{mL}$ (as compared to 0.1 $\mu\text{g}/\text{mL}$), indicating that a low concentration of LPS or a small number of attacking pathogens is enough to trigger optimal TLR-4 and other TLR signalling response in porcine AM cells. It is well established that LPS is the specific ligand for TLR-4 (Nhu et al. 2006; Takeda and Akira 2004a), but expression of other TLRs might be due to the contamination of AM cells with PMNs and lymphocytes.

The present study examined the kinetics of the relative expression pattern of TLRs 2, 4, 5 and 9 on AMs of newborn piglets and young pigs in response to LPS (Fig. 3). On AM cells, TLR (2, 4, 5 and 9) mRNA expressions were significantly increased after LPS stimulation in both ages. In order to interpret our results we created two TLR groups in relation to their specificity (Kokkinopoulos et al. 2005). The first group, named 'bacterial-LPS' nature groups involves TLR-2 and TLR-4. This group carries the characteristic of being able to recognize bacterial LPS. Our data reveal that LPS-induced AMs resulted in an increase in the levels of mRNA transcript for TLR-2 and TLR-4, and TLR-4 was significantly greater enhanced in neonate AMs than in those of young animals (Fig. 3(a)). Higher TLR-4 levels on peripheral blood monocytes was reported after LPS stimulation in neonates, compared with infants and adults (Yerkovich et al. 2007). Further studies using peripheral blood samples showed higher expression of TLR-4 level after LPS stimulation (Levy et al. 2009). These findings are consistent with the idea that TLR-4 is essential in LPS signaling. Presumably, this increases the responsiveness of immune cells to subsequent microbial insults. From the results it may be speculated that BAL containing AM cells of newborn piglets are more sensitive to LPS than

older animals which may be due to less pre-exposure to nosocomial challenges. It has been established that LPS is a specific ligand for TLR-4, but it induced TLR-2 expression and the trend was relatively higher in piglets than those of young animals. Several other explanations are also possible as to why both TLR-2 and TLR-4 responded to LPS. One could be the divergence in LPS structure among Gram-negative bacteria, and it is reasonable to presume that TLR-4 responds to certain types of LPS better than TLR-2 and vice versa. A second possibility is that TLR-2 and TLR-4 may cooperate to respond to LPS. The second group, the 'bacterial' TLR group, involves TLR-5 and TLR-9 that recognize flagellin protein and CpG motifs, respectively (Boule et al. 2004, Didierlaurent et al. 2004). In the present study, significantly higher expression of TLR-5 and 9 transcripts in AMs of both age groups in a time dependent manner was also observed. The results are consistent with previous studies in human monocytes and dendritic cells (Kokkinopoulos et al. 2005) and PBMC in cattle (Hornung et al. 2002).

Our findings demonstrated that AMs from newborn piglets and young pigs are able to express significantly higher levels of LBP molecule after stimulation with LPS, compared with those of un-treated cells. Neonatal piglet AMs are markedly more capable of expressing LBP compared with those of young pigs. Experimental data is not available to explain why AMs produced a greater level of LBP in neonates. However, our finding is supported by another study which showed that increased expression of LBP by AMs of neonatal rats is more than that of adults, and it suggests that these molecules may be a vital factor in the neonatal immunologic response to infections (Lee et al. 2000). LBP has been shown to opsonize LPS-bearing particles for recognition and uptake by macrophages (Wright et al. 1989) and enhance the effects of LPS on CD-14-bearing cells by accelerating the transfer of LPS to CD-14 (Hailman et al. 1994). In addition, the current study showed that AMs from newborn piglets expressed significantly higher levels of CD-14 after stimulation with LPS, compared with those of untreated cells. This finding is supported by a previous study which reported increased expression of CD-14 at base line and after LPS stimulation in neonates compared to adults (Levy et al. 2009). Previously, it has also been reported that LPS stimulation significantly up-regulated CD-14 gene expression in AMs of adult pigs (Sanz et al. 2007). A remarkable feature of TLR-2 and TLR-4 is its ability to cooperate with CD-14 and MD-2 on the host cell surface in sensing LPS of Gram-negative bacterial infections (Miyake 2004). Modulation of immune response by LPS and the role of LBP and CD-14 as bacterial pattern recognition receptors are important in the case of bacterial infections. Although we can not speculate on the differential role of TLR-4 and these associated molecules in lung

homeostasis, collectively our data suggest that the increased LBP and CD-14 mRNA expression on LPS stimulation in neonatal AM cells may participate in the exacerbated inflammatory response often occurring during neonatal infection. On the other hand, our findings could be a significant mechanism in the newborn animal, whereby innate immunity in neonates is compensated and strengthened at a time when humoral immunity is still relatively immature.

The findings of the present study have shown that AMs responded to the presence of LPS by up-regulating MyD-88 in neonates and young pigs, and that this was significantly greater expressed in newborn piglets than those of young pigs. MyD-88 is an important link between innate and acquired immunity since all TLRs, except TLR-3, signal through it to production of inflammatory cytokines (Takeda and Akira 2004b), and it may play the same role in swine AMs cells. MyD-88 knockout mice showed no response to the TLR-4 ligand LPS in terms of macrophage produced inflammatory mediators, B cell proliferation, or endotoxin shock (Kawai et al. 1999). These findings demonstrated that the TIR domain-containing adaptor MyD-88 is essential for the inflammatory responses mediated by TLR-4 however; there are no data about the expression of MyD-88 in swine AM cells. Hence, it could be speculated that LPS-induced AMs are more host sensitive via the MyD-88 dependent pathway in newborns than in young animals in the respiratory system against microbial infections. Moreover, the effects of LPS on the up-regulation of the IRAK-4 and TRAF-6 gene expression on AMs may play a role in further augmentation of innate immune responses. Upon LPS stimulation, MyD-88 recruits and activates a death domain-containing kinase, IL-1 receptor-associated kinase-4 (IRAK-4) to TLR-4 and is activated by phosphorylation and then associates with downstream gene TRAF6. This cascade leads to the activation of signaling transduction pathways of NF- κ B to produce inflammatory cytokines, and in addition to other immune related genes (O'Neill et al. 2009; Takeda and Akira 2005). Our findings revealed LPS-induced up-regulation of signal transduction pathway molecules, IRAK-4 and TRAF-6, in AM cells of newborn and young pigs.

In the current study, most TLRs and downstream genes were expressed significantly higher at 8 h but showed highest expression during 24 to 48 h post-stimulation of LPS. The results demonstrated that early stage cells are hyporesponsive to LPS which may lead to several assumptions such as endotoxin tolerance. Since animals for this experiment were from a conventional farming system, it is likely that these animals may be exposed to low doses of Gram-negative bacteria from their environment, which may lead to LPS tolerance. Pre-exposure to LPS resulting modulation of the expression of TLRs may account for the altered

response to a second stimulus of cellular functions. However, the expression pattern of TLRs and CD-14 in cells pre-treated with LPS or LPS tolerant cells are not always consistent (reviewed by Nahid et al. 2011, Nomura et al. 2000). The mechanism by which LPS tolerance induction affects the interaction of TLR-4 with MyD-88 remains unclear and several explanations are plausible.

Kinetics secretion of cytokines in LPS-induced porcine BAL cells

Previous reports have shown that the onset of the respiratory diseases caused by Gram-negative bacterial infections is induced by the expression of pro-inflammatory cytokines, such as TNF- α , IL-1 and IL-6 (Fossum et al. 1998; Murtaugh et al. 1996; Nakagawa et al. 2001). The measurement of cytokine production is the easiest assessment for cell responsiveness, which is used for different *in vitro* models (Jaekal et al. 2007; Matsuguchi et al. 2000). Thus, we assessed the kinetic production capacity of pro-inflammatory cytokines by AMs of 5-day-old newborn piglets and 120-day-old young pigs. Results from the present study showed that AMs from young pigs produced significantly higher levels of IL-6 and TNF- α after 8 to 48 h and 1 to 8 h of LPS stimulation respectively, compared to newborn piglets. This data indicates that LPS exerts striking time-dependent modulatory effects with age on the cytokine secretion, and it may plausible that pro-inflammatory cytokines are playing an important part of the host's defense response in the lung. In this regard, other studies reported higher IL-6 and TNF- α concentration in older piglets in comparison with those of levels determined on newborn piglets (Matteri et al. 1998; Moya 2007). Cytokines have the ability to regulate a broad range of immune and inflammatory responses, including humoral and cell-mediated immune reactions (Wood and Seow 1996). However, pro-inflammatory cytokines IL-6 and TNF- α are crucial mediators in various respiratory tract conditions that may play a role in pulmonary defense against bacterial pathogens. Overall, age-related changes in IL-6 and TNF- α levels found in pigs from the present study may reflect maturation of immune cells and immune function.

Our data reveal that LPS-induced AMs of piglets showed higher expression of mRNA transcript for TLRs and, on the contrary, lower production of pro-inflammatory cytokine (IL-6 and TNF- α) than those of young pigs. The obtained result seems somewhat contradictory. From these data, we could not explain in detail how cytokine protein release is regulated by inflammation of AMs with age. The BAL fluids containing AM populations were varied in our study which is consistent with the study age-dependent increases in the percentage of AMs in the BAL fluids (Dickie et al. 2009). The relative proportion of cell types and cellular

functional maturity may contribute to age-dependent improvement in AM performance (Weiss et al. 1985). In our study, AMs contaminated with PMN cells and lymphocytes may lead to the alterations in TLR mRNA expression and subsequent cytokine production levels between age groups. In addition, a biological factor of importance could be the post-transcriptional and post-translational regulation of cytokine production (O'Neill et al. 2011). It will be of concern to learn to what extent genetic and age-related factors contribute to the variation in TLR expression and cytokine production by AM cells in pigs.

Conclusion

In conclusion, results from this study describe age-related changes in the expression kinetics of TLRs, downstream genes and secretion of pro-inflammatory cytokine in response to LPS in porcine AMs. Our study elucidated that AMs of newborn piglets are more sensitive to LPS in regards to the expression of TLR-4, LBP, CD-14 and MyD-88 mRNA than those of young pigs. The understanding of the response of piglet AMs to LPS will provide important insights into some effect of LPS and Gram-negative bacteria on the lung. This may help to formulate therapy to prevent the over expression of TLRs and associated genes by AMs that can contribute to pathological tissue damage. On the contrast, the ability of AMs from piglets to produce pro-inflammatory cytokines IL-6 and TNF- α was reduced compared to the young pigs, and suggests that these might influence the role in the innate immune response during pulmonary infection. Further investigations to determine the precise effects of LPS on porcine AMs *in vivo* through functional study across a wider age range are necessary.

Acknowledgement

This project was supported by the Gene Dialog project, FUGATO Plus, BMBF, grant no: 0315130C, Germany. The authors are indebted to Prof. Dr. Florian M. W. Grundler, Molecular Phytomedicine, University of Bonn, Germany for provision with StepOnePlus Real-time PCR system (Applied Biosystem) during the experiment. Authors are also thankful to Ms. Nadine Leyer for her technical assistance during the experiments.

References

- Boule MW, Broughton C, Mackay F, Akira S, Marshak-Rothstein A, Rifkin IR (2004): Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes. *J. Exp. Med.* 199, 1631-1640.
- Burkey TE, Skjolaas KA, Dritz SS, Minton JE (2007): Expression of toll-like receptors, interleukin 8, macrophage migration inhibitory factor, and osteopontin in tissues from pigs challenged with *Salmonella enterica* serovar Typhimurium or serovar Choleraesuis. *Vet. Immunol. Immunopathol.* 115, 309-319.
- Choi C, Kwon D, Jung K, Ha Y, Lee YH, Kim O, Park HK, Kim SH, Hwang KK, Chae C (2006): Expression of inflammatory cytokines in pigs experimentally infected with *Mycoplasma hyopneumoniae*. *J. Comp. Pathol.* 134, 40-46.
- Dickie R, Tasat DR, Alanis EF, Delfosse V, Tsuda A (2009): Age-dependent changes in porcine alveolar macrophage function during the postnatal period of alveolarization. *Dev. Comp. Immunol.* 33, 145-151.
- Didierlaurent A, Ferrero I, Otten LA, Dubois B, Reinhardt M, Carlsen H, Blomhoff R, Akira S, Kraehenbuhl JP, Sirard JC (2004): Flagellin promotes myeloid differentiation factor 88-dependent development of Th2-type response. *J. Immunol.* 172, 6922-6930.
- Droemann D, Goldmann T, Branscheid D, Clark R, Dalhoff K, Zabel P, Vollmer E (2003): Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung. *Histochem. Cell Biol.* 119, 103-108.
- Droemann D, Goldmann T, Tiedje T, Zabel P, Dalhoff K, Schaaf B (2005): Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients. *Respir. Res.* 6, 68.
- Feng WH, Tompkins MB, Xu JS, Zhang HX, McCaw MB (2003): Analysis of constitutive cytokine expression by pigs infected in-utero with porcine reproductive and respiratory syndrome virus. *Vet. Immunol. Immunopathol.* 94, 35-45.
- Forster-Waldl E, Sadeghi K, Tamandl D, Gerhold B, Hallwirth U, Rohrmeister K, Hayde M, Prusa AR, Herkner K, Boltz-Nitulescu G, Pollak A, Spittler A (2005): Monocyte toll-like receptor 4 expression and LPS-induced cytokine production increase during gestational aging. *Pediatr. Res.* 58, 121-124.

- Fossum C, Watrang E, Fuxler L, Jensen KT, Wallgren P (1998): Evaluation of various cytokines (IL-6, IFN-alpha, IFN-gamma, TNF-alpha) as markers for acute bacterial infection in swine: a possible role for serum interleukin-6. *Vet. Immunol. Immunopathol.* 64, 161-172.
- Gabler NK, Spencer JD, Webel DM, Spurlock ME (2008): n-3 PUFA attenuate lipopolysaccharide-induced down-regulation of toll-like receptor 4 expression in porcine adipose tissue but does not alter the expression of other immune modulators. *J. Nutr. Biochem.* 19, 8-15.
- Gioannini TL, Weiss JP (2007): Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells. *Immunol. Res.* 39, 249-260.
- Hailman E, Lichenstein HS, Wurfel MM, Miller DS, Johnson DA, Kelley M, Busse LA, Zukowski MM, Wright SD (1994): Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J. Exp. Med.* 179, 269-277.
- Henneke P, Osmers I, Bauer K, Lamping N, Versmold HT, Schumann RR (2003): Impaired CD14-dependent and independent response of polymorphonuclear leukocytes in preterm infants. *J. Perinat. Med.* 31, 176-183.
- Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T, Endres S, Hartmann G (2002): Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* 168, 4531-4537.
- Jaekal J, Abraham E, Azam T, Netea MG, Dinarello CA, Lim JS, Yang Y, Yoon DY, Kim SH (2007): Individual LPS responsiveness depends on the variation of toll-like receptor (TLR) expression level. *J. Microbiol. Biotechnol.* 17, 1862-1867.
- Kawai T, Adachi O, Ogawa T, Takeda K, Akira S (1999): Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11, 115-122.
- Kawai T, Akira S (2005): Pathogen recognition with toll-like receptors. *Curr. Opin. Immunol.* 17, 338-344.
- Kokkinopoulos I, Jordan WJ, Ritter MA (2005): Toll-like receptor mRNA expression patterns in human dendritic cells and monocytes. *Mol. Immunol.* 42, 957-968.
- Lee DY, Cho YW, Kang SG, Shin NR, Choi IS, Shin SJ, Yoo HS (2004): Quantitative analysis of interleukin-6 expression in porcine spleen cells and alveolar macrophages using real-time PCR. *Vet. Res. Commun.* 28, 503-513.

- Lee PT, Holt PG, McWilliam AS (2000): Role of alveolar macrophages in innate immunity in neonates: evidence for selective lipopolysaccharide binding protein production by rat neonatal alveolar macrophages. *Am. J. Respir. Cell. Mol. Biol.* 23, 652-661.
- Levy E, Xanthou G, Petrakou E, Zacharioudaki V, Tsatsanis C, Fotopoulos S, Xanthou M (2009): Distinct roles of TLR4 and CD14 in LPS-induced inflammatory responses of neonates. *Pediatr. Res.* 66, 179-184.
- Levy O, Zarembek KA, Roy RM, Cywes C, Godowski PJ, Wessels MR (2004): Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. *J. Immunol.* 173, 4627-4634.
- Matsuguchi T, Musikachoen T, Ogawa T, Yoshikai Y (2000): Gene expressions of toll-like receptor 2, but not toll-like receptor 4, is induced by LPS and inflammatory cytokines in mouse macrophages. *J. Immunol.* 165, 5767-5772.
- Matteri RL, Klir JJ, Fink BN, Johnson RW (1998): Neuroendocrine-immune interactions in the neonate. *Domest. Anim. Endocrinol.* 15, 397-407.
- Mikami O, Muneta Y, Mori Y, Yokomizo Y, Nakajima Y (2002): Expression of proinflammatory cytokine mRNA in the lymphatic organs of adult and neonatal pigs. *Vet. Immunol. Immunopathol.* 90, 203-207.
- Miyake K (2004): Innate recognition of lipopolysaccharide by toll-like receptor 4-MD-2. *Trends Microbiol.* 12, 186-192.
- Miyake K (2007): Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Semin. Immunol.* 19, 3-10.
- Moya SL, Boyle LA, Lynch PB, Arkins S (2007): Age-related changes in proinflammatory cytokines, acute phase proteins and cortisol concentrations in neonatal piglets. *Biol. Neonate Neonatol.* 91, 44-48.
- Murtaugh MP, Baarsch MJ, Zhou Y, Scamurra RW, Lin G (1996): Inflammatory cytokines in animal health and disease. *Vet. Immunol. Immunopathol.* 54, 45-55.
- Nakagawa M, Oono H, Nishio A (2001): Enhanced production of IL-1beta and IL-6 following endotoxin challenge in rats with dietary magnesium deficiency. *J. Vet. Med. Sci.* 63, 467-469.
- Nhu QM, Cuesta N, Vogel SN (2006): Transcriptional regulation of lipopolysaccharide (LPS)-induced Toll-like receptor (TLR) expression in murine macrophages: role of interferon regulatory factors 1 (IRF-1) and 2 (IRF-2). *J. Endotoxin. Res.* 12, 285-295.

- O'Neill LA, Bryant CE, Doyle SL (2009): Therapeutic targeting of toll-like receptors for infectious and inflammatory diseases and cancer. *Pharmacol. Rev.* 61, 177-197.
- O'Neill LA, Sheedy FJ, McCoy CE (2011): MicroRNAs: the fine-tuners of Toll-like receptor signalling. *Nat. Rev. Immunol.* 11, 163-175.
- Pabst R (1996): The respiratory immune system of pigs. *Vet. Immunol. Immunopathol.* 54, 191-195.
- Rozen S, Skaletsky H (2000): Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386.
- Sanz G, Perez E, Jimenez-Marin A, Mompert F, Morera L, Barbancho M, Llanes D, Garrido JJ (2007): Molecular cloning, chromosomal location, and expression analysis of porcine CD14. *Dev. Comp. Immunol.* 31, 738-747.
- Scamurra R, Arriaga C, Sprunger L, Baarsch MJ, Murtaugh, MP (1996): Regulation of interleukin-6 expression in porcine immune cells. *J. Interferon Cytokine Res.* 16, 289-296.
- Takeda K, Akira S (2004a): Microbial recognition by toll-like receptors. *J. Dermatol. Sci.* 34, 73-82.
- Takeda K, Akira S (2004b): TLR signaling pathways. *Semin. Immunol.* 16, 3-9.
- Takeda, K., Akira, S. (2005): Toll-like receptors in innate immunity. *Int. Immunol.* 17, 1-14.
- Takeuchi O, Akira S (2010): Pattern recognition receptors and inflammation. *Cell* 140, 805-820.
- Tatad AM, Nesin M, Peoples J, Cheung S, Lin H, Sison C, Perlman J, Cunningham-Rundles S (2008): Cytokine expression in response to bacterial antigens in preterm and term infant cord blood monocytes. *Neonatology* 94, 8-15.
- Tohno M, Shimosato T, Moue M, Aso H, Watanabe K, Kawai Y, Yamaguchi T, Saito T, Kitazawa H (2006): Toll-like receptor 2 and 9 are expressed and functional in gut-associated lymphoid tissues of presuckling newborn swine. *Vet. Res.* 37, 791-812.
- Twigg HL (2004): Macrophages in innate and acquired immunity. *Semin. Respir. Crit. Care Med.* 25, 21-31.
- van Duin D, Mohanty S, Thomas V, Ginter S, Montgomery RR, Fikrig E, Allore HG, Medzhitov R, Shaw AC (2007): Age-associated defect in human TLR-1/2 function. *J. Immunol.* 178, 970-975.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002): Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3, Research0034.

- Vezina SA, Roberge D, Fournier M, Dea S, Oth D, Archambault D (1995): Cloning of porcine cytokine-specific cDNAs and detection of porcine tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-1 beta gene expression by reverse transcription PCR and chemiluminescence hybridization. *Clin. Diagn. Lab. Immunol.* 2, 665-671.
- Weiss RA, Chanana AD, Joel DD (1985): The status of pulmonary host defense in the neonatal sheep: cellular and humoral aspects. *Ann. N. Y. Acad. Sci.* 459, 40-55.
- Wood PR, Seow HF (1996): T cell cytokines and disease prevention. *Vet. Immunol. Immunopathol.* 54, 33-44.
- Wright SD, Tobias PS, Ulevitch RJ, Ramos RA (1989): Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages. *J. Exp. Med.* 170, 1231-1241.
- Yan SR, Qing G, Byers DM, Stadnyk AW, Al-Hertani W, Bortolussi R (2004): Role of MyD88 in diminished tumor necrosis factor alpha production by newborn mononuclear cells in response to lipopolysaccharide. *Infect. Immun.* 72, 1223-1229.
- Yerkovich ST, Wikstrom ME, Suriyaarachchi D, Prescott SL, Upham JW, Holt PG (2007): Postnatal development of monocyte cytokine responses to bacterial lipopolysaccharide. *Pediatr. Res.* 62, 547-552.
- ZDS (2003): Richtlinie für die Stationsprüfung auf Mastleistung, Schlachtkörperwert und Fleischbeschaffenheit beim Schwein, 10.12.2003.

Table 1. Product sizes and nucleotide sequence details of primers used for real time quantitative PCR analysis of mRNA expression of porcine TLRs, associated molecules and two house keeping genes.

Gene name	GenBank accession number	Primer sequence	Ann. tem. °C	Amp. length	Amplification efficiency	R ²
TLR-2	NM_213761.1	F:CAGTCCGGAGGTTGCATATT R:ATGCTGTGAAAGGGAACAGG	58	137	102.38%	0.996
TLR-4	NM_001113039	F:TGGAACAGGTATCCCAGAGG R:CAGAATCCTGAGGGAGTGGA	58	125	115.11%	0.996
TLR-5	NM_001123202.1	F:GTTCTCGCCCACCACATTAT R:TCGGAAGTTCAGGGAGAAGA	60	144	111.04%	0.986
TLR-9	NM_213958.1	F:TGGCCATTACTAGGGAGGTG R:GTCCAAGGTGAAGCTGAAGG	60	134	101.20%	0.989
LBP	NM_001128435.1	F:AGGCATCACCATTTTCAGTCA R:TTGCTCTCCAATGTTCTTTCG	57	186	108.05%	0.988
CD-14	NM_001097445.2	F:TGCCAAATAGACGACGAAGA R:ACGACACATTACGGAGTCTGA	59	174	102.09%	0.999
MD-2	NM_001104956.1	F:TGCAATTCCTCTGATGCAAG R:CCACCATATTCTCGGCAAAT	59	226	93.66%	0.987
MyD-88	NM_001099923.1	F:CCAGTTTGTGCAGGAGATGAR: TCACATTCCTTGCTTTGCAG	54	185	94.14%	0.997
IRAK-4	NM_001112693.1	F:CTTTGATGAGCGACCCGTT R:CCACTGCTGCAAGCTTCTTC	58	184	109.03%	0.998
TRAF-6	NM_001105286.1	F:GGAGCTAACTGCGAAAATGG R:ACGCATGCACAGTTTGTAGC	58	234	108.65%	0.993
PPIA	NM_214353.1	F:CACAAACGGTTCAGTTT R:TGTCCACAGTCAGCAATGGT	58	171	106.53%	0.995
B2M	NM_213978.1	F:ACTTTTCACACCGCTCCAGT R:CGGATGGAACCCAGATACAT	58	180	93.89%	0.997

F: Forward primer, R: Reverse primer, Ann.: Annealing, Amp.: Amplicon,

R²: correlation coefficient of the slope of the standard curve.

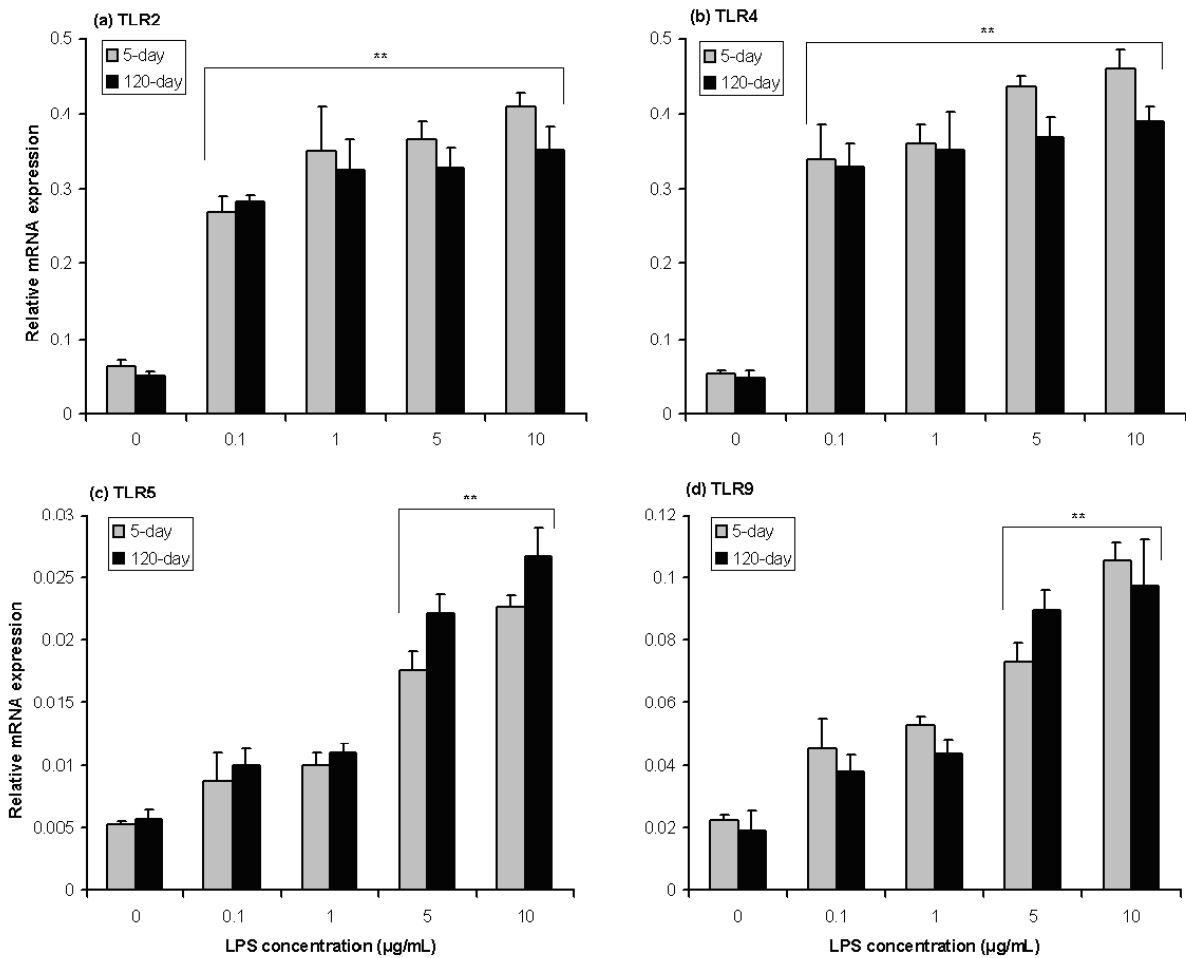


Figure 1. A dose-dependant relative expression ratios of TLRs mRNA were quantified by qRT-PCR in LPS-induced alveolar macrophage cells of 5-day-old newborn and 120-day-old young pigs. Cells (2×10^6 cells/ml/well) were isolated at 24 h after incubation with concentrations of LPS 0.01, 1.0, 5.0, and 10.0 µg/ml. (a) TLR-2, (b) TLR-4, (c) TLR-5, and (d) TLR-9. Data shown is an average of $n = 3$ biological replicates \pm standard error. Level of expression and significance is relative to 0.0 µg/ml LPS concentration (only in medium). Asterisks indicate significant differences of gene expression for all concentrations of LPS stimulation from non-stimulated cells in newborn piglets and young pigs ($P < 0.05$).

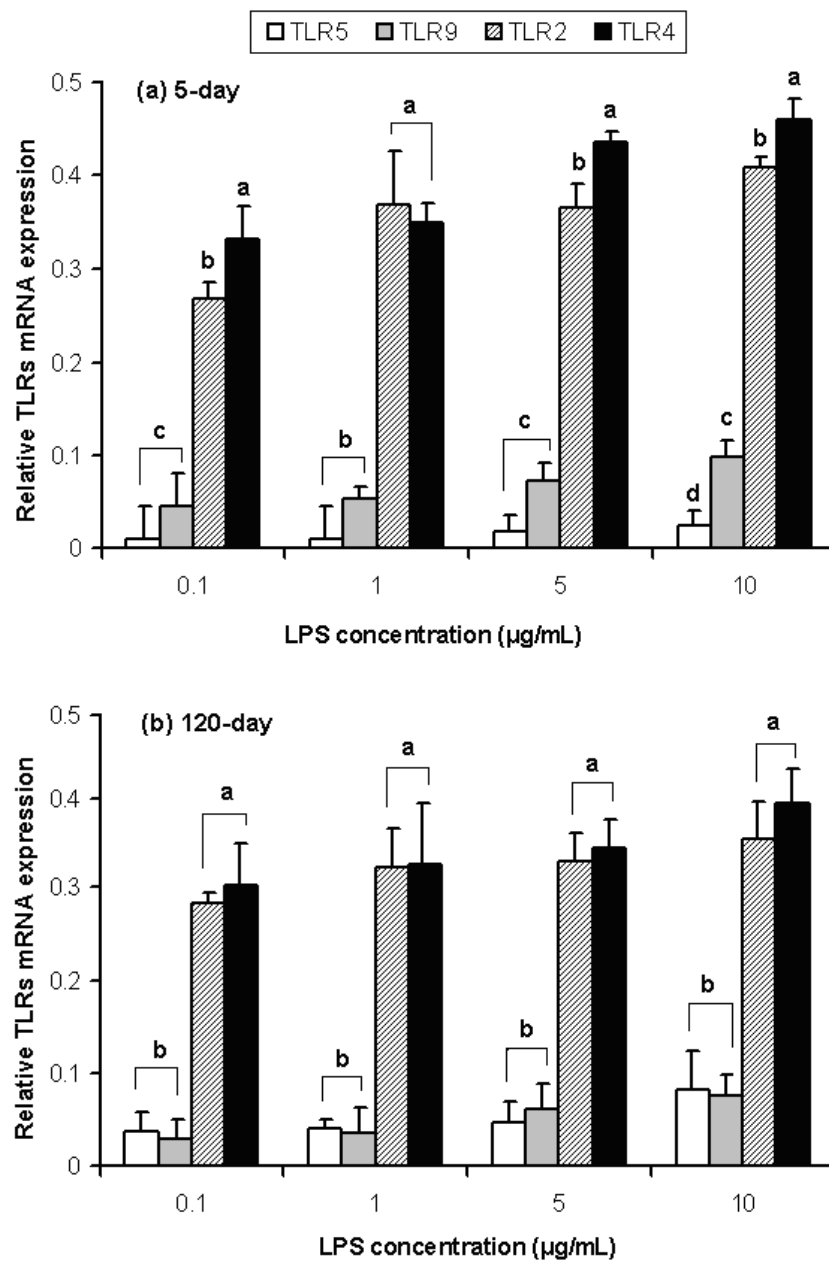


Figure 2. Age-wise differential expression of TLRs (2, 4, 5 and 9) mRNA. The data shown using the same values presented in Fig. 1. (a) 5-day-old piglets, and (b) 120-day-old young pigs. Data shown is an average of $n = 3$ biological replicates \pm standard error. Values with different letters indicate the expression difference of TLRs with same LPS concentration ($P < 0.05$).

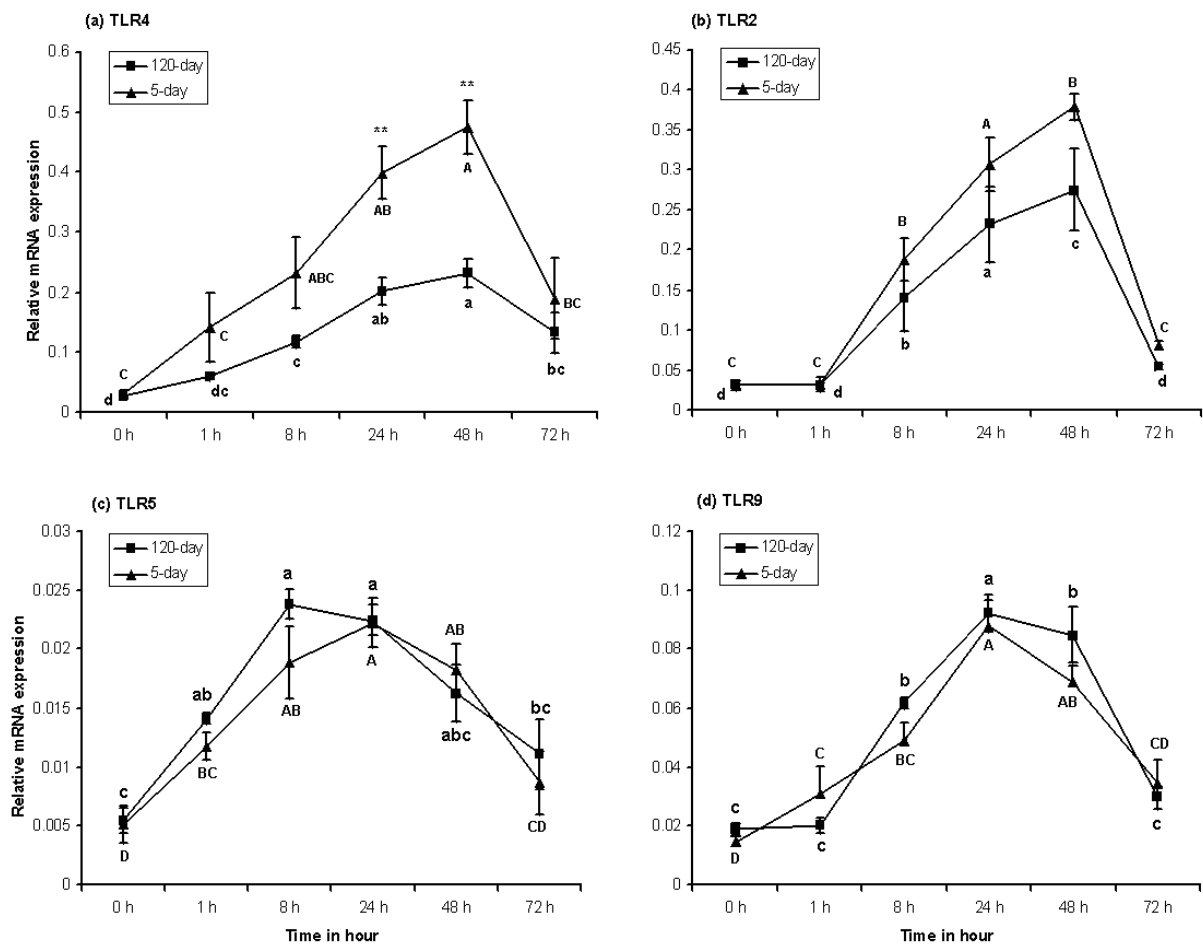


Figure 3. A time-dependant relative expression pattern of TLRs mRNA were quantified by qRT-PCR in LPS-induced alveolar macrophage cells of 5-day-old, newborn and 120-day-old, young pigs. Cells (2×10^6 cells/ml/well) were isolated at 1, 8, 24, 48 and 72 hour after LPS ($10.0 \mu\text{g/mL}$) stimulation or not (time 0 h). Data shown is an average of $n = 3$ biological replicates \pm standard error. (a) TLR-4, (b) TLR-2, (c) TLR-5, and (d) TLR-9. Values with different capital and small letters indicate significant difference at different time points for newborn and young pigs respectively ($P < 0.05$). Asterisks indicate significant differences of newborn from young, $**P < 0.01$.

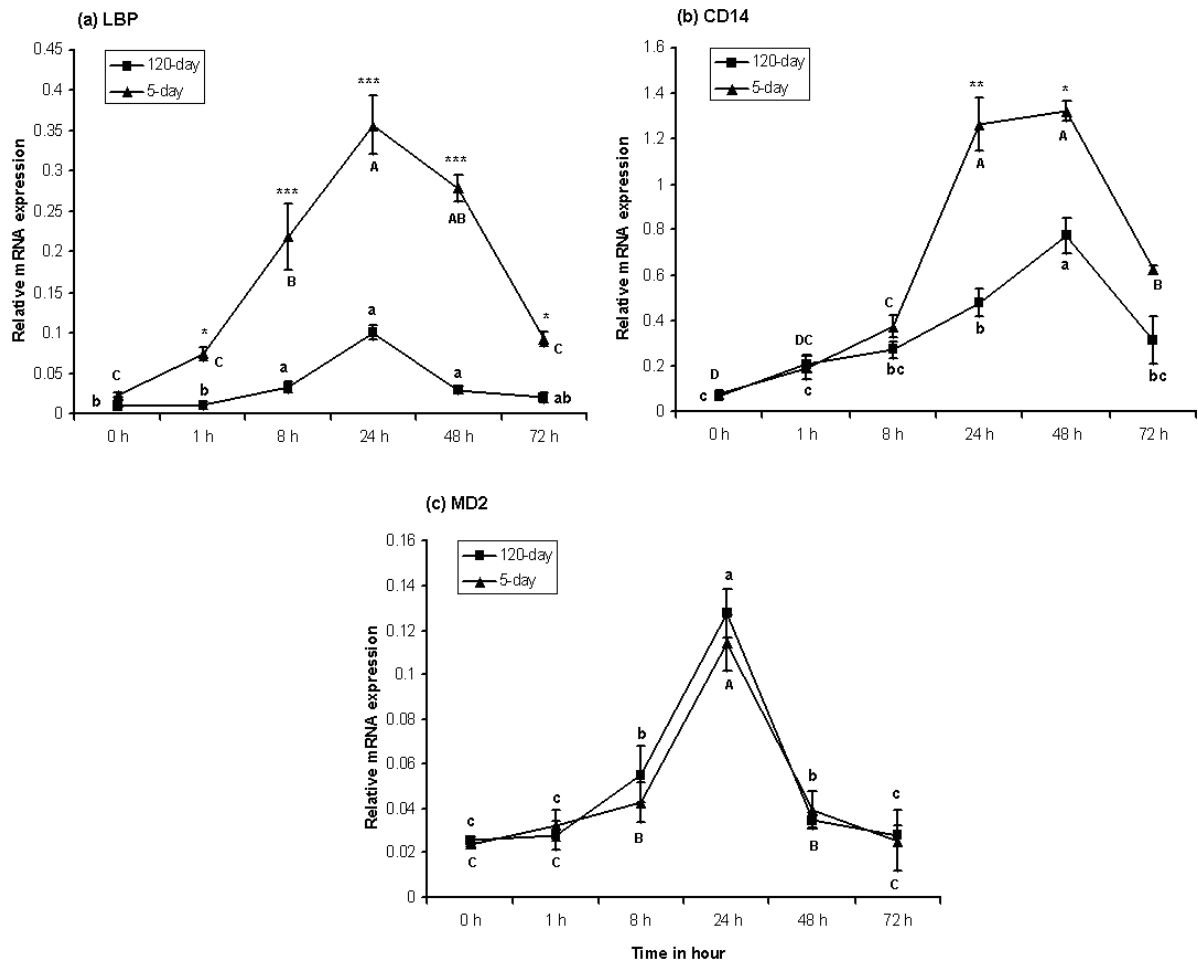


Figure 4. Kinetics relative expression of LPS-signaling TLR-4 associated molecules were performed using the same samples presented in Figure 3. (a) LBP, (b) CD-14, and (c) MD-2. Values with different capital and small letters indicate significant difference at different time points for newborn and young pigs respectively ($P < 0.05$). Asterisks indicate significant differences of newborn from young, * $P < 0.05$; ** $P < 0.01$, and * $P < 0.001$.**

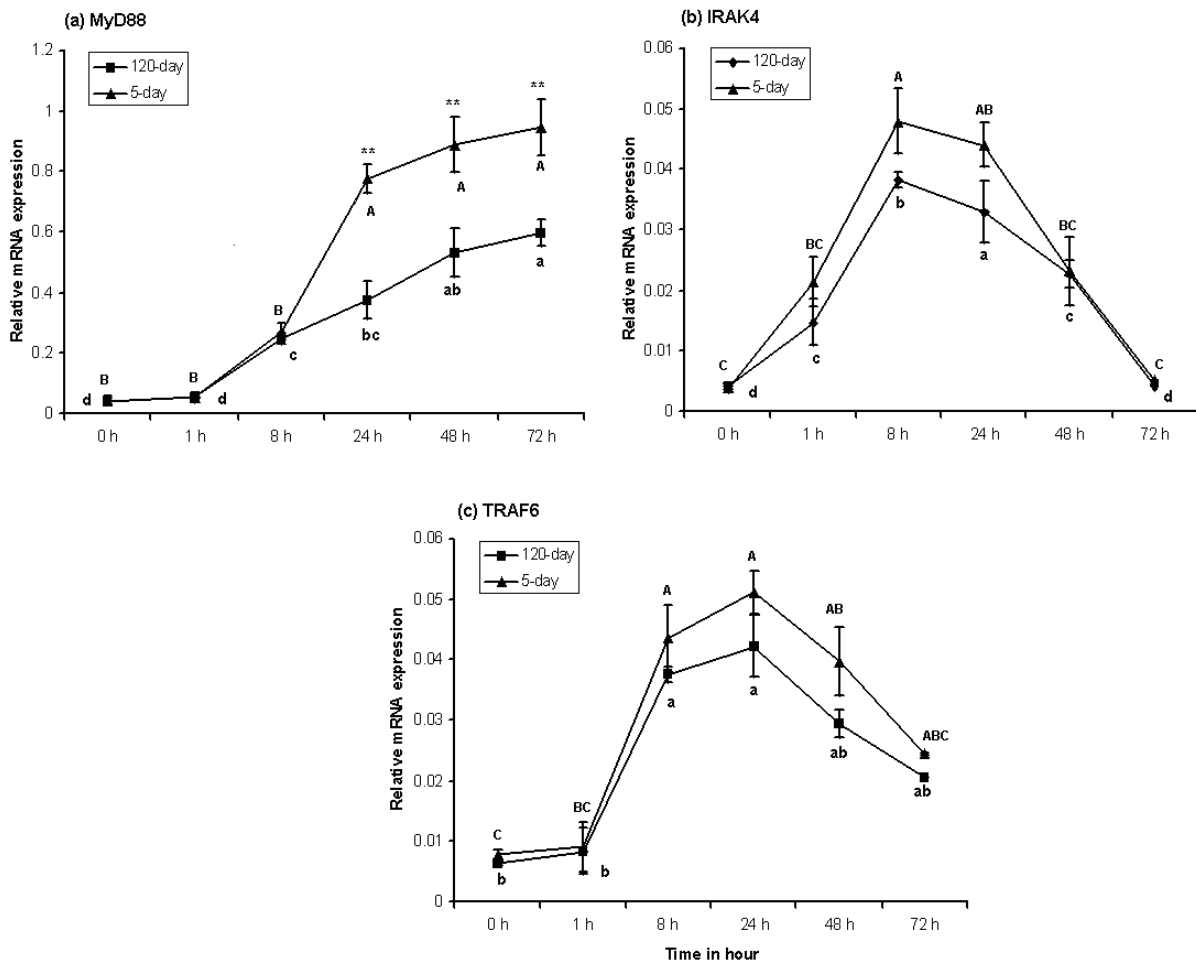


Figure 5. A time-course relative expression analysis of TLR-downstream genes was performed using the same samples presented in Figure 3. (a) MyD-88; (b) IRAK-4, and (c) TRAF-6. Values with different capital and small letters indicate significant difference at various time points for newborn and young pigs respectively ($P < 0.05$). Asterisks indicate significant differences of newborn from young, $**P < 0.01$.

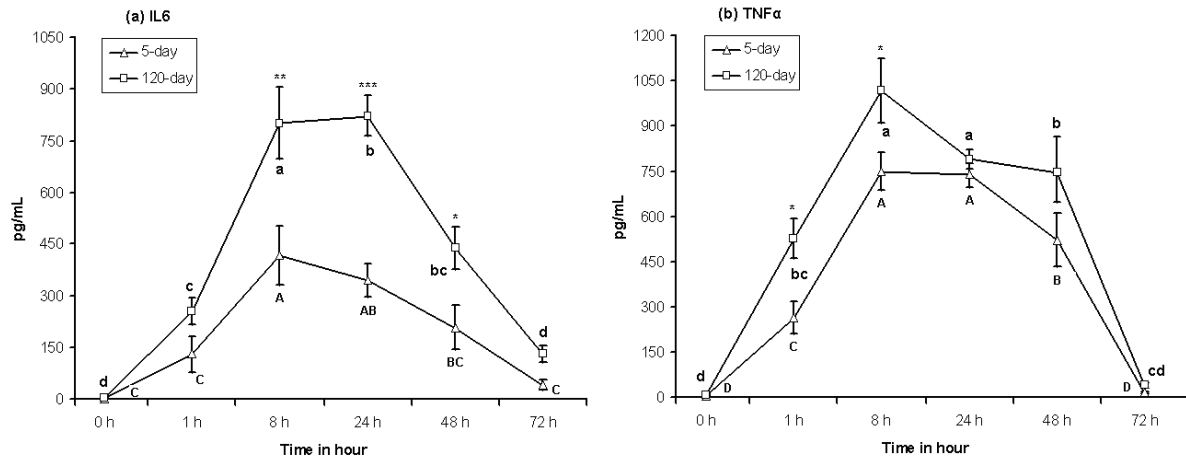


Figure 6. Concentrations level of pro-inflammatory cytokines protein by LPS-stimulated alveolar macrophage cells relative to that of un-treated cells. Alveolar macrophage cells (2×10^6 cells/ml/well) from 5-day-old newborn piglets and 120-day-old young pigs were incubated with LPS ($10 \mu\text{g/ml}$). After stimulation for 1, 8, 24, 48, and 72 h, the supernatant fluids were collected for measurement of cytokines protein levels by ELISA. Data shown is an average of $n = 3$ biological replicates \pm standard error. (a) IL-6, and (b) TNF- α . Values with different capital and small letters indicate significant difference at different time points for newborn and young pigs, respectively ($P < 0.05$). Asterisks indicate significant differences of newborn from young, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Chapter 2. (Published in : *Cytokine*, 2012, 60: 707-717)

Age-related changes in phagocytic activity and production of pro-inflammatory cytokines by lipopolysaccharide stimulated porcine alveolar macrophages

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Article type and status: research and new

Short title: Age-related cytokine production in swine alveolar macrophage

Key words: Age-dependent; alveolar macrophage; phagocytosis; lipopolysaccharide; cytokine; innate immunity

Abstract

The aim of the present study was to determine the age-related changes of phagocytic capacity and the kinetic production of cytokines in lipopolysaccharide-stimulated porcine alveolar macrophages. For this purpose, AMs were isolated from 5 (newborn), 40 (post-weaned) and 120 (young) day old pigs. Results of phagocytosis assay showed that AMs from newborn piglets had less phagocytic capacity than those of young pigs ($P < 0.05$). For the kinetics study, cells and supernatant were collected at 1, 6, 12, 24, 36 and 48 h after LPS stimulation for quantification of cytokine mRNA and protein by quantitative real-time PCR and ELISA, respectively. The kinetics results showed that AMs from newborn piglets were significantly less capable of producing IL-1 β , IL-6, IL-12 β , TNF- α and IL-8 than post-weaned piglets or young pigs. IL-18 mRNA did not show significant differences between ages. MIP-2 and MCP-1 mRNA was higher in young pigs. Hence, higher production of cytokines by AMs may be the surfactant factors in the pulmonary host defense system. These results indicate that AMs from newborn piglets might be functionally immature, which may lead to increased susceptibility to lung infections. Future studies of cytokine kinetics in more animals are clearly needed to confirm these results across a wider age range.

Introduction

The innate immune response plays a critical role in the host defense system. In general, neonates have an immature immune system compared to that of adults. Newborn and early postnatal piglets are highly susceptible to a wide range of infectious disease, especially respiratory disease complex (Cutler et al. 2006). In the respiratory tract, alveolar macrophages (AMs) have important roles in conducting the first line of defense against invading pathogens by two important and effective means. First, AMs directly bind, phagocytose, and kill pathogens, and whereas some earlier studies have demonstrated deficient phagocytic capacity of AM in the neonates compared with adults (Sherman et al. 1977; Kurland et al. 1988). In contrast, other contradictory reports suggest that neonatal and adult AMs are equivalent in their capacity to phagocytose bacteria (Conly et al. 1991; Speer et al. 1988). Second, AMs are able to secrete a large range of inflammatory mediators including cytokine and chemokine. Cytokines are acting directly on the pathogens while others such as chemokines exert their effects indirectly by recruiting other components of the immune system. The functional deficiency of neonatal AMs includes impaired phagocytosis, chemotaxis, and production of

cytokine and chemokine (Sherman et al. 1977; Cheung et al. 1986; Mills 1983). During early postnatal life there are relative deficiencies in the number and differentiation of AMs. Thus, AMs may contribute to diminish the host's defense and increased neonatal susceptibility to respiratory infection (Coonrod et al. 1987).

Recently, increasing attention has been paid to the involvement of the cytokine and chemokine response in neonatal immune defense mechanism. The ability of newborn children to produce cytokine and chemokine is different from adults (Marodi 2006). Understanding ontogeny of inflammatory mediators production in pigs is not detailed as in humans. The age-dependent variation of cytokine expression levels in blood plasma, peripheral blood phagocytes and lymphatic organs were observed in pigs (Zelnickova et al. 2008; Mikami et al. 2002; Moya et al. 2007). Also, measurement of some pro-inflammatory cytokines has shown the significant age-related differential production in pigs (Islam et al. 2012; Matteri et al. 1998). The altered ability of newborns to produce inflammatory cytokines and chemokines can be in part responsible for the higher sensitivity of newborns to infectious diseases. Moreover, it has been reported that different cytokines can be expressed by immune cells at different time points after initial stimulation with mitogens, such as LPS and phytohemagglutinin (PHA) (Choi et al. 2002; Yoo et al. 1995; Reddy et al. 2000). Determination of cytokine profiles during the time course stimulation would give important information on immunostimulatory and immunosuppressive mechanism. To the authors' knowledge, understanding the age-associated variability expression and the kinetics pattern of pro-inflammatory cytokine and chemokine production by AMs has not been well reported in pigs.

In the present study, we first attempted to compare the age-related changes in bronchoalveolar lavage (BAL) fluid cellular content and the ability of AM cell functions in terms of their phagocytic response for selected Gram-negative bacteria in different age groups. As the production of inflammatory mediators is highly dependent of the time after stimulation, we sought to determine the age-related time kinetic profiles of pro-inflammatory cytokine and chemokine production by porcine AMs in response to LPS. Generally, mRNA responses are assumed to correlate with secreted protein responses as has been confirmed by some studies (Greenbaum et al. 2003), but not in others (Nie et al. 2006; Gry et al. 2009). Hence, the kinetics of cytokine expression during stimulation with LPS was further investigated comparing cytokine mRNA (analysed by qRT-PCR) with the corresponding protein secretion (ELISA).

Materials and methods

Animals

The experiment comprised nine clinically healthy male German Landrace pigs in total. All animals were obtained from the teaching and research station of Frankenforst, University of Bonn, Germany. The animals had no history of respiratory diseases. The pigs were conventionally housed until slaughter and lungs were obtained at the following three age categories: 5 days (newborn piglets, n=3), 40 days (post-weaned piglets, n=3) and 120 days (young, n=3). The experiments were done according to the institutional guidelines and animal husbandry regulations of Germany (ZDS 2003).

Collection of AMs

Pulmonary alveolar macrophages were collected by bronchoalveolar lavage (BAL). BAL fluids from three animals of each age group were collected in separate tubes and filtered through sterile gauze and placed on wet ice. BAL fluids were mixed with RBC lysis buffer to lyse any residual erythrocytes. BAL cells were pelleted by centrifugation at 4°C for 10 minutes (min) at 400×g. Cells were re-suspended and washed twice in calcium-magnesium free Dulbecco's phosphate-buffered saline (D-PBS; pH 7.4; Sigma-Aldrich) before final re-suspension with RPMI-1640 media (Sigma-Aldrich).

Cell counts and viability test

The harvested cells were used for determination of AMs cell count, purity and viability. Assays were completed within 2 hours (h) of BAL fluid collection. The differential cell count was performed on cytopins stained with a modified commercially available Giemsa stain (Reastain Quick-Diff kit, REAGENA). AM purity was 93%, 89% and 80% in young, post-weaned and newborn piglets, respectively. Other cells were mostly polymorphonuclear cells (PMNs) and lymphocytes. The total BAL cell count was obtained for each BAL fluid using a Haemocytometer. The cell viability was determined using the Trypan blue dye exclusion method. The percentage of viable AM cells were >98 % in all cases.

Stimulation of AMs with LPS

The collected cells by bronchoalveolar lavage as described above were re-suspended in 2mM L-glutamine-containing complete RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) (Invitrogen), sodium pyruvate (1 mM) and containing antibiotic

and antimycotics (penicillin, streptomycin, amphotericin). In all experiments, AMs were cultured separately at 2×10^6 cells/well into 24-well tissue culture plates. After 1 h incubation, plates were carefully washed with D-PBS to remove any non-adherent cells and 1 mL of fresh complete medium was added for further cell culture. The remaining attached cells were >95% macrophages (as tested in the control well by 0.1% neutral dye, 10 min). Adherent cells were treated with LPS (10.0 $\mu\text{g}/\text{mL}$) of *Escherichia coli* 026:B6 (Sigma-Aldrich) and untreated wells were considered as control. All plates were incubated at 37°C with 5% CO₂ and 95% air atmosphere for 48 h. All cells and supernatants were collected at 1, 6, 12, 24, 36 and 48 h after incubation. After each time point of incubation the cells were washed with cold sterile D-PBS and stored immediately at -80°C until use.

Phagocytic assay of AMs

In vitro phagocytosis assay was performed with Vybrant Phagocytosis Assay Kit (Molecular Probes Inc.) according to manufacturer instructions. Using this method, the process of phagocytosis can be quantified by following the internalization of fluorescently labeled bacterial particles. The protocol takes advantage of the detectability of the intracellular fluorescence emitted by the engulfed bacteria and Trypan blue was shown to effectively quench the fluorescence of fluorescein-labeled *Escherichia coli* as previously described (Loike and Silverstein 1983). Briefly, 50 μl of phagocytosis effectors were seeded in a 24-well plate (1×10^6 cells/well) with 100 μl RPMI-1640 media. The cells were then left untreated with 100 μl adjusted cells and 50 μl media (positive control) and additionally, as a negative control, wells were left cell-free and filled only with 150 μl of media. Determinations were performed in five wells for experimental, positive and negative samples at each time points. After 2 h of incubation, solutions were removed from all microplate wells by vacuum aspiration. Fluorescein-labeled *Escherichia coli* (K-12 strain) bioparticles were added to the wells and phagocytic uptake was allowed to proceed for 30 min, 1 h and 2 h in a 37°C humidified incubator with 5% CO₂. Subsequently, the bioparticle suspension was removed and 100 μl of Trypan blue suspension was added for 1 min at room temperature. The excess Trypan blue was removed and the samples were measured in the fluorescence microplate reader (Thermo Electron Co.) using 480 nm for excitation and 520 nm emission wavelengths.

ELISA assays of cytokines concentration in the supernatant

The concentration of cytokine protein of IL-1 β , IL-12 β , TNF- α , IL-6 and IL-8 of cells stimulated with LPS in cultured supernatant was measured using commercially available

specific porcine ELISA Kits (R&D System), following the manufacturer instructions. The optical density (OD) value was detected using ELISA plate reader using 450 nm wavelengths. Standard and sample dilutions were added in duplicate wells for each time point to each ELISA plate and the average concentration was used as protein level (pg/ml) in cell culture supernatant. The concentrations were detected according to the standard using microplate data compliance software SoftMax Pro (Molecular Devices GmbH).

RNA extraction and cDNA synthesis

At each sampling time (1, 6, 12, 24, 36 and 48 h), harvested AM cells were washed in ice cold D-PBS and the total RNA was extracted using Pico-Pure RNA isolation kit following the manufacturer manual (Arcturus, Invitrogen). The extracted total RNA was treated with RNase-Free DNase Set (Qiagen) for 15 min at 37°C. Total RNA concentration was measured by absorption at 260 nm and purity and concentration were checked by determining the OD ratio 260/280 nm using a NanoDrop-8000 spectrophotometer (Thermo Scientific). Total RNA was then reverse transcribed into cDNA with SuperScript-II RT kit for qRT-PCR (Invitrogen). All samples were reverse transcribed under the same conditions. The synthesized cDNA was stored at -20°C and used in qRT-PCR reactions as a template.

Analysis of gene expression by qRT-PCR

The oligonucleotide gene specific primers were selected for porcine cytokine mRNA and two house keeping genes (PPIA and B2M) based on the information described in table 1. Primers were designed by using FASTA product of the GenBank mRNA sequences for *Sus scrofa* using Primer3 program (Rozen 2000). Quantitative gene expression and subsequent data analysis were performed using the StepOnePlus™ qRT-PCR System (Applied Biosystems). The qRT-PCR was set up using a 2 µl first-strand cDNA template, 7.4 µl deionized H₂O, 0.3 µM of forward and reverse gene specific primers and 10 µl 1× Power SYBR Green I (Bio-Rad) master mix with ROX as a reference dye. The thermal cycling conditions were 3 min at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. The mRNA copy numbers of the target and housekeeping gene was calculated according to the standard curve method. A standard curve was derived from the serial dilutions of plasmid DNA. Plasmid DNA was produced by cloning the amplicons of interest into the plasmids. After plasmid DNA isolation, a nine fold serial dilution was adjusted from 10⁹ copies to 10 copies. The data obtained from the qRT-PCR analysis was expressed as the mean of triplicate samples ± standard error of mean (SEM). The expression level of transcript (target gene) was normalised

relatively to the average transcript of porcine housekeeping genes (HKGs) B2M and PPIA (table 1), where the expression (copy number) of the target gene was divided by the geometric mean of the expression (copy number) of two HKGs (Vandesompele et al. 2002). Final results were reported as the relative expression level after normalization of each target cytokine gene using the HKGs.

Data analysis

Data were subjected to ANOVA procedures using the PROC GLM of SAS (ver9.2; SAS, SAS Institute Inc., Cary, NC, USA). The analysis of variance with Tukey's test was used to detect any possible effect at different time points on the production of cytokine data (mRNA and protein) within the same age group. For incubation time course study, value of $P < 0.05$ was considered to be statistical significant. Within time periods of individual age group, values with similar superscripts alphabet letters are not significantly different. Differences in phagocytic activity, differential cells in BAL fluids and cytokine production levels between age groups at each time point were determined using the student's *t*-test. Asterisks indicate significant differences among age groups at each time point. *** $P < 0.0001$, ** $P < 0.001$, and * $P < 0.05$. Comparison of results between LPS-stimulated cells and untreated cells at different time points within each age group were performed using student's *t*-test in Excel XP. All values in the figures and text are expressed as means of three animals' \pm SEM.

Results

Age-dependent increases in the percentage of AMs in the BAL fluid (Fig. 1)

Differential cell counts indicated that in all age groups, AMs were the predominant cell type present in the BAL fluids (80.33 ± 4.16 - $92.67 \pm 3.21\%$). Other cells were mostly polymorphonuclear neutrophils (PMNs) (6.33 ± 2.51 - $18.33 \pm 5.03\%$) and lymphocytes represented $\leq 1\%$ of total cells in the three age categories analyzed. The percent content of AMs in the BAL fluids increased significantly from 5-day-old newborn piglets ($80.33 \pm 4.16\%$) to 40-day-old post-weaned ($89.00 \pm 3.00\%$) and 120-day-old young pigs ($92.67 \pm 3.21\%$). The percentage of PMNs decreased significantly from newborn piglets (18.33%) and post-weaned (10.33%) to young pigs (6.33%). Between newborn and post-weaned piglets, there was no appreciable change in the percentages of PMN cells.

Phagocytosis of opsonised particles increases with age (Fig. 2)

After 30 and 120 min incubation, the percentage of phagocytic AM cells in 5-day-old piglets ($38.4 \pm 4.51\%$ and $42.8 \pm 8.26\%$, respectively) was significantly lower than that of 120-day-old young pigs ($55.4 \pm 7.37\%$ and $63.2 \pm 9.68\%$, respectively). On the contrary, after 60 min incubation, there was no statistically difference in the number of AMs that were able to ingest FITC-labeled *E. coli* ($44.2 \pm 8.35\%$ in newborn piglets compared with $58.0 \pm 7.35\%$ in young pigs). On the other hand, after 60 min incubation the phagocytic activity in 5-day-old piglets ($44.2 \pm 8.35\%$) was significantly lower than that of 40-day-old post-weaned piglets ($58.4 \pm 7.98\%$). In general, the result suggests that AMs from newborn born piglets had a significantly lower phagocytic capacity than their post-weaned and young counterparts. The phagocytic activity was not statistically different between post-weaned piglets and young pigs.

Time and age-dependent LPS effects on cytokine gene expression in AMs

In order to examine the time-course effects of LPS on cytokine and chemokine mRNA expression, AM cells from 5-day (newborn piglets), 40-day (post-weaned piglets) and 120-day (young) old pigs were incubated for 1, 6, 12, 24, 36 and 48 h. In general, quantitative cytokine mRNA expression was significantly affected by the time of incubation and the age of animals (Fig. 3-6).

In newborn piglets and young pigs the expression of IL-1 β mRNA was significantly increased, with a peak at 6 h after induction (Fig. 3a), and declined thereafter. The IL-1 β gene expression level increased between 6 and 24 h with a peak at 12 h in post-weaned piglets (Fig. 3a). LPS initiated the significant up-regulation of the IL-1 β gene in young pigs during 6 to 36 h and post-weaned piglets at 6 h compared with those of newborn piglets (Fig. 3b). The IL-6 gene rose significantly as early as 6 h, peaked at 12 h, and remained at higher levels up to 36 h in young and post-weaned pigs (Fig. 3c). In newborn piglets, IL-6 mRNA expression was significantly increased only at 6 and 24 h compared to untreated cells but time-course expression was not significantly different (Fig. 3c). After stimulation, IL-6 mRNA level remained significantly higher from 12 to 24 h in young compared to newborn piglets. No significant difference was observed between newborn and post-weaned piglets.

The IL-12 β gene expression was significantly elevated in all age groups as early as 6 h. The expression was highest at 12 h in young and post-weaned pigs and 24 h in newborn piglets (Fig. 4a). In young animals the IL-12 β mRNA expression was significantly higher than newborns and post-weaned piglets at 6 to 12 h, and 12 h, respectively (Fig. 4b). The TNF- α mRNA peaked at 6 h and remained at significantly higher levels up to 24 h in all age groups

(Fig. 4c). Only at 6 h of post LPS exposure, TNF- α expression from AMs of young pigs was higher than those of newborn piglets. No significant difference was observed between newborn and post-weaned piglets (Fig. 4d). The IL-18 mRNA level was first evident at 6 h in young, and at 12 h in newborn and post-weaned piglets (Fig. 5a). In all age groups, the expression peaked at 12 h and decreased thereafter. The expression of IL-18 was not significantly different among the age groups.

Time and age-dependent LPS effects on chemokine mRNA expression by AMs

The chemokine IL-8 mRNA expression underwent a massive elevation at 1 h and peaked at 6 h in post-weaned piglets. In young and newborn pigs, IL-8 showed sensitivity to LPS as early as 6 h with peaked levels, and declined thereafter. Significant upregulation was maintained up to 36 and 48 h in both piglets and young pigs, respectively (Fig. 5c). The IL-8 expression trend showed significant differences from 6 to 24 h between young and newborn animals. At only 24 h, the young group showed higher expression than those of post-weaned piglets.

An increase of MIP-2 mRNA expression was only observed between 24 and 36 h in post-weaned pigs (Fig. 6a). No statistically significant expression of MIP-2 by AMs in young pigs and newborn piglets was observed, as compared to un-treated cells (Fig. 6a). The relative level of MIP-2 was significantly higher during 24-36 h in young, and at 24 h in post-weaned piglets than those of newborn piglets (Fig. 6b). The MCP-1 mRNA expression showed a slight increase at 6 and 24 h in young animals but was not significantly detected in newborn and post-weaned piglets (Fig. 6c). In age-related expression, the MCP-1 gene was significantly higher in young pigs only at 36 h after LPS exposure, compare to newborn piglets.

Time and age-dependent LPS effects on cytokine release by AMs

In order to assay the time-course effects of LPS on cytokine and chemokine release, cell cultured supernatant fluids were collected at 1, 6, 12, 24, 36 and 48 h from the three age groups. Overall, the concentration of protein in supernatant was significantly affected by the time of incubation and the age of animals (Fig. 7-9).

Following LPS exposure for 48 h, AMs released IL-1 β at 6 h with a peak at 24 h in all age groups (Fig. 7a). Except newborn piglets, IL-1 β remained at a higher level up to 48 h. In age-related changes, IL-1 β secretion was significantly higher from 6-24 h and 6-36 h in young animals than those of post-weaned and newborn piglets, respectively (Fig. 7b). Secretion of IL-1 β between newborn and post-weaned piglets was not significantly different. LPS

stimulated AMs from all ages secreted significantly at higher levels of IL-6 as early as 1 h and plateaued between 6-24 h, compared to those of untreated cells. The highest concentration was observed at 24 h in young, and at 6 h in newborn and post-weaned piglets. The IL-8 secretion from AMs of young pigs showed significant differences from 6-24 h and 12-36 h than newborn and post-weaned piglets, respectively.

Release of IL-12 β was significantly detected as early as 6 h, peaked at 24 h and was maintained at higher concentrations up to 48 h in young, newborn and post-weaned pigs (Fig. 8a). All pigs released IL-12 β early at 1 h, but not in a significant quantity. AMs of young pigs released significantly more IL-12 β from 6-24 h than newborn and post-weaned piglets (Fig. 8b). At 12 h, the concentration was significantly higher in post-weaned than newborn piglets (Fig. 8b). The TNF- α concentration was first detected at 1 h and remained significantly higher up to 48 h, with peak levels at 6 h in both young and newborn animals as compared to untreated cells (Fig. 8a). Release of TNF- α was significantly detected as early as 1 h, peaked at 12 h and was maintained at higher concentrations up to 48 h in post-weaned piglets (Fig. 8b). Between 6-12 h, the concentration was significantly higher in young than newborn piglets (Fig. 8d). Secretion of TNF- α between 12-24 h, the concentration was significantly higher in post-weaned piglets than newborn piglets (Fig. 8d).

Time and age-dependent LPS effects on chemokine secretion by AMs

The CXC motif chemokine IL-8 secretion by LPS-stimulated AMs of young pigs showed a significant increase as early as 1 h, peaked at 6 h and continued at higher levels up to 48 h (Fig. 9a). In both piglets, IL-8 release significantly increased at 6 h and remained at a higher level, compared with untreated cells, until the end of incubation period (Fig. 9a). In young pigs, a significantly increasing trend was observed from 1-24 h as compared to both newborn and post-weaned piglets (Fig. 9b). IL-8 secretion was significantly higher at 6 h in post-weaned piglets in comparison with newborn piglets (Fig. 9b).

Comparison between mRNA expression and protein secretion levels

In order to examine whether cytokine mRNA expression was associated with the secretion of the respective protein, we analysed the time-dependent expression manner between mRNA and protein levels (supplementary Fig. 1-3). The IL-1 β mRNA expression and protein secretion curve peaked at 6 h and 24 h, respectively, and decreased thereafter up to 48 h in young pigs. In post-weaned piglets, production (mRNA and protein) pattern was similar with a higher peak between 12-24 h post-induction. In newborn piglets, the production curve was

same between 6-12 h; thereafter a discrepancy was observed (supplementary Fig. 1). The IL-6 distribution pattern of mRNA expression and protein secretion was similar with a highest peak between 12-24 h in young pigs. In post-weaned piglets, IL-6 production (mRNA and protein) curve was somewhat consistent during 6-24 h with a mild drop of the protein level at 12 h. In newborn piglets, the expression pattern (mRNA and protein) clearly achieved its highest level at 6 h; but thereafter was not consistent (supplementary Fig. 1).

The pro-inflammatory cytokine IL-12 β mRNA expression and protein secretion was similar with a peak between 12-24 h in post-weaned and young pigs. On the other hand, the peaked curve of IL-12 β (mRNA and protein) was clearly similar at 6 h in newborn piglets (supplementary Fig. 2). The TNF- α mRNA expression and protein secretion curve was similar with a peak at 6 h and decreased thereafter in post-weaned and young pigs. In newborn piglets, the expression curve shows highest peak at between 6-12 h with decreasing and increasing tendency at 24 and 36 h post-induction, respectively (supplementary Fig. 2). The chemokine IL8 distribution pattern of mRNA and protein was almost similar in all age groups with a higher peak between 6-12 h and decreased thereafter up to 48 h (supplementary Fig. 3).

Discussion

It is becoming increasingly clear that many factors contribute to the age-related susceptibility of neonates to bacterial infections of the respiratory system. In particular, the innate immune response plays a critical role in the host's defense. This strenuous host reaction to bacteria is influenced by the complex interplay between genetics, epigenetics and the environment, and can be organ specific in regards of functional maturation (Barnes 2005; Garcia and Moreno 2006; Raz 2007; Sokka et al. 2008). Herein, we have shown that a major component of the innate arm of the immune system in the lung of piglets, i.e. the AM population, is not completely efficient in function when compared with young animals. In particular, several key functions of AMs such as phagocytosis, production of pro-inflammatory cytokine and chemokine in response to bacterial products appear to be less efficient in newborns than in older animals.

In the present study, first we describe the age-related changes in BAL fluid cellular content and phagocytic capacity of AMs for selected Gram-negative bacteria (fluorescein-labeled *E. coli*, K-12) in newborn, post-weaned piglets and young pigs. Alveolar macrophages were relatively less abundant in structurally immature lungs in newborn piglets. As AM cell density in the lung increased with advancement of age, however, phagocytic activity also increased

with maturational changes of AMs in young pigs. In general, the phagocytic activity results suggest that AMs from newborn piglets had a significantly lower phagocytic capacity than their post-weaned and young counterparts. Consistent with earlier investigations (Dickie et al. 2009; Zeidler et al. 1985), we observed that the phagocytosis percentage of opsonised particles was significantly lower in 5-day-old piglets than older pigs. There was also a tendency of age-related change in the proportion of phagocytic cells between post-weaned and young pigs, but statistically this was not significantly different. Thus, it may not be surprising that cells of the same lineage, namely AMs, present at a site of high antigenic challenge (the lungs), would be highly efficient in bacterial clearance. Alveolar macrophage maturation, moreover, is likely influenced by the many changes occurring in the neonatal lung environment (Farver et al. 1999). For example, surfactant production and composition changes post-natally in pigs (Rau et al. 2004). Moreover, changes both in the relative proportions of cell types and in cellular functional maturity may contribute to age-dependent improvement in AM performance (Weiss et al. 1985). Early impairment of AM function may be one factor underlying the enhanced susceptibility to pulmonary infections found in early born neonates, as well as in newborn of other species.

Little is known so far about the age-related kinetic production of pro-inflammatory cytokines and chemokines (both mRNA and protein) by porcine AMs in response to LPS. In the present study, increased IL-1 β and IL-12 β mRNA expressions were accompanied by increased production of protein in post-weaned and young pigs than in newborn piglets in a time-dependent manner. This result is somewhat consistent with a previous report of decreased production of IL-1 β in newborn human babies compared to adults (Peters et al. 1993). IL-1 β and IL-12 β cytokines are secreted by monocytes and macrophages in response to bacteria and bacterial products. However, these cytokines have pleiotropic effects, which influence many physiological processes throughout the whole body (Dinarello et al. 2001). The present work showed that age had an affect on IL-6 and TNF- α at mRNA and protein levels in a time-dependent manner, indicating a local response in the lung. This result was consistent with other reports that showed higher IL-6 and TNF- α concentrations in older piglets in comparison with those of newborn piglets (Moya et al. 2007; Islam et al. 2012; Matteri et al. 1998). The ability of newborn children to produce pro-inflammatory cytokines (like IL-6 and TNF- α) was found to be lower compared to adults by many researchers (Peters et al. 1993; Chang et al. 1994; Schibler et al. 1993). In contrast, LPS did not differentially regulate IL-18 mRNA expression among age groups. This result is somewhat consistent with earlier reports; that IL-18 shows to LPS response in porcine AMs (Oem et al. 2000). However, it has been

suggested that the onset of the respiratory diseases or endotoxemia caused by Gram-negative bacterial infection is induced by the expression of pro-inflammatory cytokine, such as IL- β , IL-6, IL-12 β , TNF- α (Murtaugh et al. 1996; Fossum et al. 1998; Nakagawa et al. 2001).

From the present study, it also appears that the ability of AMs to produce chemokines increased with advancement of age in a time-course manner. Following exposure to LPS, the production of IL-8 (both mRNA and protein) significantly increased as very early as 1 h in all ages. This result is consistent with a previous study; that porcine AMs are able to produce IL-8 within 1 h after stimulation (Lin et al. 1994). Induction of IL-8 mRNA expression in porcine macrophages by LPS has been reported (Sacco et al. 1996), but this author did not include different ages. On the other hand, the kinetics mRNA level of MIP-2 and MCP-1 was also significantly higher in older pigs than in newborn piglets. In general, the chemokines are involved in the onset of inflammation; it mediates mainly the chemotaxis of macrophages and monocytes by controlling leukocyte recruitment and clearance of pathogens (Dawson et al. 2000; Dessing et al. 2007; Harada et al. 1994; Melgarejo et al. 2009).

It is generally accepted that newborn and early postnatal pigs are analogously to newborn children more susceptible to a wide range of infectious diseases compared to adults (Cutler et al. 2006). The discussion of inflammatory mediators production in relation to sensitivity of postnatal piglets and young pigs to LPS is difficult. However, the findings of the present study may be plausible that age-associated production of pro-inflammatory cytokine and chemokine by AMs may be a significant factor in pulmonary host defense and in the pathogenesis of pulmonary infections. The reasons for differences in cytokine and chemokine production in pigs may reflect maturation of immune cells which indicate functional differences of cell population. At present there are very limited reports on genetic variation in pig inflammatory mediator production levels. Apart from genetic differences in immune capacity, epigenetic and environment can also be considered as an important determinant of cytokine and chemokine production.

Furthermore, we observed that the time-dependent comparison between cytokine/chemokine mRNA expression (by qRT-PCR) and corresponding protein secretion (by ELISA) was not always consistent over the incubation time of cells in all ages (supplementary Fig. 1, 2 and 3). In general, almost similar production curves between the levels of mRNA transcription and the respective secreted proteins were found during 6-24 h of stimulation period. From this data, we could not explain in detail how protein release is regulated by cytokine mRNA with age. However, a good relation between mRNA expression and protein secretion would be expected for cytokines and chemokine when both are assayed at the same time of culture and

within 24 h after stimulation (Verfaillie et al. 2001). It has been reported that kinetics TNF α mRNA (qRT-PCR) was not followed by a protein response (ELISA) in LPS-induced porcine blood mononuclear cells (Sorensen et al. 2011). The differences observed could be explained by the test system used and variability among samples must be taken into consideration. On the other hand, biological factors of importance could be the post-transcriptional and post-translational regulation of protein production, for example, through processes that influence mRNA stability and efficiency of translation. The miRNA directly or indirectly regulate the expression of cytokine genes, post-translational regulation of protein synthesis and influence the innate immune response of the host (Asirvatham et al. 2009; Bi et al. 2009). From the present study, it also appeared that age and incubation time has a significant effect on mRNA expression and protein secretion in pigs. It can not be excluded that age and individual animal variation-related factors affect the post-transcriptional regulation of cytokine production.

In conclusion, the phagocytic activity and the ability of AMs from newborn piglets to produce pro-inflammatory cytokines and chemokines were reduced compared to older pigs in a time-dependent manner. These may reflect the development of the relatively immature immune system and influence the pathogenesis of pulmonary infection in pigs. Besides cytokines, other components of innate immunity could play a role in defense against infection (Elahi et al. 2006). The reasons for genetic differences and influences in cytokine and chemokine production in pigs are unknown with respect to the present state of knowledge. However, we can only theorize that many factors are responsible including the increasing ability of phagocytes to respond to the stimulators, maturation of signaling pathways and a numerical decrease and an increase in maturity of phagocytes from the newborn to young BAL fluid. These factors were all observed in the present study. These mechanisms exist in the age-dependent changes (Levy 2007), the understanding of ontogeny of the cytokine and chemokine production could help to explain the practical questions regarding a higher susceptibility of newborn piglets to infectious diseases. It will be of concern to learn to what extent genetic and age-related factors contribute to the variation in cytokine and chemokine production in pigs. Such knowledge will unwrap an era in obtaining better immunological resistance through breeding programmes.

Acknowledgement

This project was supported by the Gene Dialog project, FUGATO Plus, BMBF, grant no: 0315130C, Germany. The authors are indebted to Ludger Buschen in the Research Station “Frankenforst” at University of Bonn for providing pigs. Authors are also thankful to Ms. Maren Pröll, Ms. Christiane Neuhoff and Ms. Nadine Leyer for their assistance during the experiment.

References

- Cutler RS, Fahy VA, Cronin GM, Soicer EM (2006): Prewaning mortality. In: Straw, B.E., Zimmerman, J.J., D’Allaire, S., Taylor, D.J. (Eds.), Diseases of swine. Blackwell publishing, IA, USA, pp. 993-1011.
- Sherman M, Goldstein E, Lippert W, Wennberg R (1977): Neonatal lung defense mechanisms: a study of the alveolar macrophage system in neonatal rabbits. *Am. Rev. Respir. Dis.* 116, 433-40.
- Kurland G, Cheung AT, Miller ME, Ayin SA, Cho MM, Ford EW (1988): The ontogeny of pulmonary defenses: alveolar macrophage function in neonatal and juvenile rhesus monkeys. *Pediatr Res.* 23, 293-7.
- Conly ME, Speert DP (1991): Human neonatal monocyte-derived macrophages and neutrophils exhibit normal nonopsonic and opsonic receptor-mediated phagocytosis and superoxide anion production. *Biol. Neonate.* 60, 361-6.
- Speer CP, Gahr M, Wieland M, Eber S (1988): Phagocytosis-associated functions in neonatal monocyte-derived macrophages. *Pediatr. Res.* 24, 213-6.
- Cheung AT, Kurland G, Miller ME, Ford EW, Ayin SA, Walsh EM (1986): Host defense deficiency in newborn nonhuman primate lungs. *J. Med. Primatol.* 15, 37-47.
- Mills EL (1983): Mononuclear phagocytes in the newborn: their relation to the state of relative immunodeficiency. *Am. J. Pediatr. Hematol. Oncol.* 5, 189-98.
- Coonrod JD, Jarrells MC, Bridges RB (1987): Impaired pulmonary clearance of pneumococci in neonatal rats. *Pediatr. Res.* 22, 736-42. ():
- Marodi L (): Neonatal innate immunity to infectious agents. *Infect. Immun.* 2006; 74:1999-2006.

- Zelnickova P, Leva L, Stepanova H, Kovaru F, Faldyna M (2008): Age-dependent changes of proinflammatory cytokine production by porcine peripheral blood phagocytes. *Vet. Immunol. Immunopathol.* 124, 367-78.
- Mikami O, Muneta Y, Mori Y, Yokomizo Y, Nakajima Y (2002): Expression of proinflammatory cytokine mRNA in the lymphatic organs of adult and neonatal pigs. *Vet. Immunol. Immunopathol.* 90, 203-207.
- Moya SL, Boyle LA, Lynch PB, Arkins, S (2007): Age-related changes in proinflammatory cytokines, acute phase proteins and cortisol concentrations in neonatal piglets. *Biol. Neonate. Neonatol.* 91, 44-8.
- Islam MA, Cinar MU, Uddin MJ, Tholen E, Tesfaye D, Looft C, Schellander K (2012): Expression of Toll-like receptors and downstream genes in lipopolysaccharide-induced porcine alveolar macrophages. *Vet. Immunol. Immunopathol.* 146, 62-73.
- Matteri RL, Klir JJ, Fink BN, Johnson RW (1998): Neuroendocrine-immune interactions in the neonate. *Domest. Anim. Endocrinol.* 15, 397-407.
- Choi IS, Shin NR, Shin SJ, Lee DY, Cho YW, Yoo HS (2002): Time course study of cytokine mRNA expression in LPS-stimulated porcine alveolar macrophages. *J. Vet. Sci.* 3, 97-102.
- Yoo HS, Maheswaran SK, Lin G, Townsend EL, Ames TR (1995): Induction of inflammatory cytokines in bovine alveolar macrophages following stimulation with *Pasteurella haemolytica* lipopolysaccharide. *Infect. Immun.* 63, 381-8.
- Reddy NR, Borgs P, Wilkie BN (2000): Cytokine mRNA expression in leukocytes of efferent lymph from stimulated lymph nodes in pigs. *Vet. Immunol. Immunopathol.* 74, 31-46.
- Greenbaum D, Colangelo C, Williams K, Gerstein M (2003): Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome. Biol.* 4, 117.
- Nie L, Wu G, Zhang W (2006): Correlation of mRNA expression and protein abundance affected by multiple sequence features related to translational efficiency in *Desulfovibrio vulgaris*: a quantitative analysis. *Genetics.* 174, 2229-43.
- Gry M, Rimini R, Stromberg S, Asplund A, Ponten F, Uhlen M, Nilsson P (2009): Correlations between RNA and protein expression profiles in 23 human cell lines. *BMC Genomics.* 10, 365.
- ZDS (2003): Richtlinie für die Stationsprüfung auf Mastleistung, Schlachtkörperwert und Fleischbeschaffenheit beim Schwein, 10.12.2003.

- Loike JD, Silverstein SC (1983): A fluorescence quenching technique using trypan blue to differentiate between attached and ingested glutaraldehyde-fixed red blood cells in phagocytosing murine macrophages. *J. Immunol. Methods.* 57, 373-9.
- Rozen S SH (2000): Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Humana Press, Totowa, NJ, pp 365-86.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002): Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome. Biol.* 3, RESEARCH0034.
- Barnes KC (2005): Genetic determinants and ethnic disparities in sepsis-associated acute lung injury. *Proc. Am. Thorac. Soc.* 2, 195-201.
- Garcia JG, Moreno Vinasco L (2006): Genomic insights into acute inflammatory lung injury. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 291, L1113-7.
- Raz E (2007): Organ-specific regulation of innate immunity. *Nat. Immunol.* 8, 3-4.
- Sokka T, Abelson B, Pincus T (2008): Mortality in rheumatoid arthritis: 2008 update. *Clin. Exp. Rheumatol.* 26, S35-61.
- Dickie R, Tasat DR, Alanis EF, Delfosse V, Tsuda A (2009): Age-dependent changes in porcine alveolar macrophage function during the postnatal period of alveolarization. *Dev. Comp. Immunol.* 33, 145-51.
- Zeidler RB, Kim HD (1985): Phagocytosis, chemiluminescence, and cell volume of alveolar macrophages from neonatal and adult pigs. *J. Leukoc. Biol.* 37, 29-43.
- Farver CF, Kobzik L (1999): Lung macrophage differentiation antigens in developing fetal and newborn rat lungs: A quantitative flow cytometric analysis with immunohistochemistry. *Lung* 177, 205-17.
- Rau GA, Vieten G, Haitzma JJ, Freihorst J, Poets C, Ure BM, Poets C, Ure BM, Bernhard W (2004): Surfactant in newborn compared with adolescent pigs: adaptation to neonatal respiration. *Am. J. Respir. Cell. Mol. Biol.* 30, 694-701.
- Weiss RA, Chanana AD, Joel DD (1985): The status of pulmonary host defense in the neonatal sheep: cellular and humoral aspects. *Ann. N. Y. Acad. Sci.* 459, 40-55.
- Peters AM, Bertram P, Gahr M, Speer CP (1993): Reduced secretion of interleukin-1 and tumor necrosis factor-alpha by neonatal monocytes. *Biol. Neonate.* 63, 157-62.
- Dinarelli CA, Muegge K, Durum SK (2001): Measurement of soluble and membrane-bound interleukin 1 using a fibroblast bioassay. *Curr. Protoc. Immunol.* Chapter 6, Unit 6 2.

- Chang M, Suen Y, Lee SM, Baly D, Buzby JS, Knoppel E, Wolpe S, Cairo MS (1994): Transforming growth factor-beta 1, macrophage inflammatory protein-1 alpha, and interleukin-8 gene expression is lower in stimulated human neonatal compared with adult mononuclear cells. *Blood* 84, 118-24.
- Schibler KR, Trautman MS, Liechty KW, White WL, Rothstein G, Christensen RD (1993): Diminished transcription of interleukin-8 by monocytes from preterm neonates. *J. Leukoc. Biol.* 53, 399-403.
- Oem JK, Song HJ, Kang SW, Jeong WS (2000): Cloning, sequencing, and expression of porcine interleukin-18 in *Escherichia coli*. *Mol. Cells.* 10, 343-7.
- Murtaugh MP, Baarsch MJ, Zhou Y, Scamurra RW, Lin G (1996): Inflammatory cytokines in animal health and disease. *Vet. Immunol. Immunopathol.* 54, 45-55.
- Fossum C, Watrang E, Fuxler L, Jensen KT, Wallgren P (1998): Evaluation of various cytokines (IL-6, IFN-alpha, IFN-gamma, TNF-alpha) as markers for acute bacterial infection in swine--a possible role for serum interleukin-6. *Vet. Immunol. Immunopathol.* 64, 161-72.
- Nakagawa M, Oono H, Nishio A (2001): Enhanced production of IL-1beta and IL-6 following endotoxin challenge in rats with dietary magnesium deficiency. *J. Vet. Med. Sci.* 63, 467-9.
- Lin G, Pearson AE, Scamurra RW, Zhou Y, Baarsch MJ, Weiss DJ, Murtaugh MP (1994): Regulation of interleukin-8 expression in porcine alveolar macrophages by bacterial lipopolysaccharide. *J. Biol. Chem.* 269, 77-85.
- Sacco RE, Nibbelink SK, Baarsch MJ, Murtaugh MP, Wannemuehler MJ (1996): Induction of interleukin (IL)-1beta and IL-8 mRNA expression in porcine macrophages by lipopolysaccharide from *Serpulina hyodysenteriae*. *Infect. Immun.* 64, 4369-72.
- Dawson TC, Beck MA, Kuziel WA, Henderson F, Maeda N (2000): Contrasting effects of CCR5 and CCR2 deficiency in the pulmonary inflammatory response to influenza A virus. *Am. J. Pathol.* 156, 1951-9.
- Dessing MC, van der Sluijs KF, Florquin S, van der Poll T (2007): Monocyte chemoattractant protein 1 contributes to an adequate immune response in influenza pneumonia. *Clin. Immunol.* 125, 328-36.
- Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, Matsushima K (1994): Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J. Leukoc. Biol.* 56, 559-64.

- Melgarejo E, Medina MA, Sanchez-Jimenez F, Urdiales JL (2009): Monocyte chemoattractant protein-1: a key mediator in inflammatory processes. *Int. J. Biochem. Cell. Biol.* 41, 998-1001.
- Verfaillie T, Cox E, To LT, Vanrompay D, Bouchaut H, Buys N, Goddeeris BM (2001): Comparative analysis of porcine cytokine production by mRNA and protein detection. *Vet. Immunol. Immunopathol.* 81, 97-112.
- Sorensen NS, Skovgaard K, Heegaard PM (2011): Porcine blood mononuclear cell cytokine responses to PAMP molecules: comparison of mRNA and protein production. *Vet. Immunol. Immunopathol.* 139, 296-302.
- Asirvatham AJ, Magner WJ, Tomasi TB (2009): miRNA regulation of cytokine genes. *Cytokine* 45, 58-69.
- Bi Y, Liu G, Yang R (2009): MicroRNAs: novel regulators during the immune response. *J. Cell. Physiol.* 218, 467-72.
- Elahi S, Buchanan RM, Attah-Poku S, Townsend HG, Babiuk LA, Gerds V (2006): The host defense peptide beta-defensin 1 confers protection against *Bordetella pertussis* in newborn piglets. *Infect. Immun.* 74, 2338-52.
- Levy O (2007): Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat. Rev. Immunol.* 7, 379-90.

Table 1. Oligonucleotide sequences designed for the quantitative real-time PCR analysis of mRNA expression of porcine cytokines and two house keeping genes, with the respective annealing temperature, amplicon products (bp) and GenBank accession number.

Gene	Primer set	Annealing temperature (°C)	Amplicon size (bp)	GenBank accession number
IL-1 β	F: GTACATGGTTGCTGCCTGAA R: CTAGTGTGCCATGGTTTCCA	58	137	NM_001005149.1
IL-6	F: GGCAGAAAACAACCTGAACC R: GTGGTGGCTTTGTCTGGATT	58	125	NM_214399.1
IL-12 β	F: GAATCTGCAGCTGAATCCAT R: TCCTTGTGGCATGTAACCTT	57	186	NM_214013.1
TNF- α	F: ACTGCACTTCGAGGTTATCG R: GCTGGTTGTCTTTCAGCTTC	58	226	NM_214022.1
IL-8	F: TAGGACCAGAGCCAGGAAGA R: CAGTGGGGTCCACTCTCAAT	58	137	NM_213867.1
IL-10	F: GTGGAGGAGGTGAAGAGTGCC R: GAGGTACAGCAGGGTTTCCCA	60	266	L20001
IFN- γ	F: AGAGCCAAATTGTCTCCTTCTAC R: TCTCTGGCCTTGGAACATAG	60	285	S63967
MIP2	F: TGCTGTAGCTTTAGCGGAAT R: GACCTGCCAAACACATTCAT	57	186	NM_001001861.1
MCP1	F: ATCCTCCAGCATGAAGGTCT R: ACTTGCTGCTGGTGACTCTT	56	125	NM_214214.1
PPIA	F: CACAAACGGTTCCCAGTTT R: TGTCCACAGTCAGCAATGGT	58	171	NM_214353.1
B2M	F: ACTTTTCACACCGCTCCAGT R: CGGATGGAACCCAGATACAT	58	180	NM_213978.1

F: Forward primer; R: Reverse primer; bp: base pair

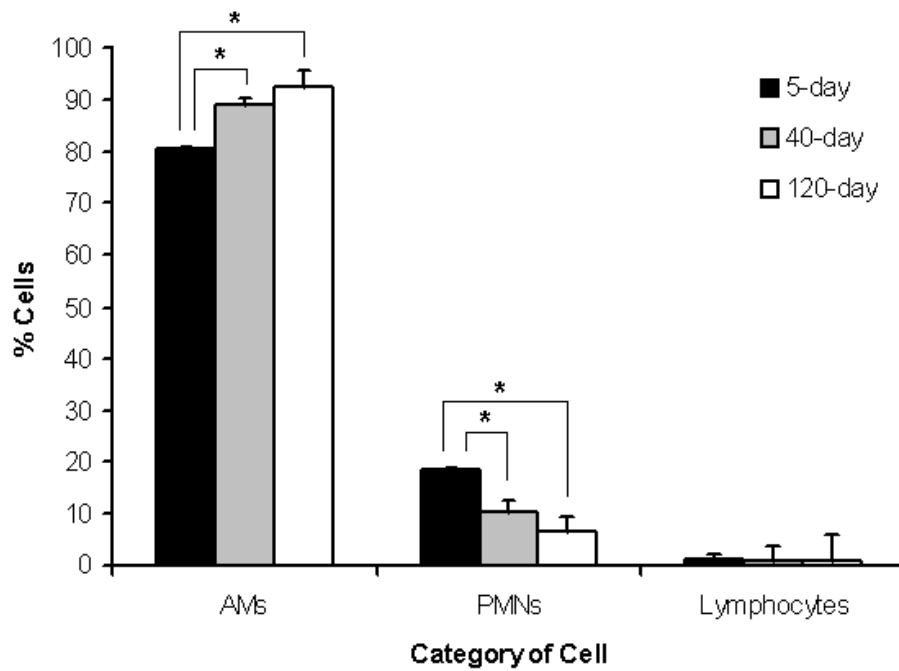


Figure 1. Differential cell count percentages from BALF of 5-day-old newborn piglets, 40-day-old post-weaned piglets and 120-day-old young pig lungs. The percentage of AMs in the BALF increased with the advancement of age and the percentage of neutrophils decreased. Asterisks indicates statistical differences of AMs and PMNs cells between age groups ($*P<0.05$). Each age group comprised three animals and counts were made from three slides/pig.

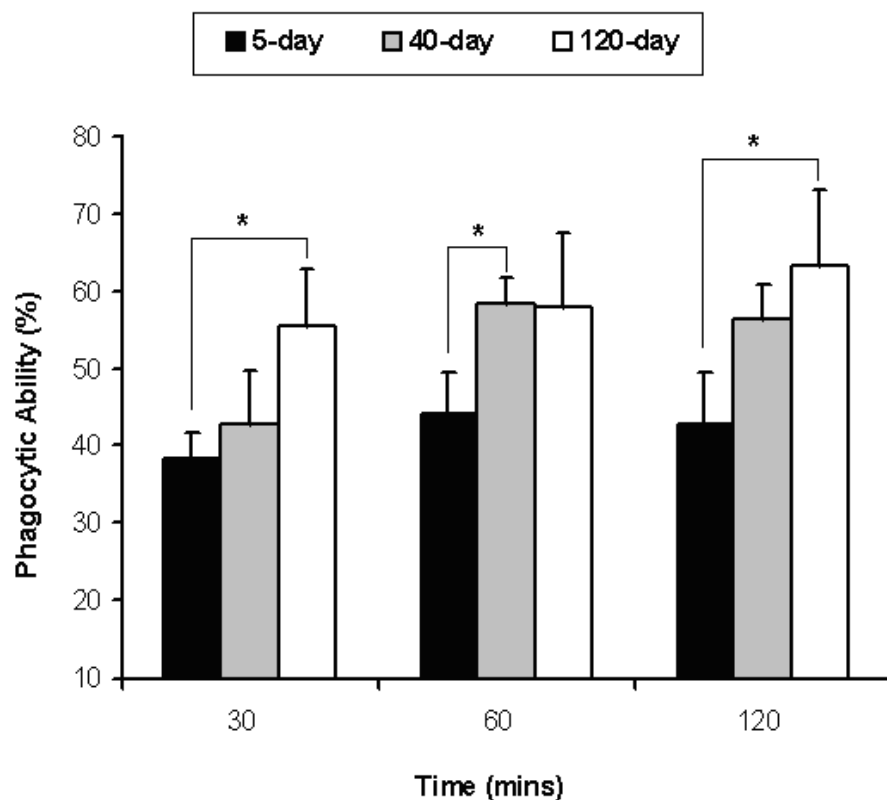


Fig. 2. Phagocytic ability of AMs from pigs of different ages. Alveolar macrophages from 5-day-old newborn piglets, 40-day-old post-weaned piglets and 120-day-old young pigs were incubated with FITC-labeled *E. coli* for 30, 60 and 120 mins at 37°C with CO₂. Fluorescence as a result of bacteria adherent to the outside of the AMs was quenched with trypan blue. The phagocytic capacity of this population is represented as percentage, which is indicative of the total number of bacteria each cell had ingested. Asterisk indicates statistical differences from 5-day-old early born piglets (* $P < 0.05$). Each age group comprised three animals and read made from five replicates/pig.

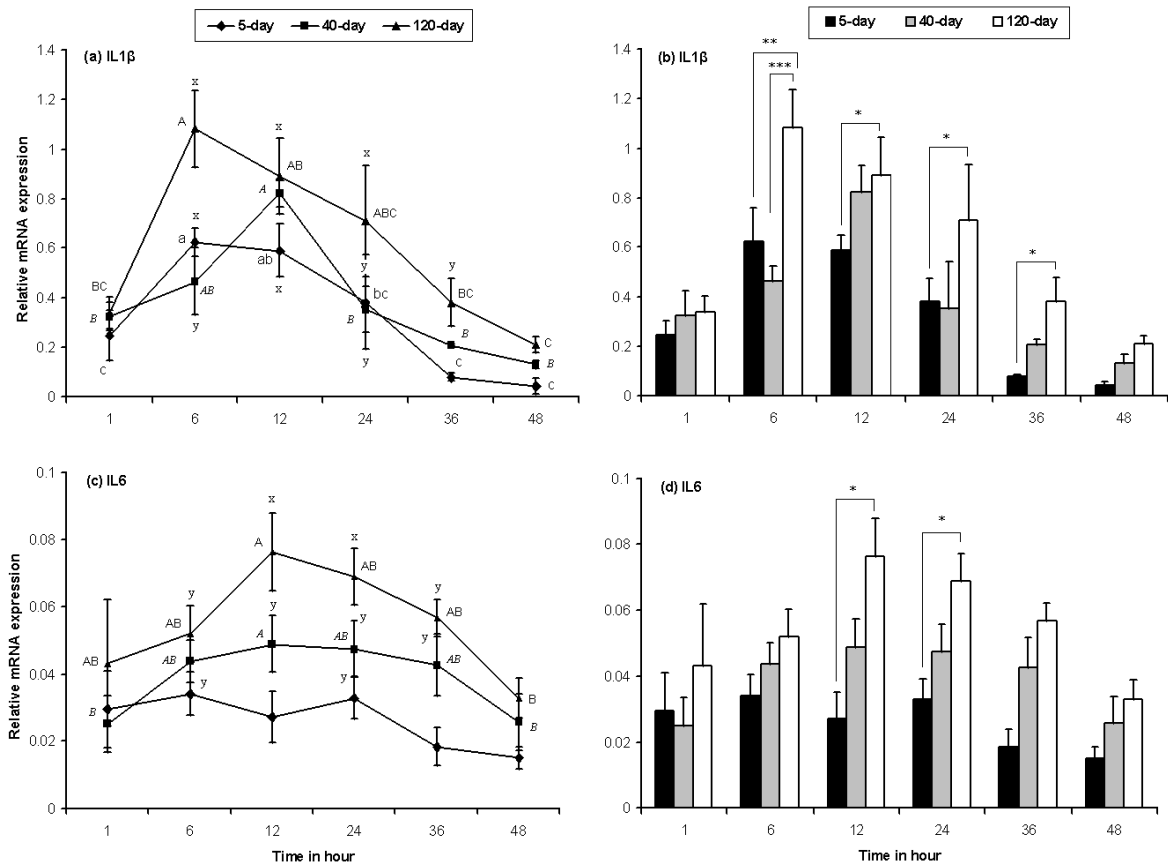


Figure 3. Expression levels of proinflammatory cytokines mRNA by LPS-stimulated alveolar macrophages relative to that of untreated cells. Alveolar macrophages (2×10^6 cells/ml/well) from 5-day-old newborn piglets, 40-day-old post-weaned piglets and 120-day-old young pigs were incubated with LPS ($10 \mu\text{g/ml}$). After stimulation for 1, 6, 12, 24, 36, and 48 h, the cells were harvested for quantification of cytokines mRNA by qRT-PCR. Data shown is an average of three pigs of each age group \pm standard error. Time-dependent expression, (a) IL-1 β and (c) IL-6. Values with different capital, capital italic and small letters denote a statistical difference among time points within 120 days, 40 days and 5 days old animals, respectively ($P < 0.05$). The letter x ($P < 0.001$) and y ($P < 0.05$) indicate significant differences between LPS-treated and untreated cells at each time point. Age-related changes, (b) IL-1 β and (d) IL-6. Asterisks indicate significant differences among age groups at each time point. *** $P < 0.0001$, ** $P < 0.001$, and * $P < 0.05$.

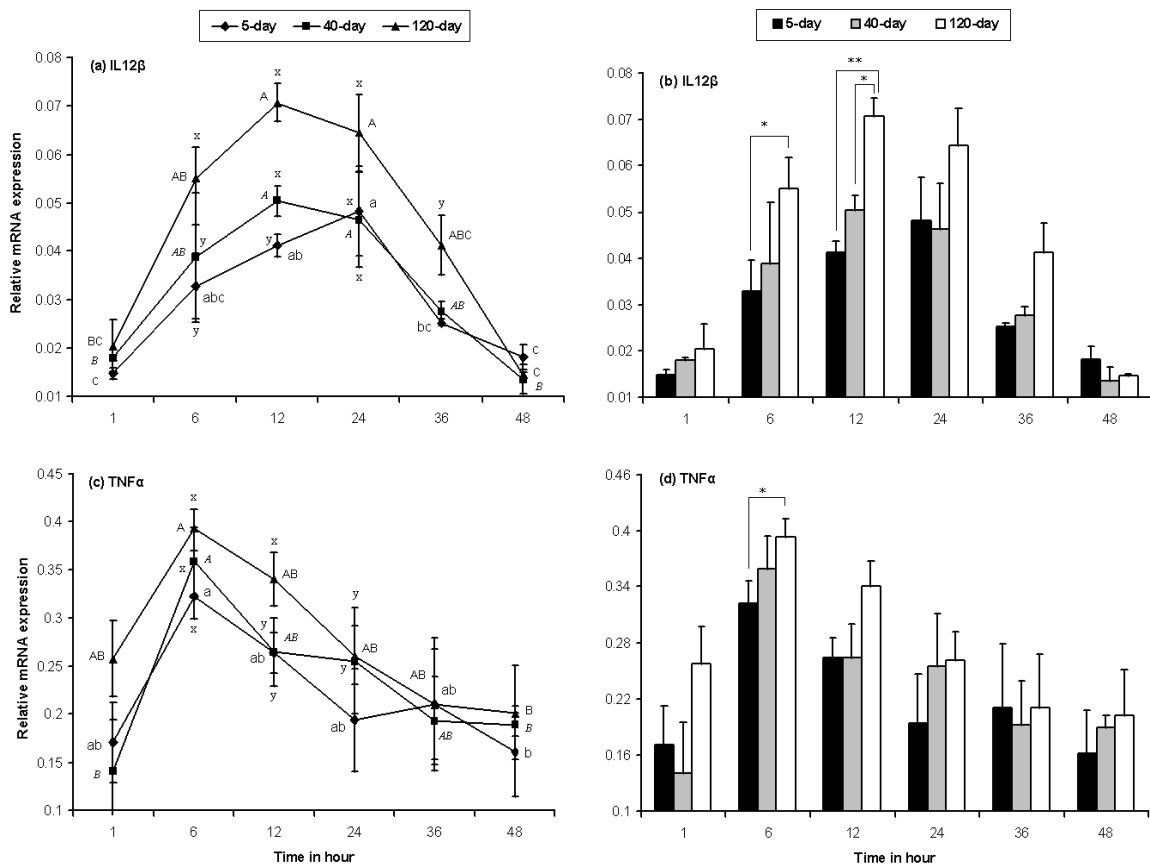


Figure 4. Expression levels of IL12 β and TNF α were quantified using the same samples presented in Figure 3. Data shown is an average of three pigs \pm standard error. Kinetics expression, (a) IL-12 β , and (c) TNF- α . Values with different capital, capital italic and small letters denote a statistical difference among time points within 120 days, 40 days and 5 days old animals, respectively ($P < 0.05$). The letter x ($P < 0.001$) and y ($P < 0.05$) indicate significant differences between LPS-treated and un-treated cells at each time point. Age-related changes, (b) IL-12 β , and (d) TNF- α . Asterisks indicate significant differences among age groups at each time point. ** $P < 0.001$, and * $P < 0.05$.

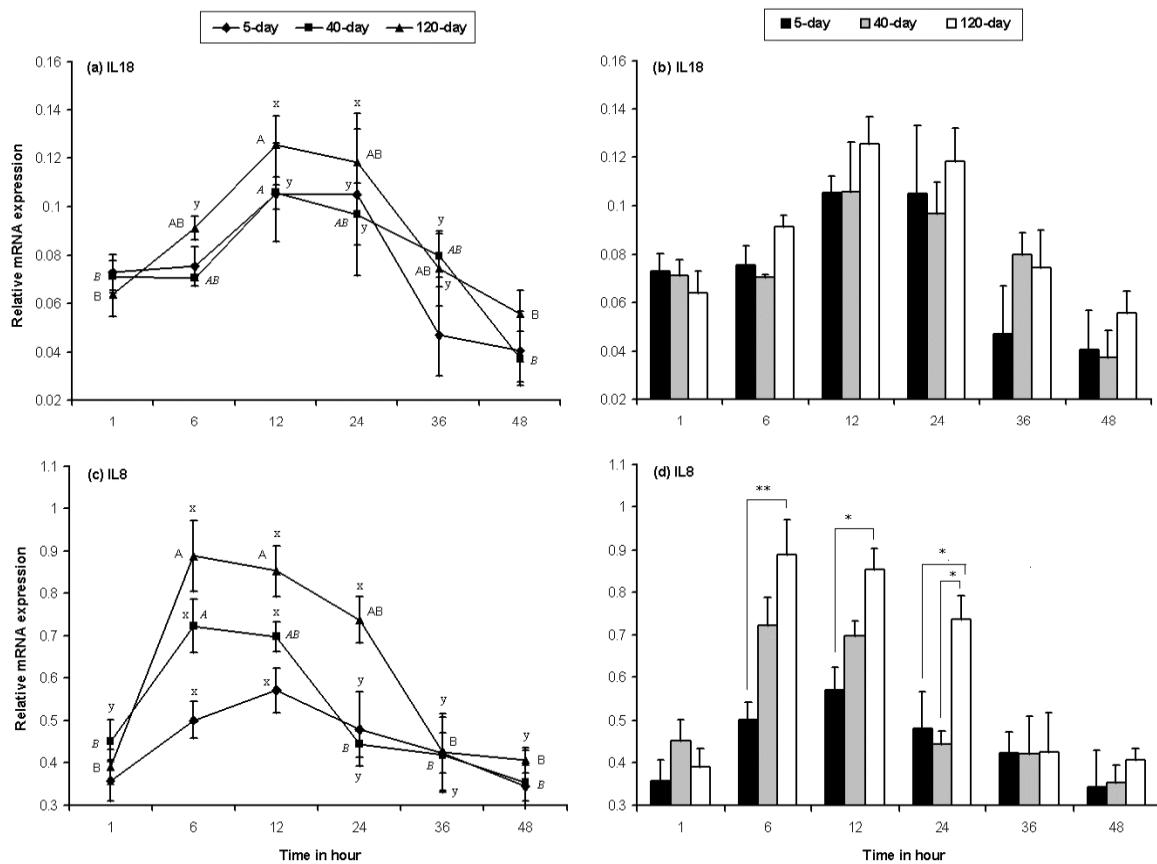


Figure 5. Expression levels of IL18 and IL8 were quantified using the same samples presented in Figure 3. Data shown is an average of three pigs \pm standard error. Kinetics expression, (a) IL-18 and (c) IL-8. Values with different capital, capital italic and small letters denote a statistical difference among time points within 120 days, 40 days and 5 days old animals, respectively ($P < 0.05$). The letter x ($P < 0.001$) and y ($P < 0.05$) indicate significant differences between LPS-treated and un-treated cells at each time point. Age-related changes, (d) IL-8. Asterisks indicate significant differences among age groups at each time point. ** $P < 0.001$, and * $P < 0.05$.

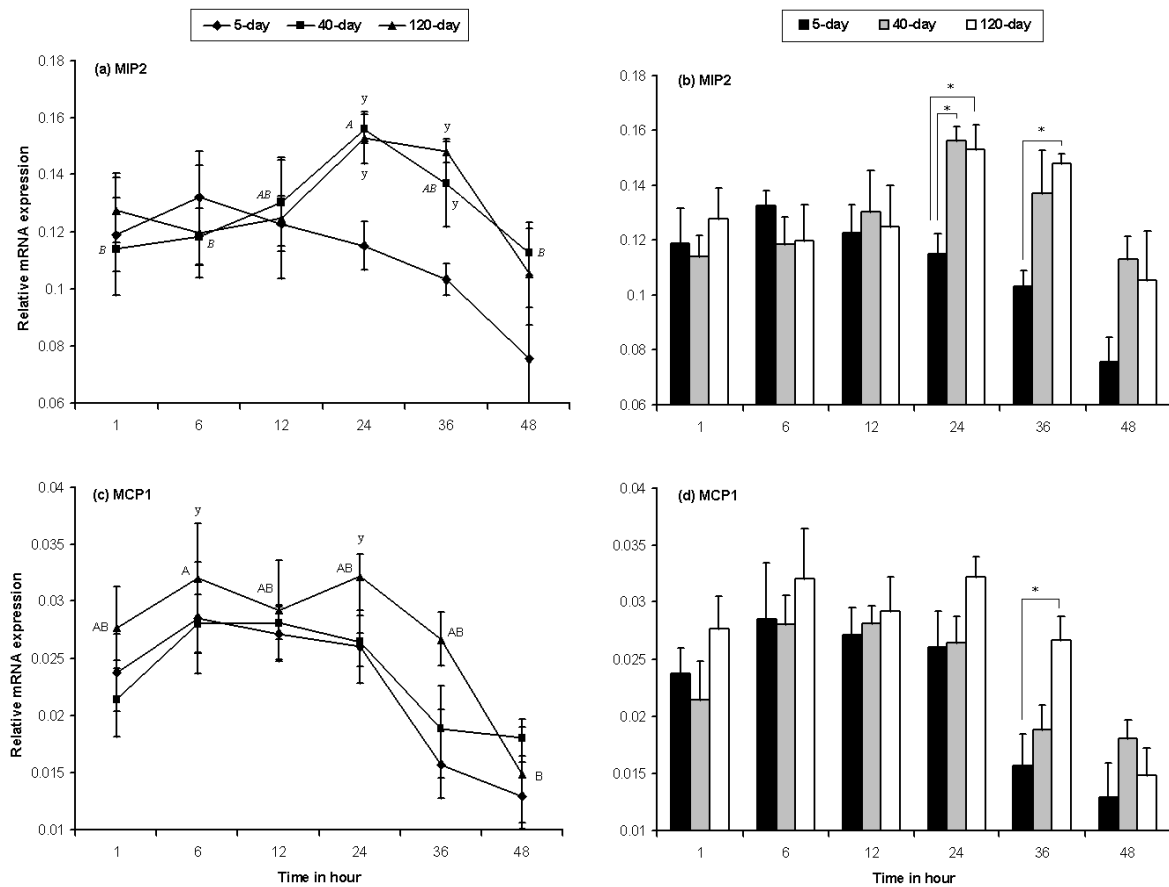


Figure 6. Expression levels of MIP2 and MCP1 were quantified using the same samples presented in Figure 3. Data shown is an average of three pigs \pm standard error. Kinetics expression, (a) MIP-2 and (c) MCP-1. Values with different capital and capital italic letters denote a statistical difference among time points within 120 days, 40 days and 5 days old animals, respectively ($P < 0.05$). The letter x ($P < 0.001$) and y ($P < 0.05$) indicate significant differences between LPS-treated and un-treated cells at each time point. Age-related changes, (b) MIP-2 and (d) MCP-1. Asterisks indicate significant differences among age groups at each time point. * $P < 0.05$.

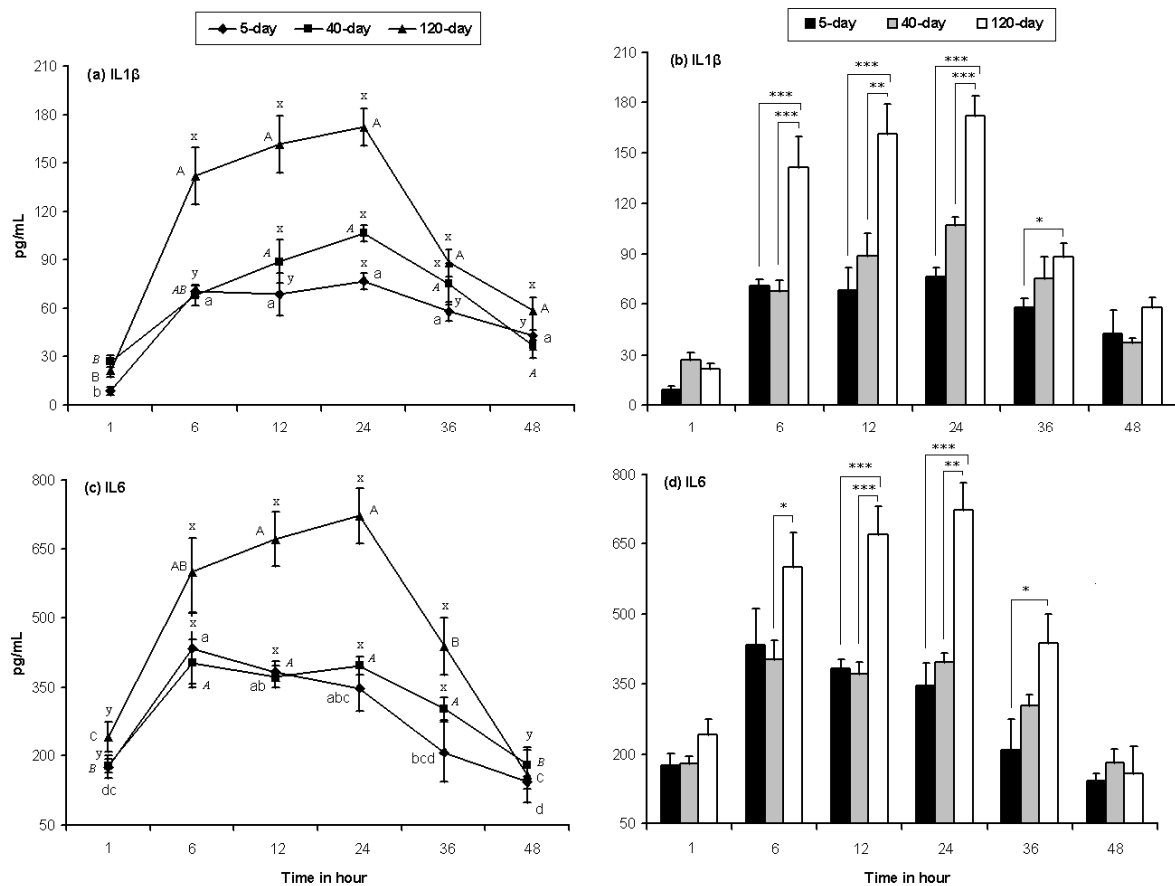


Fig. 7. Concentration levels of pro-inflammatory cytokines protein by LPS-stimulated alveolar macrophages relative to that of un-treated cells. Alveolar macrophages (2×10^6 cells/ml/well) from 5-day-old newborn piglets, 40-day-old post-weaned piglets and 120-day-old young pigs were incubated with LPS ($10 \mu\text{g/ml}$). After stimulation for 1, 6, 12, 24, 36, and 48 h, the supernatant fluids were collected for measurement of cytokines protein levels by ELISA. Data shown is an average of three pigs of each age group \pm standard error. Kinetics concentrations, (a) IL-1 β and (c) IL-6. Values with different capital, capital italic and small letters denote a statistical difference among time points within 120 days, 40 days and 5 days old animals, respectively ($P < 0.05$). The letter x ($P < 0.001$) and y ($P < 0.05$) indicate significant differences between LPS-stimulated and un-stimulated cells at each time point. Age-related changes, (b) IL-1 β and (d) IL-6. Asterisks indicate significant differences among age groups at each time point. *** $P < 0.0001$, ** $P < 0.001$, and * $P < 0.05$.

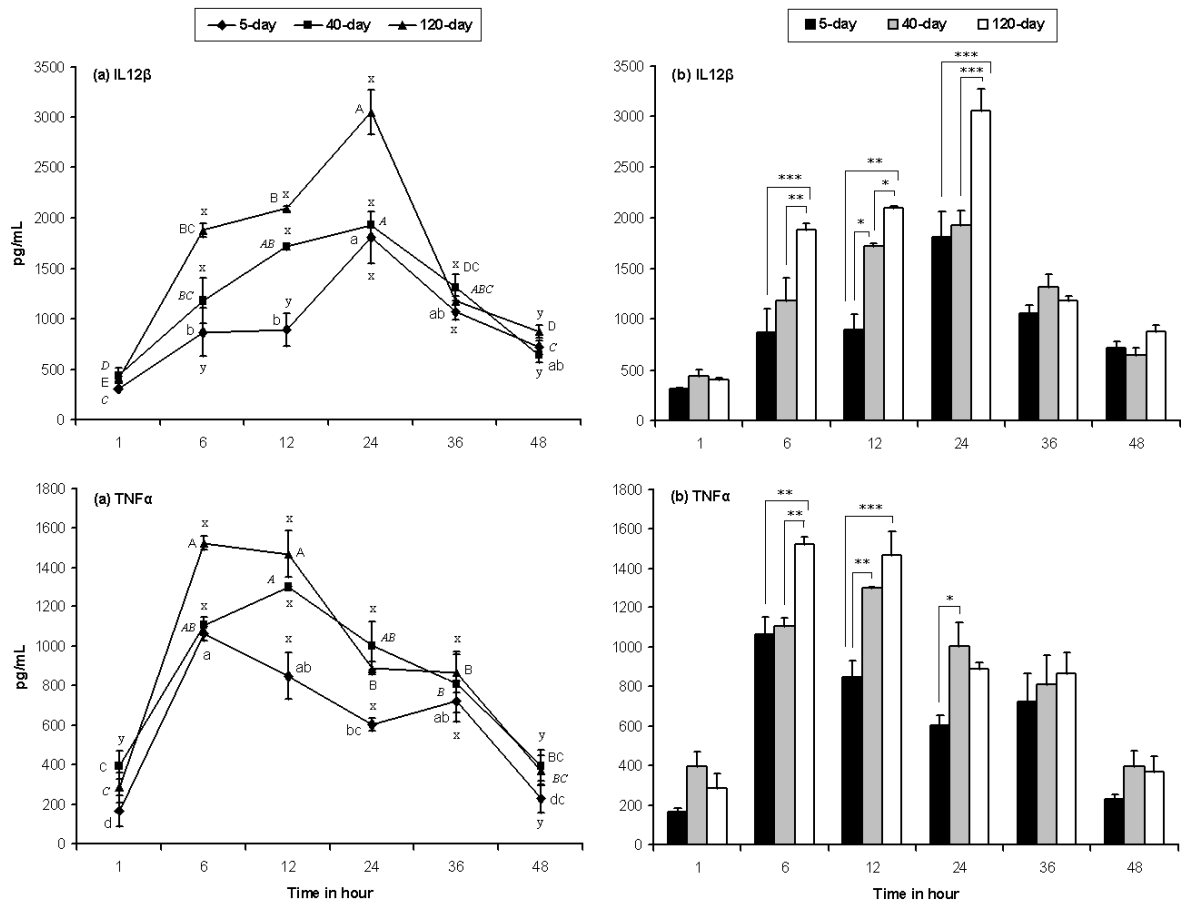


Fig. 8. Concentrations of cytokines protein IL-12 β and TNF- α was assayed using the same samples presented in Fig. 7. Data shown is an average of three pigs \pm standard error. Kinetics concentration, (a) IL-1 β and (c) IL-6. Values with different capital, capital italic and small letters denote a statistical difference among time points within 120 days, 40 days and 5 days old animals, respectively ($P < 0.05$). The letter x ($P < 0.001$) and y ($P < 0.05$) indicate significant differences between LPS-treated and un-treated cells at each time point. Age-related changes, (b) IL-1 β and (d) IL-6. Asterisks indicate significant differences among age groups at each time point. *** $P < 0.0001$, ** $P < 0.001$, and * $P < 0.05$.

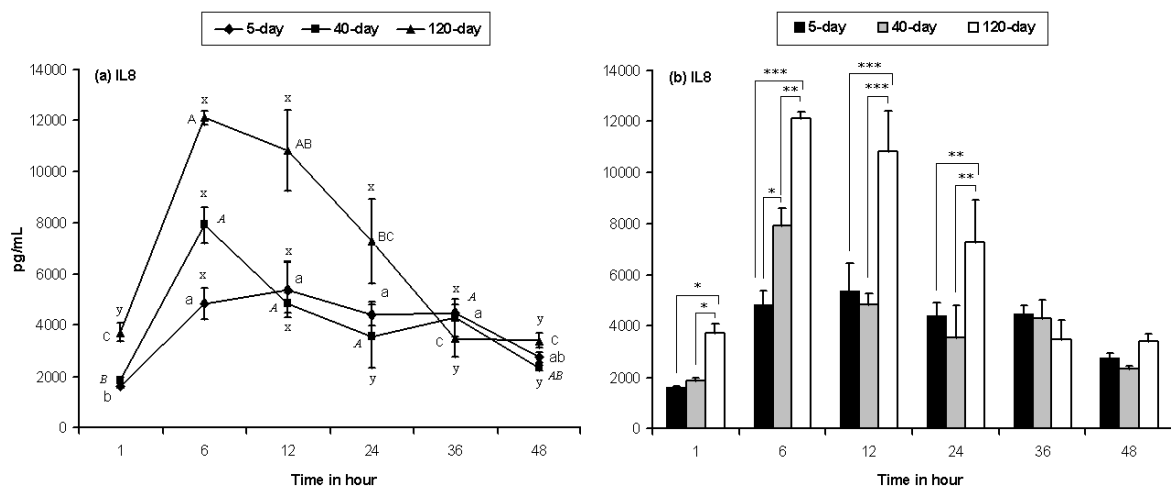
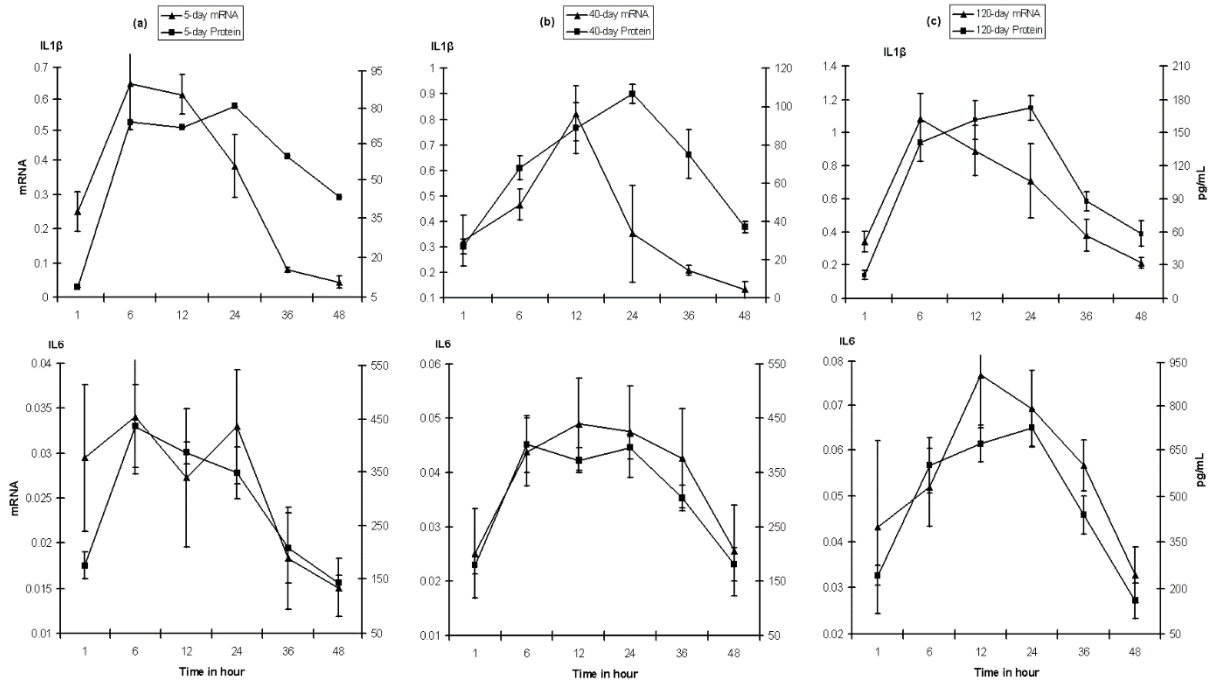
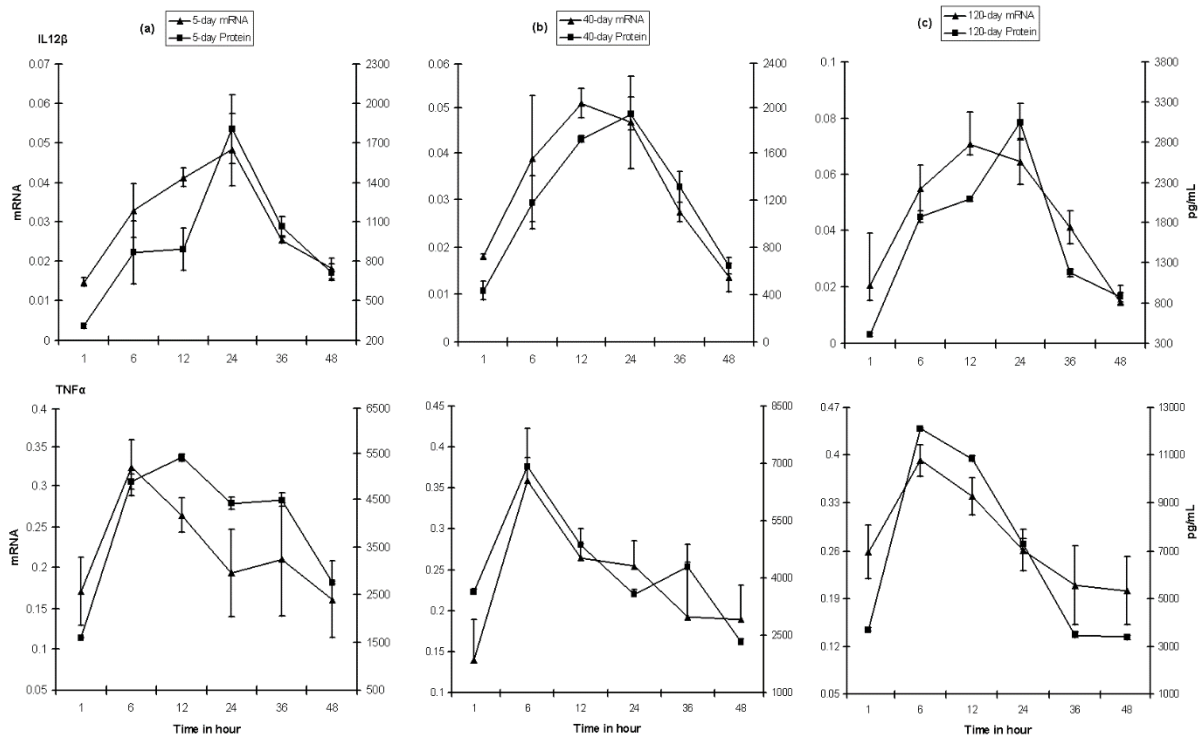


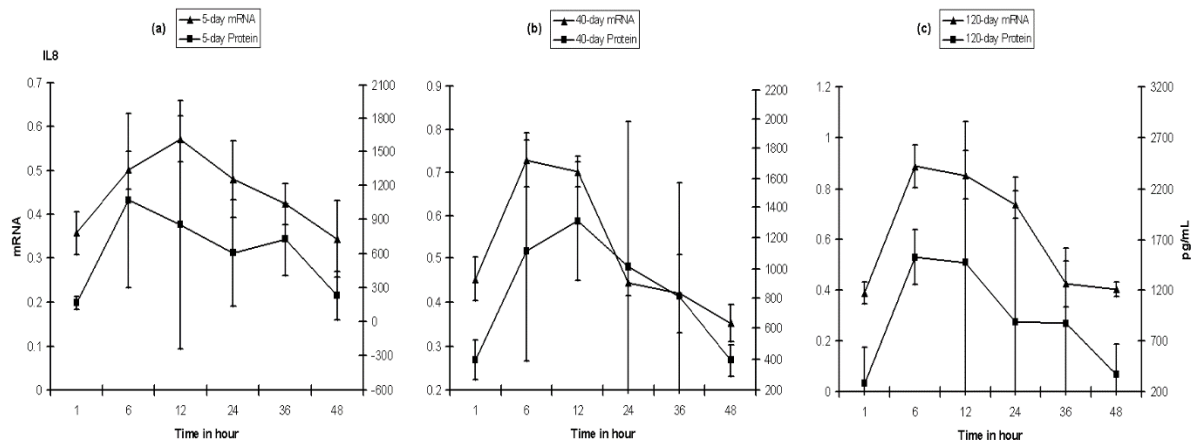
Figure 9. Concentration of chemokine IL-8 was assayed using the same samples presented in Figure 7. Data shown is an average of three pigs \pm standard error. Kinetics concentration, (a) IL-8. Values with different capital, capital italic and small letters denote a statistical difference among time points within 120 days, 40 days and 5 days old animals, respectively ($P < 0.05$). The letter x ($P < 0.001$) and y ($P < 0.05$) indicate significant differences between LPS-treated and un-treated cells at each time point. Age-related changes, (b) IL-8. Asterisks indicate significant differences among age groups at each time point. *** $P < 0.0001$, ** $P < 0.001$, and * $P < 0.05$.



Supplementary Figure 1. Kinetics profiles of the curves for IL-1 β and IL-6 mRNA expression in comparison with secreted IL-1 β and IL-6 mRNA, respectively. (a) 5 days, (b) 40 days, and (c) 120 days. Alveolar macrophages were isolated from pigs and incubated with LPS. After stimulation for 1, 6, 12, 24, 36, and 48 h, the cells and supernatants were collected for assessment of mRNA and protein by qRT-PCR and ELISA, respectively. The results are the mean of three pigs \pm standard error.



Supplementary Figure 2. Kinetics profiles of the curves for IL-12 β and TNF- α mRNA expression in comparison with secreted IL-12 β and TNF- α mRNA, respectively. (a) 5 days, (b) 40 days, and (c) 120 days. Alveolar macrophages were isolated from pigs and incubated with LPS. After stimulation for 1, 6, 12, 24, 36, and 48 h, the cells and supernatants were collected for assessment of mRNA and protein by qRT-PCR and ELISA, respectively. The results are the mean of three pigs \pm standard error.



Supplementary Figure 3. Kinetics profiles of the curves for chemokine IL-8 expression in comparison with secreted IL-8 mRNA. (a) 5 days, (b) 40 days, and (c) 120 days. Alveolar macrophages were isolated from pigs and incubated with LPS. After stimulation for 1, 6, 12, 24, 36, and 48 h, the cells and supernatants were collected for assessment of mRNA and protein by qRT-PCR and ELISA, respectively. The results are the mean of three pigs \pm standard error.

Chapter 3. (Published in: *The Veterinary Journal*, 2013, 198: 245-251)

Age-associated differential production of IFN- γ , IL-10 and GM-CSF by porcine alveolar macrophages in response to lipopolysaccharide

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Article type and status: research and new

Keywords: Alveolar macrophage; Lipopolysaccharide; Cytokine; Piglet; Immune system.

Abstract

The aim of the present study was to investigate the age-related production variation of T helper (Th)-type cytokines (IL-2, IL-4, IFN- γ and IL-10), granulocyte macrophage-colony stimulating factor (GM-CSF) and nitric oxide (NO) by lipopolysaccharide (LPS)-stimulated porcine alveolar macrophages (AMs) in a time-dependent manner. For this purpose, AMs were isolated from 5-days (newborn), 40-days (post-weaned) and 120-days (young) old pigs. Cells were incubated for 24 h in the absence or presence of increasing concentrations of LPS (0.0, 0.01, 1.0, 5.0 and 10.0 $\mu\text{g/ml}$). IL-10, IFN- γ and GM-CSF mRNA expression was upregulated in a dose-dependent manner for all age groups ($P < 0.05$). Age-related differences included a significantly increased IL-10 mRNA and protein production in newborn piglets compared to post-weaned and young pigs. IL-10 production pattern was similar with a higher peak between 12-36 h post-induction in all age groups. In contrast, IFN- γ mRNA and protein level was significantly elevated in young pigs 12 h and 24 h post-induction, respectively, while the time course production of IFN- γ was mostly consistent in newborn and post-weaned piglets. GM-CSF mRNA expression was significantly lower in newborn piglets than in post-weaned and young pigs. The kinetic GM-CSF expression peaked at 12 h in young and post-weaned pigs and at 24 h in newborn piglets. IL-4 mRNA levels were very low and no apparent change of IL-2 expression was observed following LPS stimulation in all age groups. Only very low levels of NO were detected in the cell supernatants of young pigs. Collectively, these studies suggest age-related differences in time-dependent production of IL-10, IFN- γ and GM-CSF by porcine AMs with potential immunoregulatory consequences to be explored further.

Introduction

The innate immune response plays a critical role in the host defence system. In general, neonates have an immature immune system compared to adults. The immune response has been associated with age and functional maturity of the host system that may lead to susceptibility to infectious diseases. Neonatal piglets are highly susceptible to infectious diseases, especially the respiratory disease complex (Cutler 2006). Alveolar macrophages (AMs) are specialized macrophages that have a critical role in pulmonary

host defence against invading pathogens and surfactant homeostasis (Pabst 1996). AMs express various inflammatory cytokine genes in response to respiratory diseases (Murtaugh et al. 1996).

Cytokines are important mediators of immune and inflammatory responses in humans and animals (McWilliam et al. 1996). They regulate immunity at low concentrations and interact with each other to keep the homeostasis in many physiological responses (Balkwill and Burke 1989). They are categorized into T helper 1 (Th1) and T helper 2 (Th2)-type cytokines. The Th1 type cytokines are represented by interleukin (IL)-2, interferon gamma (IFN- γ) and lymphokine, whereas IL-4, IL-5 and IL-10 belong to Th2 type cytokines (Hernandez et al. 2001; McGuirk and Mills 2002). The production and the imbalance of Th1 and Th2-type cytokines by immune cells is associated with disease susceptibility or pathogenesis. Th1 and Th2-type cytokines are produced by T cells as well as other immune cells. In various in vitro studies, Th1 and Th2-type cytokine transcripts were detected in concanavalin A (ConA)- and lipopolysaccharide (LPS)-stimulated porcine spleen cells, peripheral blood mononuclear cells (PBMC) and AMs (Choi et al. 2002a; Choi et al. 2002b). The kinetics of cytokine expression responses to LPS and phytohaemagglutinin (PHA) in lymphocytes derived from lymph nodes was also determined in pigs (Reddy et al. 2000). In addition, the age-related changes in cytokine expression were observed in plasma, lymphoid organs and peripheral blood phagocytes of pigs (Feng et al. 2003; Mikami et al. 2002; Moya 2007). Recently, we have reported an age-dependent ability of porcine AMs to produce pro-inflammatory cytokines (Islam et al. 2012a; Islam et al. 2012b). To our knowledge, the extent of age-related differential production of Th-type cytokines (IL-2, IL-4, IFN- γ and IL-10) produced by AMs has not been investigated in pigs.

Granulocyte macrophage-colony stimulating factor (GM-CSF) regulates diverse functions of AMs critical to surfactant homeostasis and innate pulmonary immune response (Chen et al. 1988; Shibata et al. 2001). GM-CSF is produced by different cell types, including macrophages and lymphocytes (Cho et al. 2003; Choi et al. 2002b; Metcalf and Nicola 1984). Macrophages are also involved in the innate immune response through the production of nitric oxide (NO) and cytokines (Dempsey et al. 2003). Functional maturity and production of NO by AMs is beneficial in killing microorganisms entering the lung. However, relatively little information exist in the

age-associated variation of GM-CSF gene expression and NO production by porcine AMs in response to LPS.

To gain insight into the role of cellular pulmonary immune system in response to LPS, we incubated and propagated in vitro AMs from 5-days (newborn), 40-days (post-weaned) and 120-days (young) old pigs. The objective of the study was to investigate the age-related production patterns of Th-type cytokines, GM-CSF and NO in a time-dependent manner following exposure to different concentration of LPS.

Materials and methods

Animals

Nine clinically healthy German Landrace pigs (male) were used for isolation of AMs. The pigs were conventionally housed and euthanised at the following ages for AM isolation: 5 days (newborn piglets, $n = 3$), 40 days (post-weaned piglets, $n = 3$) and 120 days (young, $n = 3$). All animals were obtained from the teaching and research station of Frankenforst, University of Bonn, Germany. After birth, piglets were housed with the sow for 4 weeks. After weaning, six piglets were selected randomly and transferred to a different pen with ad libitum food and water available. No substrate was provided in the pen and no medication was administered to the piglets in this study. The animals had no history of respiratory diseases. The experiments were approved and conducted according to the institutional guidelines and animal husbandry regulations of Germany (ZDS 2003).

Isolation of alveolar macrophages

AMs were obtained by bronchoalveolar lavage (BAL) as previously described (Islam et al. 2012a). BAL fluids from three animals of each age group were collected in separate tubes. The lavage fluid was passed through a layer of sterile gauze to remove gross mucus and then centrifuged at 4°C for 10 min at 400×g to separate cells from fluid. The cell pellet was washed twice in calcium-magnesium free Dulbecco's phosphate-buffered saline (D-PBS; pH 7.4; Sigma-Aldrich) before final resuspension with RPMI-1640 media (Sigma-Aldrich).

Identification of bronchoalveolar lavage cells

BAL cells were identified as previously described (Islam et al. 2012b). The differential cell count was performed on cytopins stained with a modified commercially available Giemsa stain (Reastain Quick-Diff kit, REAGENA). Overall, AM purity was 80-93% irrespective of age and other cells were mostly polymorphonuclear cells (PMNs) and lymphocytes. The total BAL cell count was obtained using a haemocytometer. Cell viability was determined using the Trypan blue dye exclusion method. The percentage of viable AMs was > 98 % in all cases.

In vitro stimulation of cells with LPS

AM culture was performed as previously described (Islam et al. 2012b). The collected BAL cells were resuspended in complete RPMI-1640 medium (Sigma-Aldrich). First, AMs (2×10^6 cells/well) were treated for 24 h in triplicate with 0.0, 0.01, 1.0, 5.0 and 10.0 $\mu\text{g/ml}$ of LPS to determine their response to different LPS concentrations. For kinetics study, 2×10^6 cells were cultured in a final volume of 1 ml medium in each well in ultra-low attachment polystyrene 24-well plate (CellStar). For each time point, adherent macrophages were stimulated in triplicate wells with LPS of *Escherichia coli* 055:B5 (Cat. L-2880, Sigma Aldrich) (10 $\mu\text{g/ml}$). All plates were incubated at 37°C with 5% CO₂ and 95% air atmosphere for 48 h. Cells and supernatants were collected at 0, 6, 12, 24, 36 and 48 h after stimulation and stored immediately at -80°C until use.

RNA extraction and cDNA synthesis

Total RNA was extracted from harvested cells at each sampling time (0, 6, 12, 24, 36 and 48 h) using Pico-Pure RNA isolation kit following the manufacturer's manual (Arcturus, Invitrogen). Details of RNA isolation and cDNA synthesis are provided elsewhere (Islam et al., 2012a). The concentration of total RNA was measured spectrophotometrically by using the NanoDrop ND-8000 instrument (Thermo Scientific). Approximately 1.5 μg of total RNA for each sample was transcribed into cDNA by using SuperScript-II RT kit (Invitrogen) for RT-qPCR analysis.

Quantification of cytokine mRNA

Quantitative RT-PCR analysis was performed as previously described (Islam et al., 2012a). Briefly, 9-fold serial dilution of plasmids DNA were prepared and used as

template for the generation of the standard curve. Experiment was performed by the StepOnePlus qRT-PCR System (Applied Biosystems). Primer sequences were designed using FASTA product of the GenBank mRNA sequences for *Sus scrofa* using Primer3 program (Skaletsky 2000). Two housekeeping genes (*PPIA* and *B2M*) were selected from previously published data (Cinar et al. 2012). The features of the primers used for this study are presented in Table 1. Normalization of data was performed according to the Vandesompele's procedure (Vandesompele et al. 2002).

Measurement of cytokine concentrations

Concentrations of IL-10 and IFN- γ were measured using commercially available specific porcine enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D System). Results are reported as the mean values of duplicate wells of protein concentrations (pg/ml) in the cell culture supernatant.

Nitric oxide assay

The Griess Reagent assay system (Promega) was used to measure nitrite formed by the spontaneous oxidation of NO as previously described (Cho and Chae 2003). Briefly, 50 μ L of cell supernatant was added to each well of the 96-well microplate and incubated with 50 μ L of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) at room temperature for 5 to 10 min in darkness. Then, 50 μ L of 0.1% N-1-naphthylethylenediamine dihydrochloride in water was added to each well and incubated at room temperature for 5 to 10 min in darkness. The optical density was measured at 530 nm wavelengths. Concentrations were calculated from a standard sodium nitrite curve. All samples were analyzed in duplicate.

Cell viability assay

In a separate set of experiments, macrophages were cultured as described above and treated with LPS (0.1, 1.0, 5.0 and 10.0 μ g μ g/ml) or medium (control). After 24 h incubation, cells were collected, washed twice with D-PBS and resuspended in RPMI-1640 medium. Cell viability was determined using Trypan blue dye exclusion in duplicate for each concentration group. The results are presented as percentage of viable cells and in general, at least 200 cells per vial were scored for viability.

Statistical analysis

The data were analyzed using the SAS software package v.9.2 (SAS Institute). All values are expressed as means of triplicates data \pm standard error of mean (SEM). The one-way factorial analysis of variance with Tukey's test was used to detect any possible effect of LPS concentration and incubation time on the production of cytokines within the same age group. For the incubation time course study, $P < 0.05$ was considered statistically significant. Differences between age groups were determined using student's *t* test.

Results

Cell viability

There was no significant difference in cell viability between samples treated with a range of LPS concentrations and controls after 24 h incubation. The mean (\pm SEM) percentages of viable cells were 89.4 ± 3.4 , 82.62 ± 6.4 , 84.03 ± 4.07 and 87.25 ± 3.6 for the control, early born piglets, post-weaned piglets and young pig groups, respectively.

LPS concentration response of cytokine mRNA levels

In the first phase of the experiment, the dose-dependent responses to LPS of Th1-type (IL-2 and IFN- γ) and Th2-type cytokines (IL-4 and IL-10), and GM-CSF in AMs of newborn piglets, post-weaned piglets and young pigs were measured after 24 h of incubation. IL-2 and IL-4 mRNA was not detected in AMs following stimulation by the various LPS concentrations (0, 0.1, 1.0, 5.0 and 10.0 $\mu\text{g/ml}$), except for a very low expression of IL-4 in young pigs with the highest dosages (data not shown).

Overall, IFN- γ mRNA was moderately upregulated with the higher LPS concentrations in comparison to untreated cells. IFN- γ mRNA expression at the highest LPS concentration was significantly higher in young pigs and post-weaned piglets compared to newborn piglets (Fig. 2A, $P < 0.05$). The mRNA expression levels of IL-10 and GM-CSF mRNA were significantly increased following stimulation by all LPS concentrations when compared to the controls in the respective age groups (Figs. 2B and C; $P < 0.05$). The expression level of IL-10 mRNA following exposure to LPS at 5.0 and 10.0 $\mu\text{g/ml}$ was significantly higher in newborn piglets than in post-weaned and

young pigs, (Fig. 2B; $P < 0.05$). In contrast, the young pigs showed significantly higher GM-CSF mRNA levels at 5.0 $\mu\text{g/ml}$ of LPS than newborn piglets. (Fig. 2C; $P < 0.05$).

Time and age-dependent LPS effects on cytokine production

In order to examine the time-course effects of LPS on cytokine expression, AMs from 5-days (newborn piglets), 40-days (post-weaned piglets) and 120-days (young) old pigs were incubated for 0, 6, 12, 24, 36 and 48 h with 10.0 $\mu\text{g/ml}$ LPS. In general, production of IFN- γ , IL-10 and GM-CSF was significantly affected by incubation time and the age of animals (Figs. 3, 4 and 5).

IFN- γ production tended to be higher in young pigs than in newborn and post-weaned piglets. The baseline mRNA detection level was very low and protein concentration was undetected (Fig. 3A) irrespective of the age. IFN- γ mRNA expression was significantly elevated in young pigs and post-weaned piglets only at 12 h. In contrast, IFN- γ protein concentration was lower in newborn piglets compared to young pigs only at 24 h (Fig. 3B). Production of IFN- γ was not significantly different between young pigs and post-weaned piglets.

In newborn piglets, IL-10 mRNA and protein levels were highest at the 24 h time point and maintained higher levels up to 48 h. In post-weaned piglets and young pigs, both mRNA and protein levels peaked at the 12 h and 36 h time points, respectively (Figs. 4A and B). LPS induced a significant upregulation of IL-10 mRNA expression in newborn piglets during between 24-36 h time points compared to post-weaned piglets and young pigs (Fig. 4A). In contrast, IL-10 protein release from newborn piglets was significantly higher between 12 and 36 h compared to post-weaned piglets and young pigs (Fig. 4B). No significant difference was observed between young pigs and post-weaned piglets.

GM-CSF mRNA was significantly upregulated at 6 h and up to 36 h, before returning to baseline levels by 48 h in all age groups. GM-CSF mRNA expression was highest at 12 h in young and post-weaned pigs and at 24 h in newborn piglets. In young animals, GM-CSF gene expression was higher during the 12-36 h period than in newborn and post-weaned piglets. In addition, GM-CSF mRNA levels were significantly higher at 12 h in post-weaned piglets compared to newborn piglets (Fig. 5).

Comparison between mRNA and protein levels

To determine whether kinetic changes in cytokine mRNA expression were paralleled by changes in corresponding protein secretion, LPS-activated macrophage culture supernatants were assayed for IFN- γ and IL-10 concentrations. In newborn piglets, the IFN- γ mRNA expression and protein secretion curved peaked at 12 h and 24 h, respectively, and thereafter a discrepancy was observed (Appendix A, Supplementary Fig. 1). In post-weaned piglets, IFN- γ mRNA and protein secretion levels were highest at 12 h and 36 h, respectively, and decreased thereafter (Appendix A, Supplementary Fig. 1). The expression pattern of IL-10 mRNA and protein was similar with a highest peak at 24 h in newborn piglets, between 12-24 h in post-weaned piglets, and at 12 h in young pigs (Appendix A, Supplementary Fig. 2).

NO assay

There was no significant difference in NO production between the age groups and controls (data not shown). Very low levels of NO ($< 2.5 \mu\text{M}$) were detected in the culture supernatants of AMs in young pigs (data not shown).

Discussion

Little is known about the age-related production changes of Th-type cytokines, GM-CSF and NO production in porcine AMs following stimulation by LPS. In this study, we showed that there are differences in cytokine response that are age- and time-dependent and also affected by age. Notably, there was no evidence that the LPS concentrations used affected cell viability.

Our results showed that IFN- γ , IL-10 and GM-CSF mRNA levels were upregulated by all concentrations of LPS in all age groups in a time-dependent manner compared to non-stimulated cells. In contrast, IL-2 and IL-4 mRNA expression was not detected in our cell samples. This is consistent with other published data (Choi et al. 2002b). The absence or low level of IL-4 mRNA expression in these cells may be due to the low stability of IL-4 mRNA in AMs as demonstrated in Th1 cells compared with Th2 cells (Butler et al. 2002). It is also possible that IL-4 and IL-2 mRNA was degraded during mRNA isolation, although this appears unlikely given the detection of other mRNA types. However, since IL-2 and IL-4 are typically produced from T lymphocytes (de

Groot et al., 2005) and have not been detected in leukocytes of efferent lymph from stimulated lymph nodes in pigs (Reddy et al. 2000), it is more likely that AMs do not express at significant levels these two cytokines.

Our previous study showed that various concentrations of LPS were able to induce production of pro-inflammatory cytokines in porcine AMs (Islam et al. 2012a). However, in the present study, increasing the concentration of LPS did not result in changes in IL-10 and GM-CSF mRNA levels, except for a slight, but non-significant increase at 10.0 µg/ml (as compared to 0.1 µg/ml). The lack of dose response suggests that a low concentration of LPS or a small number of pathogens is enough to elicit optimal production of cytokines by porcine AMs.

In the current study, baseline IFN- γ mRNA levels were very low and the IFN- γ protein was undetected in all age groups. Following stimulation, IFN- γ mRNA levels were low in newborn piglets and only slightly higher in post-weaned and young pigs. This is somewhat consistent to previous studies (Choi et al. 2002a; La Pine et al. 2003), although another study could not demonstrate IFN- γ upregulation in LPS-stimulated porcine AMs (Choi et al. 2002b). It has been shown that foals produce relatively less IFN- γ at birth, both by peripheral blood mononuclear cells (PBMCs) and pulmonary cells (Breathnach et al. 2006), which is consistent with our findings in pigs. Our results suggest that LPS-stimulated porcine AMs are only producing low levels of IFN- γ . This is in contrast to IL-10 in newborn piglets, but not in post-weaned piglets and young pigs. The reasons for these age-associated differences in IL-10 production could be due to functional differences of AMs. Similar differences have been noted in horses, where activation of PBMC results in more robust production of IL-10 in neonatal foals compared with adult horses (Sponseller et al. 2009). IL-10 downregulates many of the effector functions of macrophages, including the release of reactive nitrogen and oxygen intermediates (Cenci et al. 1993; Kuga et al. 1996; Wu et al. 1993). IL-10 is a pleiotropic cytokine with a broad spectrum of immunosuppressive and anti-inflammatory effects, which is produced predominantly by macrophages (Moore et al. 2001). Hence, it can be speculated that elevated levels of IL-10 could suppress the production of pro-inflammatory mediators, particularly in early born piglets which may have an impact on the cellular pulmonary immune system.

The GM-CSF gene expression level was significantly decreased in a time-dependent manner in newborn piglets compared to young pigs and post-weaned piglets. GM-CSF is produced by different cell types, including macrophages and lymphocytes (Metcalf and Nicola 1984) and is expressed by porcine AMs and spleen cells (Cho et al. 2003; Choi et al. 2002b). GM-CSF regulates diverse functions of AMs critical to surfactant homeostasis and the innate pulmonary immune response in mice (Chen et al. 1988; Shibata et al. 2001), hence, it may play the same role in swine AMs. Overall these findings provide some evidence of the functional immaturity of neonatal AMs. Although these in vitro data may not fully reflect the in vivo situation, they suggest that differences in cytokine production may play a role for the increased susceptibility of neonatal animals. Whether and to what extent susceptibility to disease is related to variation in immune response parameters in pigs is at present unknown (Stear et al. 2001).

NO is involved in a wide range of physiologic and pathologic processes in mammals and its production by macrophages is fundamental for immune defence (MacMicking et al. 1997). In the present study, production of NO by AMs was not detected in any age groups, except for very low levels in young pigs. Our results confirmed previous studies, which also failed to induce NO production in porcine AMs, Kupffer cells (KC) and PBMC following stimulation with LPS and IFN- γ (Akunda et al. 2001; Pampusch et al. 1998; Zelnickova et al. 2008). This is in contrast to findings in a rat model (Lee et al. 2001; Tasat et al. 2003), and in LPS-activated rat and bovine AMs (Zelnickova et al. 2008). These species differences in NO production by AMs are not understood.

There were several limitations to the current study. Firstly, this is an in vitro study and thus it is unclear if these findings reflect the in vivo situation. Secondly, only few individuals were used per group and only one isolation was conducted, which may not reflect the real population. Thirdly, a limited concentration range of LPS was used, which may limit the immunostimulatory effects on AMs. Finally, we used a single breed and therefore our results may not represent the inflammatory response of other pig breeds, as immune capacity in pigs is known to be influenced by genetic factors (Edfors-Lilja et al. 1998; Uddin et al. 2011).

Conclusion

The current results showed time-dependent differences in cytokine production by porcine AMs between age groups, which may explain the increased susceptibility of newborn piglets to respiratory pathogens. Future investigations to evaluate the in vivo relevance of these results are warranted.

Acknowledgement

The authors are indebted to Research Station “Frankenforst” at University of Bonn for providing pigs. Authors are also thankful to Ms. Nadine Leyer and Ms. Birgit Koch-Fabritius for their technical assistance during the experiments.

References

- Akunda JK, Ahrens FA, Kramer TT (2001): Evaluation of phagocytosis, bactericidal activity, and production of superoxide anion, nitric oxide, and tumor necrosis factor-alpha in Kupffer cells of neonatal pigs. *Am. J. Vet. Res.* 62, 1040-1045.
- Balkwill FR, Burke F (1989): The cytokine network. *Immunol. Today* 10, 299-304.
- Breathnach CC, Sturgill-Wright T, Stiltner JL, Adams AA, Lunn DP, Horohov DW (2006): Foals are interferon gamma-deficient at birth. *Vet. Immunol. Immunopathol.* 112, 199-209.
- Butler NS, Monick MM, Yarovinsky TO, Powers LS, Hunninghake GW (2002): Altered IL-4 mRNA stability correlates with Th1 and Th2 bias and susceptibility to hypersensitivity pneumonitis in two inbred strains of mice. *J. Immunol.* 169, 3700-3709.
- Cenci E, Romani L, Mencacci A, Spaccapelo R, Schiaffella E, Puccetti P, Bistoni F (1993): Interleukin-4 and interleukin-10 inhibit nitric oxide-dependent macrophage killing of *Candida albicans*. *Eur. J. Immunol.* 23, 1034-1038.
- Chen BD, Mueller M, Chou TH (1988): Role of granulocyte/macrophage colony-stimulating factor in the regulation of murine alveolar macrophage proliferation and differentiation. *J. Immunol.* 141, 139-144.

- Cho WS, Chae C (2003): In vitro effects of *Actinobacillus pleuropneumoniae* on inducible nitric oxide synthase and cyclooxygenase-2 in porcine alveolar macrophages. *A. J. Vet. Res.* 64, 1514-1518.
- Cho YW, Lee DY, Shin SJ, Shin NR, Kang SG, Choi IS, Yoo HS (2003): Kinetic study of porcine GM-CSF expression in porcine alveolar macrophages and spleen cells. *FEMS Immunol. Med. Microbiol.* 39, 61-67.
- Choi IS, Collisson EW, Maheswaran SK, Yoo HS (2002a): Evaluation of cytokine gene expression in porcine spleen cells, peripheral blood mononuclear cells, and alveolar macrophages by competitive RT-PCR. *FEMS Immunol. Med. Microbiol.* 34, 119-126.
- Choi IS, Shin NR, Shin SJ, Lee DY, Cho YW, Yoo HS (2002b): Time course study of cytokine mRNA expression in LPS-stimulated porcine alveolar macrophages. *J. Vet. Sci.* 3, 97-102.
- Cinar MU, Islam MA, Uddin MJ, Tholen E, Tesfaye D, Looft C, Schellander K (2012): Evaluation of suitable reference genes for gene expression studies in porcine alveolar macrophages in response to LPS and LTA. *BMC Res. Notes* 5, 107.
- Cutler RS, Fahy VA, Cronin GM, Soicer EM (2006): Prewaning mortality. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.), *Diseases of swine*. Blackwell publishing, IA, USA, pp. 993-1011.
- de Groot J, Kruijt L, Scholten JW, Boersma WJ, Buist WG, Engel B, van Reenen CG (2005): Age, gender and litter-related variation in T-lymphocyte cytokine production in young pigs. *Immunology* 115, 495-505.
- Dempsey PW, Vaidya SA, Cheng G (2003): The art of war: Innate and adaptive immune responses. *Cell. Mol. Life Sci.* 60, 2604-2621.
- Edfors-Lilja I, Wattrang E, Marklund L, Moller M, Andersson-Eklund L, Andersson L, Fossum C (1998): Mapping quantitative trait loci for immune capacity in the pig. *J. Immunol.* 161, 829-835.
- Feng WH, Tompkins MB, Xu JS, Zhang HX, McCaw MB (2003): Analysis of constitutive cytokine expression by pigs infected in-utero with porcine reproductive and respiratory syndrome virus. *Vet. Immunol. Immunopathol.* 94, 35-45.

- Hernandez J, Garfias Y, Nieto A, Mercado C, Montano LF, Zenteno E (2001): Comparative evaluation of the CD4+CD8+ and CD4+CD8- lymphocytes in the immune response to porcine rubulavirus. *Vet. Immunol. Immunopathol.* 79, 249-259.
- Islam MA, Cinar MU, Uddin MJ, Tholen E, Tesfaye D, Looft C, Schellander K (2012a): Expression of Toll-like receptors and downstream genes in lipopolysaccharide-induced porcine alveolar macrophages. *Vet. Immunol. Immunopathol.* 146, 62-73.
- Islam MA, Uddin MJ, Tholen E, Tesfaye D, Looft C, Schellander K, Cinar MU (2012b): Age-related changes in phagocytic activity and production of pro-inflammatory cytokines by lipopolysaccharide stimulated porcine alveolar macrophages. *Cytokine* 60, 707-717.
- Kuga S, Otsuka T, Niuro H, Nunoi H, Nemoto Y, Nakano T, Ogo T, Umei T, Niho Y (1996): Suppression of superoxide anion production by interleukin-10 is accompanied by a downregulation of the genes for subunit proteins of NADPH oxidase. *Exp. Hematol.* 24, 151-157.
- La Pine TR, Joyner JL, Augustine NH, Kwak SD, Hill HR (2003): Defective production of IL-18 and IL-12 by cord blood mononuclear cells influences the T helper-1 interferon gamma response to group B Streptococci. *Pediatr. Res.* 54, 276-281.
- Lee PT, Holt PG, McWilliam AS (2001): Ontogeny of rat pulmonary alveolar macrophage function: evidence for a selective deficiency in IL-10 and nitric oxide production by newborn alveolar macrophages. *Cytokine* 15, 53-57.
- MacMicking J, Xie QW, Nathan C (1997): Nitric oxide and macrophage function. *Annu. Rev. Immunol.* 15, 323-350.
- McGuirk P, Mills KH (2002): Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases. *Trends Immunol.* 23, 450-455.
- McWilliam AS, Napoli S, Marsh AM, Pemper FL, Nelson DJ, Pimm CL, Stumbles PA, Wells TN, Holt PG (1996): Dendritic cells are recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli. *J. Exp. Med.* 184, 2429-2432.
- Metcalf D, Nicola NA (1984): The regulatory factors controlling murine erythropoiesis in vitro. *Prog. Clin. Biol. Res.* 148, 93-105.

- Mikami O, Muneta Y, Mori Y, Yokomizo Y, Nakajima Y (2002): Expression of proinflammatory cytokine mRNA in the lymphatic organs of adult and neonatal pigs. *Vet. Immunol. Immunopathol.* 90, 203-207.
- Moya SL, Boyle LA, Lynch PB, Arkins S (2007): Age-related changes in proinflammatory cytokines, acute phase proteins and cortisol concentrations in neonatal piglets. *Neonatology* 91, 44-48.
- Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A (2001): Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 19, 683-765.
- Murtaugh MP, Baarsch MJ, Zhou Y, Scamurra RW, Lin G (1996): Inflammatory cytokines in animal health and disease. *Vet. Immunol. Immunopathol.* 54, 45-55.
- Pabst R (1996): The respiratory immune system of pigs. *Vet. Immunol. Immunopathol.* 54, 191-195.
- Pampusch MS, Bennaars AM, Harsch S, Murtaugh MP (1998): Inducible nitric oxide synthase expression in porcine immune cells. *Vet. Immunol. Immunopathol.* 61, 279-289.
- Reddy NR, Borgs P, Wilkie BN (2000): Cytokine mRNA expression in leukocytes of efferent lymph from stimulated lymph nodes in pigs. *Vet. Immunol. Immunopathol.* 74, 31-46.
- Shibata Y, Berclaz PY, Chroneos ZC, Yoshida M, Whitsett JA, Trapnell BC (2001): GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1. *Immunity* 15, 557-567.
- Skaletsky SR.a. HJ (2000): Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386.
- Sponseller BA, de Macedo MM, Clark SK, Gallup JM, Jones DE (2009): Activation of peripheral blood monocytes results in more robust production of IL-10 in neonatal foals compared to adult horses. *Vet. Immunol. Immunopathol.* 127, 167-173.
- Stear MJ, Bishop SC, Mallard BA, Raadsma H (2001): The sustainability, feasibility and desirability of breeding livestock for disease resistance. *Res. Vet. Sci.* 71, 1-7.
- Tasat DR, Mancuso R, O'Connor S, Molinari B (2003): Age-dependent change in reactive oxygen species and nitric oxide generation by rat alveolar macrophages. *Aging Cell* 2, 159-164.

- Uddin MJ, Cinar MU, Grosse-Brinkhaus C, Tesfaye D, Tholen E, Juengst H, Looft C, Wimmers K, Phatsara C, Schellander K (2011): Mapping quantitative trait loci for innate immune response in the pig. *Int. J. Immunogenet.* 38, 121-131.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002): Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3, RESEARCH0034.
- Wu J, Cunha FQ, Liew FY, Weiser WY (1993): IL-10 inhibits the synthesis of migration inhibitory factor and migration inhibitory factor-mediated macrophage activation. *J. Immunol.* 151, 4325-4332.
- ZDS (2003): Richtlinie für die Stationsprüfung auf Mastleistung, Schlachtkörperwert und Fleischbeschaffenheit beim Schwein, 10.12.2003.
- Zelnickova P, Matiasovic J, Pavlova B, Kudlackova H, Kovaru F, Faldyna M (2008): Quantitative nitric oxide production by rat, bovine and porcine macrophages. *Nitric Oxide* 19, 36-41.

Table 1. Oligonucleotide sequences designed for the quantitative real-time PCR analysis of mRNA expression of porcine cytokines and two house keeping genes, with the respective annealing temperature, amplicon products (bp), GenBank accession number and references.

Gene	Primer set	Annealing temp. (°C)	Amplicon size (bp)	GenBank accession number
IL-2	F: CTAACCCTTGCACTCATGGCA R: AATTCTGTAGCCTGCTTGGGC	58	185	X56750
IL-4	F: GCGACATCACCTTACAAGAGAT R: CAGCTTCAACACTTTGAGTATTTC	61	328	X68330
IL-10	F: GTGGAGGAGGTGAAGAGTGCC R: GAGGTACAGCAGGGTTTCCCA	60	266	L20001
IFN- γ	F: CAGAGCCAAATTGTCTCCTTCTAC R: TCTCTGGCCTTGGAACATAG	60	285	S63967
GM-CSF	F: CAAGCACTATGAGCAGCACT R: GGTGAAGATCCTGAGTTTGG	55	133	NM_214118.1
PPIA	F: CACAAACGGTTCCTCAGTTT R: TGTCCACAGTCAGCAATGGT	58	171	NM_214353.1
B2M	F: ACTTTTCACACCGCTCCAGT R: CGGATGGAACCCAGATACAT	58	180	NM_213978.1

F: Forward primer; R: Reverse primer; bp: base pair; temp.: temperature

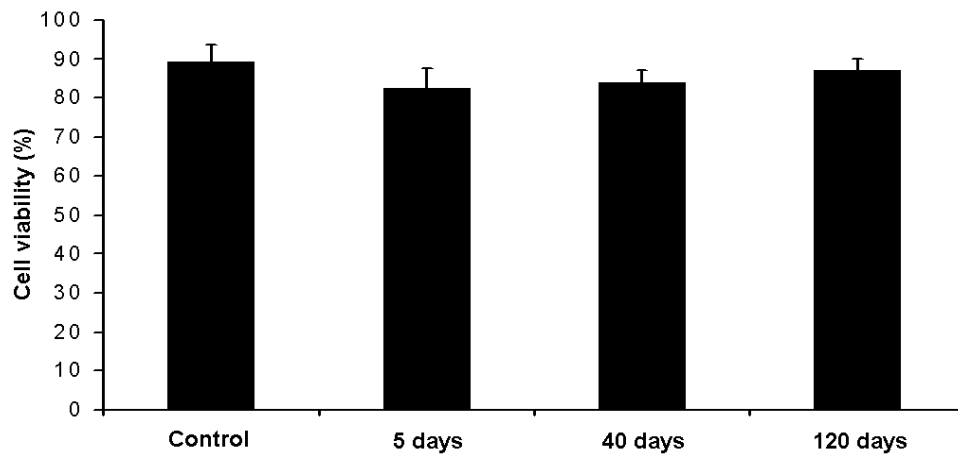


Figure 1. Effects of LPS stimulation on alveolar macrophage cell viability. Macrophages were stimulated with LPS at 10 $\mu\text{g/ml}$ for 24 h. Cell viability was measured by Trypan blue exclusion. The results are expressed as the mean (\pm SEM, $n = 3$) percent cell viability.

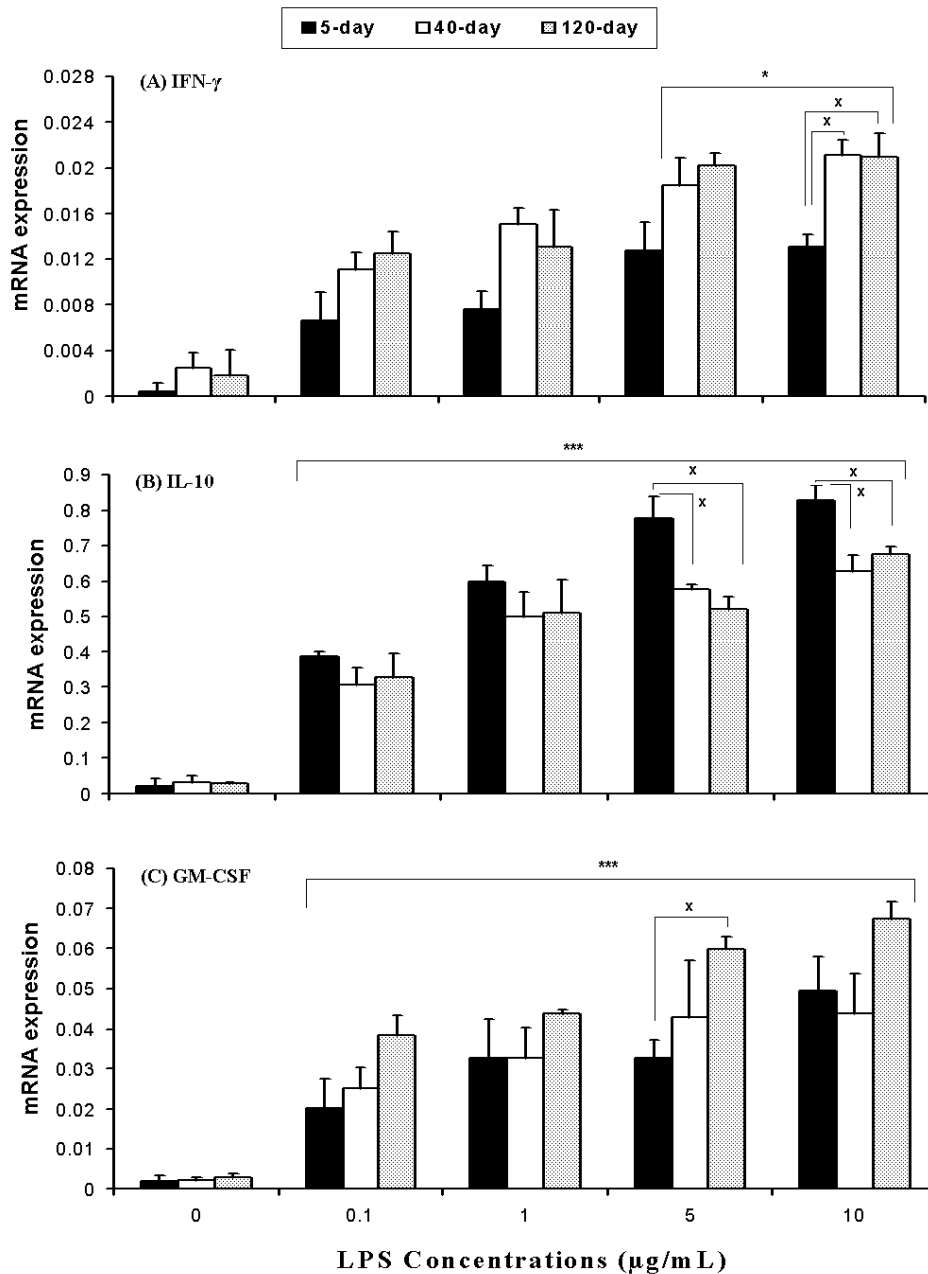


Figure 2. Dose-dependant cytokine mRNA expression was quantified by qRT-PCR in LPS-induced alveolar macrophage cells of 5-day-old newborn piglets, 40-day-old post-weaned piglets, and 120-day-old young pigs. Cells (2×10^6 cells/ml/well) were incubated with concentrations of LPS 0, 0.01, 1, 5, and 10 $\mu\text{g/ml}$ for 24 h. (A) IFN- γ , (B) IL-10, and (C) GM-CSF. Data shown is an average of $n = 3$ biological replicates \pm SEM. Asterisk indicate the significant difference to control (0 $\mu\text{g/ml}$) ($*P < 0.05$ and $***P > 0.001$). The letter x indicate significant differences of mRNA expression between the age groups ($P < 0.05$).

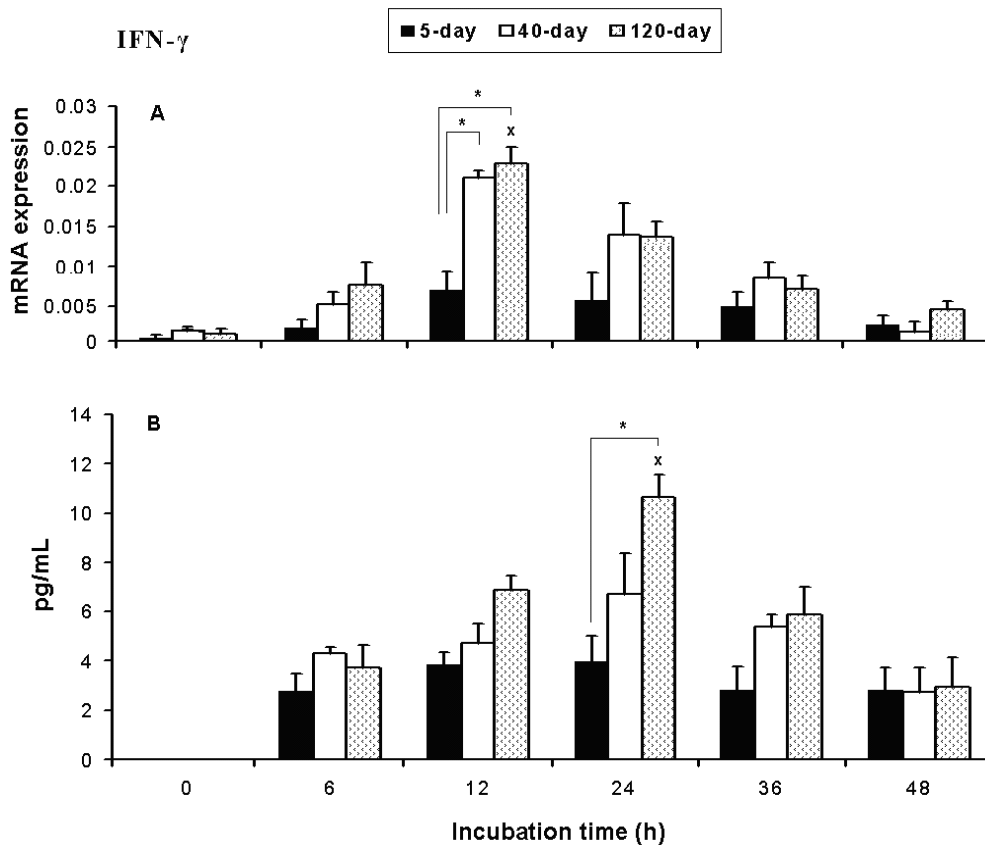


Figure 3. Age-related production of IFN- γ (mRNA and protein) by LPS-stimulated alveolar macrophages in a time-dependent manner. Macrophages (2×10^6 cells/ml/well) from 5 days (newborn piglets), 40 days (post-weaned piglets) and 120 days (young pigs) old animals were incubated with LPS ($10 \mu\text{g/ml}$). After stimulation for 0, 6, 12, 24, 36, and 48 h, the cells and supernatant fluids were collected for quantification of mRNA by qRT-PCR and protein concentration by ELISA, respectively. Data shown is an average of three replications \pm SEM. Graph panel A and B are indicated at the kinetics mRNA and protein level, respectively in different age groups. Asterisk indicate the significant difference among age groups ($*P < 0.05$). The letter x indicate the time-dependent up-regulation in young pigs from its control (0 h) ($P < 0.05$).

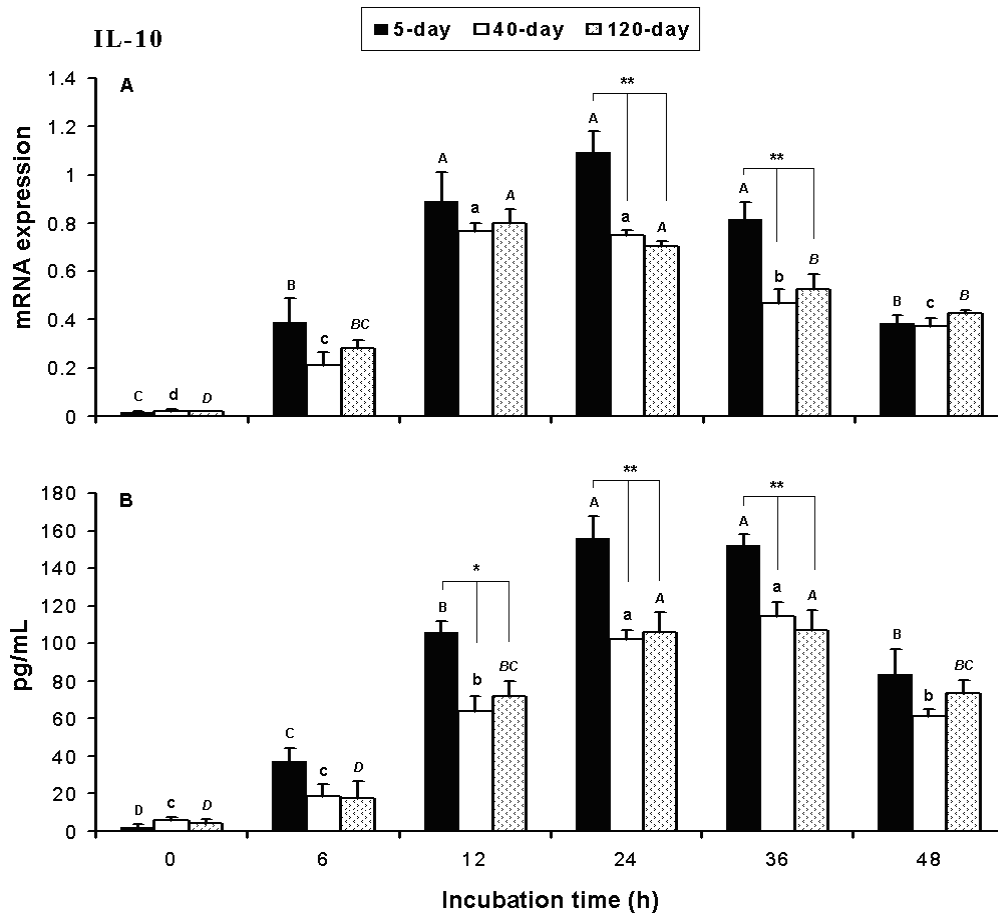


Figure 4. Production level of IL-10 mRNA and protein was quantified using the same samples presented in Figure 3. Data shown is an average of three replications \pm SEM. Graphs panel A and B are demonstrated at the mRNA and protein level, respectively in a time-dependent manner. Values with different capital, small and capital italic letters denote a statistical difference among time points within newborn piglets, post-weaned piglets and young pigs, respectively ($P < 0.05$). Asterisks indicate significant differences among age groups at each time point (** $P < 0.001$, and * $P < 0.05$).

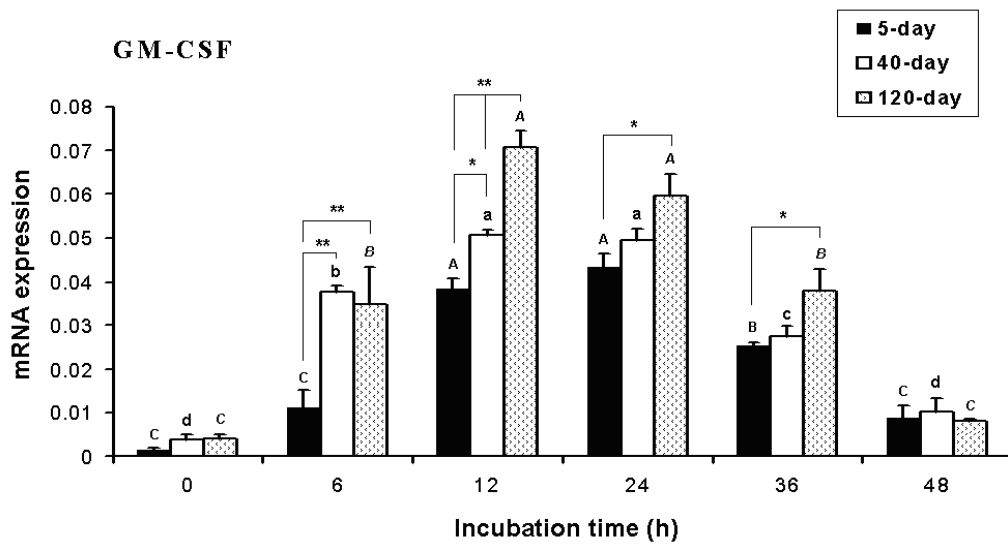
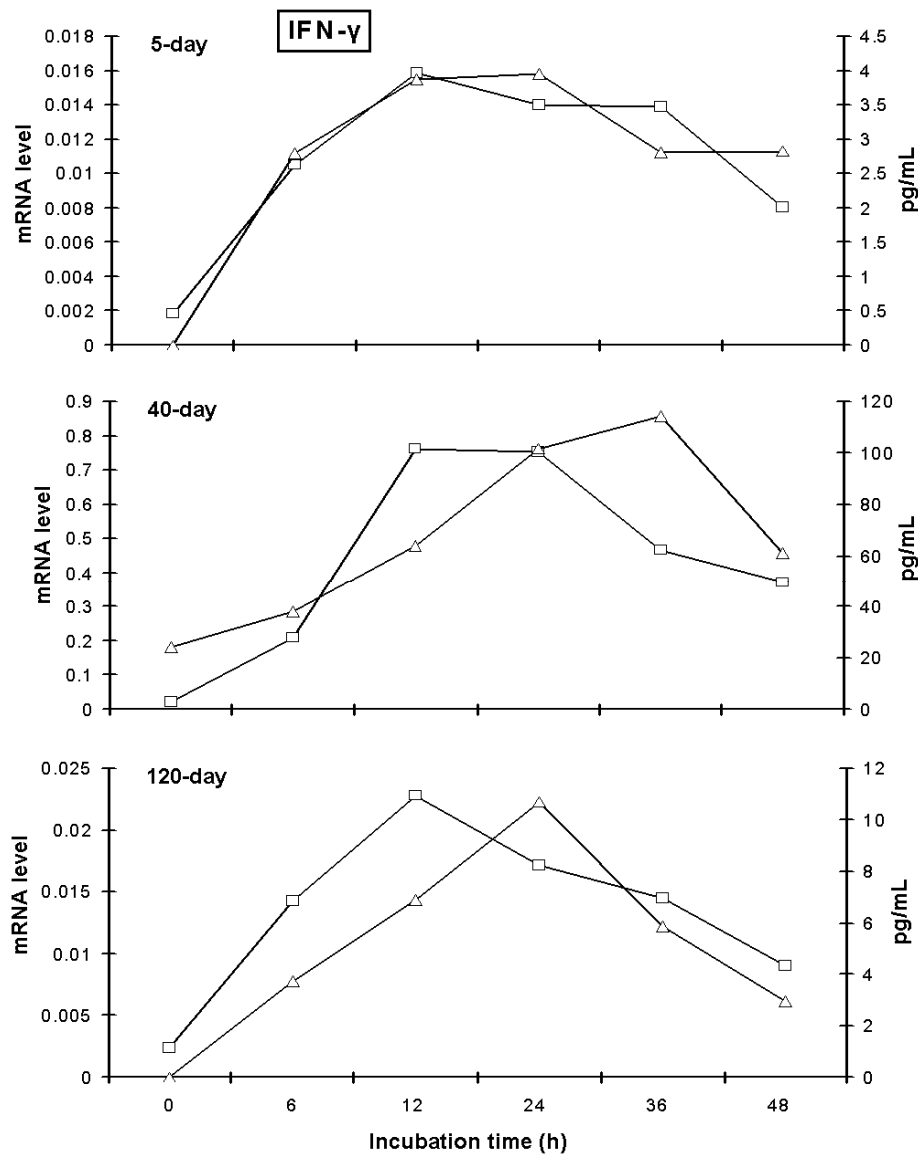
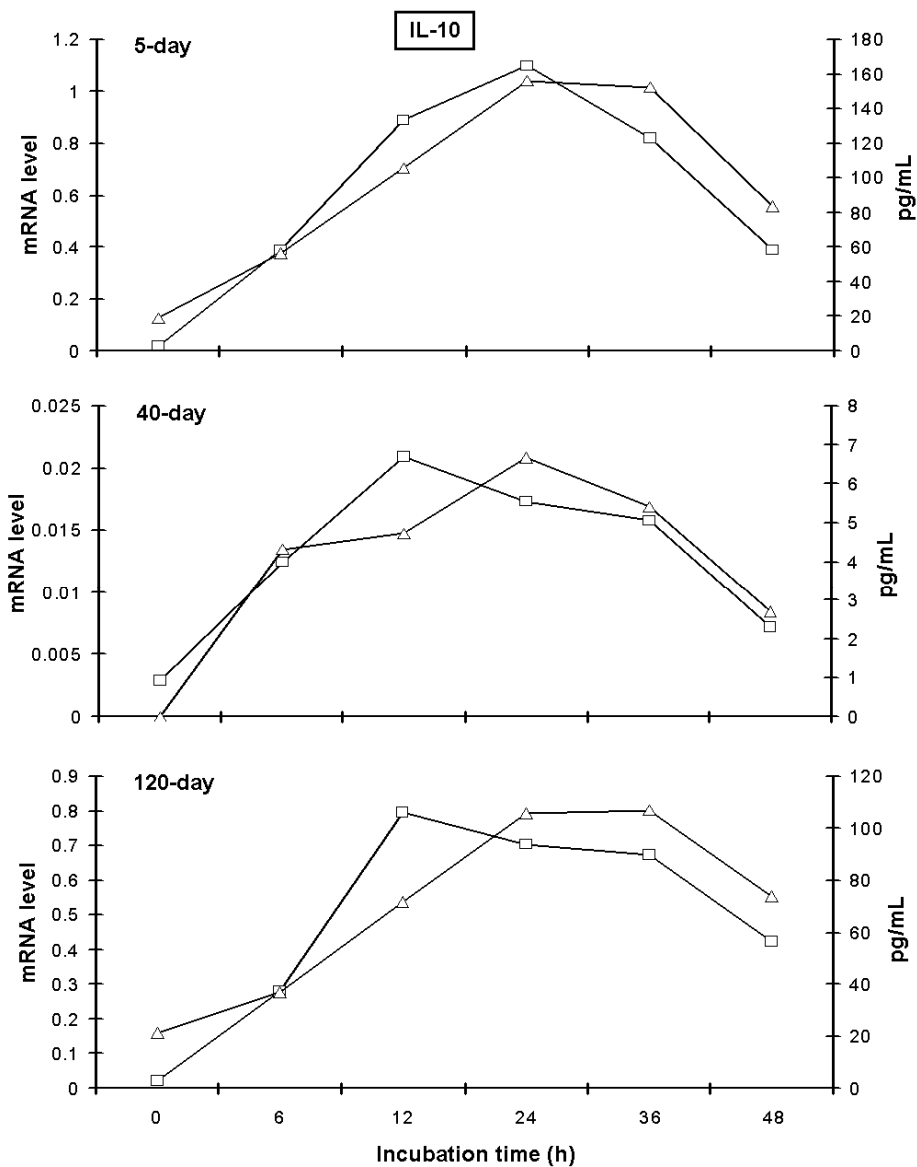


Figure 5. Expression level of GM-CSF mRNA was quantified using the same samples presented in Figure 3. Data shown is an average of three replications \pm SEM. Graphs are indicated the mRNA level in a time-dependent manner. Values with different capital, small and capital italic letters denote a statistical difference among time points within newborn piglets, post-weaned piglets and young pigs, respectively ($P < 0.05$). Asterisks indicate significant differences among age groups at each time point (** $P < 0.001$, and * $P < 0.05$).



Supplementary Figure 1. Kinetics comparison of IFN- γ mRNA and protein release by alveolar macrophages were assessment by qRT-PCR and ELISA, respectively. Alveolar macrophages of 5 days old newborn piglets, 40 days old post-weaned piglets, and 120 days old young pigs were incubated with LPS for 48 h. Comparison graph panels of IFN- γ are prepared using the same data presented in Fig. 3.

The mRNA (\square) and protein (\triangle) levels are plotted against time.



Supplementary Figure 2. Kinetics comparison of IL-10 mRNA and protein production by alveolar macrophages were assessment by qRT-PCR and ELISA, respectively. Alveolar macrophages of 5 days old newborn piglets, 40 days old post-weaned piglets, and 120 days old young pigs were incubated with LPS for 48 h. Comparison graph panels of IL-10 are prepared using the same data presented in Fig. 4. The mRNA (—□—) and protein (—△—) levels are plotted against time.

Chapter 4. (Published in: *Innate Immunity*, 2013, 19: 631-643)

Alveolar macrophage phagocytic activity is enhanced with LPS priming and combined stimulation of LPS and LTA synergistically induce pro-inflammatory cytokines in pigs

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Article type and status: original research and new

Short title: Alveolar macrophages phagocytosis and cytokine production

Key words: Alveolar macrophages; cytokine; innate immunity; LPS and LTA; phagocytosis.

Abstract

The objective of the present study was to investigate the lipopolysaccharide (LPS) and lipoteichoic acid (LTA)-induced TLRs, associated signaling molecules and inflammatory mediators, as well as to compare their combined effect in porcine alveolar macrophages. Macrophages were incubated for 24 h with various concentrations of LPS, LTA, LPS plus LTA or control. Multiple concentrations of LPS elicited the marked up-regulation in mRNA for TLR-2 and-4, CD-14, MD-2, MyD-88, IRAK-4, TRAF-6, as compared to control. LTA had no effect on TLR-4 and MD-2; only higher doses up-regulated TLR-2, CD-14, MyD-88, IRAK-4 and TRAF-6 mRNA. LPS-activated cells released IL-1 β , IL-12 β , TNF- α , IL-6, IL-8, IFN- γ and IL-10 in a dose-dependent manner while LTA had no effect on IL-1 β , IL-6 and IFN- γ . Higher doses of LTA induced IL-12 β , TNF- α , IL-8 and IL-10. Combined stimulation augmented TLR-2, CD-14 and MyD-88 mRNA, and subsequently produced elevated levels of IL-6, TNF- α and IL-8 when compared to LPS and LTA alone. Additionally, phagocytosis of macrophages was significantly increased following low concentration of LPS treatment. Only low levels of nitric oxide were detected in LPS group. Overall, compared to LPS, LTA was a relatively weak inducer, and co-stimulation accelerated genes and cytokines production associated with pulmonary innate immune function.

Introduction

Toll-like receptors (TLRs) are key receptors in innate immunity and trigger responses following interaction with pathogen-associated molecular patterns (PAMPs) (Akira 2009; Krishnan et al. 2007). Well-described PAMPs include Gram-negative bacterial cell walls elements such as lipopolysaccharide (LPS), inducing signal transduction predominantly via TLR-4, whereas Gram-positive bacterial elements, lipoteichoic acid (LTA) triggers cells mainly via TLR-2 (Akira et al. 2006; Beutler et al. 2006). Lipoteichoic acid produces share pathophysiological properties and recognition mechanisms such as CD-14 and TLRs, with LPS (Ginsburg 2002). Activated TLRs transmit signals through MyD88-dependent or independent pathways which in turn activate intra-cellular signaling cascades and

ultimately result in the induction of innate immune inflammatory mediators (Akira 2009; Krishnan et al. 2007; Takeda et al. 2003).

Macrophages are involved in the innate immune response through phagocytosis and the production of a variety of compounds, like cytokines and nitric oxide (NO) (Dempsey et al. 2003). PAMP-stimulated immune cells secrete pro-inflammatory cytokines, such as TNF- α , IL1- β and IL-6, initiating both innate and adaptive defense responses, and also release the anti-inflammatory cytokine, IL-10 (Ebersole and Cappelli 2000; O'Garra and Murphy 2009; Pulendran and Ahmed 2006). Nitric oxide synthase gene expression is up-regulated in response to PAMPs through activation of NF- κ B and mitogen-activated protein kinases (Chow et al. 2009). Over-production of these cytokines and NO might cause inflammatory diseases (Bogdan 2001).

Infections of the respiratory tract are frequently caused by co-infections of Gram-positive and Gram-negative bacteria. Specially, bacterial pneumonia, often caused by Gram-positive pathogens, is the most frequent source of sepsis (Laterre et al. 2005). As the primary inflammatory cell found within the airway, alveolar macrophages (Daly et al. 2009) play a central role in the constant recognition of potentially pathogenic organisms entering the lung (Medzhitov 2007; Twigg 2004). Despite the importance of AM cells in host defense, there is limited knowledge of the factors that are crucial for their effector functions. Recently, we have reported that different cytokines can be produced by porcine AM cells after initial stimulation with LPS (Islam et al. 2012a; Islam et al. 2012b). In addition, some investigations have been published on the effects of LPS and other PAMPs in porcine immune cells (Ramjeet et al. 2005; Sorensen et al. 2011; Verfaillie et al. 2001; Yancy et al. 2001), but the AMs responses to LTA and co-stimulation with LPS has been never studied in pig. The synergistic interactions between bacterial components and their activation of TLR signalling have been suggested to contribute to the pathophysiology of sepsis (Takada and Galanos 1987; Wray et al. 2001). It has been reported that combinations of TLR ligands synergistically augment various cytokine production by macrophages (Bagchi et al. 2007) and appears to be crucial for the innate immune defense against a variety of pathogens (Trinchieri and Sher 2007). We, therefore, hypothesized that LPS and LTA would induce differential inflammatory mediators from porcine AMs and that combination of these TLR-2 and TLR-4 ligands have a synergistic effect. It is likely that LTA is

recognized by TLR-2, whereas LPS is mediated through TLR-4 (Akira et al. 2006). On the other hand it has been reported, using transient-transfection experimental system, that TLR-2 mediates signals from other bacterial components, including peptidoglycan (PGN), and lipoproteins and/or lipopeptides (Hirschfeld et al. 1999). Indeed, highly purified LTA, as well as chemically synthesized LTA analogs, can stimulate TLR-2 (Morath et al. 2002).

In the present study, we first examined the phagocytic ability of AMs with priming of a range of concentrations of LPS for the selected Gram-negative bacteria. Thereafter, we sought to investigate the comparative induction of TLRs, the associated pathway genes and their effector molecules in porcine AMs following stimulation with LPS and LTA in a dose-dependent manner. Furthermore, we evaluated whether combined stimulation of LPS and LTA would result in amplified induction of these genes and inflammatory molecules from porcine AM cells.

Materials and methods

Animals

Three 40-days old Pietrain piglets (post-weaned) were used in this study. The pigs were clinically healthy and no respiratory diseases based at the time of this investigation on their health history were found. Animals were conventionally housed in the teaching and research station of Frankenforst, University of Bonn, Germany. The experiment was done according to the institutional guidelines and animal husbandry regulations of Germany (ZDS 2003).

Isolation of alveolar macrophages

Porcine alveolar macrophages from lungs were obtained by bronchoalveolar lavage (BAL) using ice-cold sterile calcium-magnesium free Dulbecco's phosphate-buffered saline (D-PBS, pH 7.4; Sigma Aldrich) as described elsewhere (Islam et al., 2012a). BAL fluids were filtered through sterile gauze and placed on wet ice. The cells were washed twice with D-PBS by centrifugation at 4°C for 10 min at 400×g before final re-suspension with RPMI-1640 media (Sigma-Aldrich). BAL fluids were mixed with RBC lysis buffer to lyse any residual erythrocytes. Cell differentials were performed on cytopspins stained with a

modified Giemsa stain (Reastain Quick-Diff kit, REAGENA). Macrophage purity was 92%, and other cells were mostly polymorphonuclear cells (PMNs) and lymphocytes. The total BAL cell count was obtained for each BAL fluid using a Haemocytometer. The cell viability was determined using the Trypan blue dye exclusion method. The percentage of viable AM cells were >98 % in all cases.

Assay of phagocytic activity

The *in vitro* phagocytic activity was performed with Vybrant phagocytosis assay kit (Molecular Probes Inc.) according to the manufacturer's instructions. Using this method, the process of phagocytosis can be quantified by following the internalization of fluorescently labeled bacterial particles. The protocol takes advantage of the detectability of the intracellular fluorescence emitted by the engulfed bacteria and Trypan blue was shown to effectively quench the fluorescence of fluorescein-labeled *Escherichia coli* as previously described (Loike and Silverstein 1983). Briefly, 150 μ l of the adjusted cell suspension (10^6 cells/mL) were seeded in a 24-well plate and treated with various concentrations of LPS (0.1, 1, 5 and 10 μ g/well) for 4 h. The positive and negative controls were prepared by adding 150 μ l of adjusted cells and 150 μ l of RPMI-1640 media, respectively to the wells on the microplate. Determinations were performed in five replications for each experimental group (at different LPS dose tested), positive and negative control wells. Following 4 h of incubation, solutions were removed from all microplate wells by vacuum aspiration. After that, fluorescein-labeled *Escherichia coli* (K-12 strain) bioparticles were added to all the positive, negative control and experimental wells. All plates were incubated for 2 h at 37°C in a humidified 5% CO₂ incubator to allow phagocytic uptake of cells. Subsequently, the *E. coli* bioparticle suspension was aspirated and extracellular fluorescence was quenched with 100 μ l of Trypan blue suspension for 1 min at room temperature. The excess Trypan blue was removed and the samples were measured in the fluorescence microplate reader (Thermo Electron Co.) using 480 nm for excitation and 520 nm emission wavelengths. The results are expressed as percentage of phagocytosis effect according to the calculation formula of assay kit.

Cell culture and stimulation

The collected porcine AM cells were re-suspended in 2mM L-glutamine-containing complete RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) (Invitrogen), sodium pyruvate (1 mM) and antibiotic and antimycotics (penicillin, streptomycin, amphotericin). For each experiment, AMs were cultured separately at 2×10^6 cells/mL/well into 24-well cell culture plates (Cell-Star). All plates were incubated at 37°C in a humidified 5% CO₂ incubator to allow macrophages attach to the plate bottom. After 2 h incubation, plates were carefully washed with D-PBS to remove any non-adherent cells and 1 mL of fresh complete medium was added for further cell culture. Adherent cell populations were >95% macrophages and >97% viable as determined by non-specific esterase staining and Trypan blue dye exclusion, respectively.

In this study, first, adherent macrophages were treated in triplicate with various concentrations (0, 0.1, 1, 5 and 10 µg/mL) of LPS from *Escherichia coli* 026:B6 (Sigma-Aldrich) and LTA from *Staphylococcus aureus* (Sigma-Aldrich). Secondly, a combination of LPS (at 1 µg/mL) and LTA (at 10 µg/mL) was added to the triplicates wells. Medium in triplicate wells were added in the same culture plate as control. All plates were incubated at 37°C, in 5% CO₂ for 24 h. Cells and supernatants were collected at 24 h of incubation. All samples were kept immediately at -80°C until use.

RNA extraction and cDNA synthesis

Harvested cells were washed in ice cold PBS, and the total RNA was extracted using PicoPure RNA isolation kit following the manufacturer's manual (Arcturus, Invitrogen). The extracted total RNA was treated with RNase-Free DNase Set (Qiagen) for 15 min at 37°C. Total RNA concentration was measured by absorption at 260 nm and purity and concentration were checked by determining the OD ratio 260/280 nm using a NanoDrop-8000 spectrophotometer (Thermo Scientific). Total RNA was then reverse transcribed into cDNA with SuperScript-II RT kit for qRT-PCR (Invitrogen). All samples were reverse transcribed under the same conditions. The synthesized cDNA was stored at -20°C and used in quantitative real-time (qRT) PCR reactions as a template.

Quantification of mRNA

Quantitative gene expression and subsequent data analysis was performed using the StepOnePlus™ qRT-PCR System (Applied Biosystems). The oligonucleotide gene specific primers for porcine TLRs and associated downstream molecules were as described in our previously published paper (Islam et al. 2012a). Two reference genes (PPIA and B2M) were selected as described previously (Cinar et al. 2012). The features of the primers used for this study are presented in Table 1. Briefly, triplicate reactions were performed using a 2 µl first-strand cDNA template, 7.4 µl deionized H₂O, 0.3 µM of forward and reverse gene specific primers and 10 µl 1× Power SYBR Green I (Bio-Rad) master mix with ROX as a reference dye. The thermal cycling conditions were 3 min at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. The mRNA copy numbers of the target and housekeeping gene were calculated according to the standard curve method. A standard curve was derived from the 9-fold serial dilutions of plasmid DNA. The data obtained from the qRT-PCR analysis was expressed as the mean of triplicate samples ± standard errors of mean (SEM). Normalization of data was performed by calculating the ratio between the gene of interest and the geometric mean of two housekeeping genes according to the Vandesompele et al. procedure (Vandesompele et al. 2002). The variations were observed between two house keeping genes (HKG) (PPIA and B2M) with various concentrations of LPS and LTA was not statistically significant.

Cytokine assays

The concentrations of IL1-β, IL12-β, TNF-α, IL-6, IL-8, IFN-γ and IL-10 in cell culture supernatants were measured using commercially available specific porcine ELISA Kits (R&D System), following the manufacturer's instructions. The optical density (OD) value was detected using ELISA plate reader at 450 nm wavelength. Standard and sample dilutions were added in duplicate wells. Concentration was calculated from a standard curve and used as protein level (pg/mL). The concentrations were detected according to the standard using microplate data compliance software SoftMax Pro (Molecular Devices GmbH).

Nitric Oxide assay

The Griess Reagent assay system (Promega) was used to measure nitrite formed by the spontaneous oxidation of NO (Cho and Chae 2003). Briefly, 50 μ L of cell supernatant was added to each well of the 96-well microplate and incubated with 50 μ L of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) at room temperature for 5 to 10 min in darkness. Then, 50 μ L of 0.1% N-1-naphthylethylenediamine dihydrochloride in water was added to each well and incubated at room temperature for 5 to 10 min in darkness. The OD was measured at 530 nm wavelengths. Concentrations were calculated from a standard sodium nitrite curve. All samples were analyzed in duplicate.

Assay of cell viability

In a separate set of experiments, AMs were cultured as described above and treated with LPS (1 μ g/mL), LTA (10 μ g/mL) and LPS (1 μ g/mL) plus LTA (10 μ g/mL) or medium (control). Following 24 h incubation, cells were collected, washed twice with D-PBS and resuspended in RPMI-1640 medium. Cell viability was determined using the principle of Trypan blue dye exclusion. The cells from each well were stained with Trypan blue, and the number of dye-excluding (live) cells and positively staining (dead) cells were counted using a Hemocytometer. The assays were performed in duplicate for each group. The results presented as the percentage of viable cells and in general, at least 200 cells per vial were scored for viability.

Statistical analysis

The data were analyzed using the SAS software package ver9.2 (SAS Institute Inc., Cary, NC). All values are expressed in the figure as means of triplicates data \pm standard errors of mean (SEM). Statistical difference of various concentrations of PAMPs on the mRNA expression, cytokine production and phagocytosis were determined by one-way factorial analysis of variance (ANOVA) followed by Tukey's multiple comparison procedure as appropriate. The significance of difference between PAMP-stimulated groups was determined using student's *t*-test. All results were deemed significant for $P < 0.05$.

Results

Phagocytosis assay

We first examined whether treatment with various concentrations of LPS would affect phagocytosis in AM cells *in vitro*. As shown in Fig. 1, treatment of cultured macrophages with low doses of LPS (0.1 $\mu\text{g}/\text{mL}$) resulted in a statistically significant increase in the rate of phagocytosis compared with untreated and highest doses of LPS-treated cells ($22 \pm 0.07\%$ and $20 \pm 0.04\%$ increase from control and highest dose of LPS, respectively). Although LPS at 1 $\mu\text{g}/\text{mL}$ ($51 \pm 3.4\%$) and 5 $\mu\text{g}/\text{mL}$ ($48 \pm 3.7\%$) primed macrophages were able to phagocytose more bio-particles than the control, but there was no significant difference between them.

Cell viability

Trypan blue exclusion demonstrated that there were no significant effects in AMs viability between samples treated with PAMPs or in the controls after 24 h incubation. As can be seen in Fig. 2, the mean viable percentages for the four samples were 90.6 ± 4.1 , 88.72 ± 5.4 , 87.03 ± 5.07 and 87.65 ± 2.7 for control, LPS, LTA and LPS plus LTA, respectively.

Expression of TLRs and their pathway signalling molecules in a dose-dependent manner

Exposure of cells with LPS resulted in significant upregulation of TLR-4, TLR-2, CD-14, MD-2, IRAK-4 and TRAF-6 compared to controls in a dose-dependent way. In contrast, at the concentrations evaluated, LTA had no effect on TLR-4 and MD-2. Only higher doses of LTA (5-10 $\mu\text{g}/\text{mL}$) caused a significant expression of TLR-2, CD-14, IRAK-4 and TRAF-6, as compared to control. In general, it is indicated that LPS was a more potent inducer than LTA and increasing concentrations of both LPS and LTA induced increasing expression of these genes.

Combined stimulation of LPS and LTA synergistically up-regulated TLR-2, CD-14 and MyD-88 genes

Having shown that various doses of LPS and LTA treatment of macrophages leads to an increase in mRNA expression of TLRs and their pathway molecules, we next assessed whether co-stimulation caused an increase of expression of these genes. Co-stimulation of AMs with LPS and LTA resulted in a significant up-regulation of TLR-2, CD-14 and MyD-88 mRNAs levels compared to both stimulator groups alone. LPS at 1 $\mu\text{g}/\text{mL}$ and co-stimulation induced significant and similar expression of TLR-4 and MD-2, but LTA at 10 $\mu\text{g}/\text{mL}$ alone had no effect. Although there were some synergism effects of co-stimulation on IRAK-4 and TRAF-6 mRNA, but this was not difference compared to LPS and LTA alone.

Cytokine production in response to LPS and LTA in a dose-dependent manner

Stimulation of cells with LPS resulted in significant IL-1 β , IL-12 β , IL-6, TNF- α , IL-8 and IL-10 production compared to control in a dose-dependent manner. In contrast, LTA stimulation failed to induce IL-1 β , IL-6 and IFN- γ production, regardless of the concentrations used. LTA at 5 and 10 $\mu\text{g}/\text{mL}$ did stimulate IL-12 β , TNF- α and IL-8 production compared to the control. The highest concentration of LTA and LPS resulted in significant production of IL-10 and IFN- γ , respectively when it is compared to control (Fig. 5).

Co-stimulation of LPS and LTA synergistically increases cytokine production

Given the results that LPS and LTA-stimulated cells lead to different patterns of cytokine production in a dose-dependent fashion, we assessed whether combined stimulation of PAMPs would result in amplified induction of cytokines. Stimulation of AMs with the combination of LPS at 1 $\mu\text{g}/\text{mL}$ and LTA at 10 $\mu\text{g}/\text{mL}$ resulted in significant production of TNF- α and IL-8, as compared to LPS and LTA alone. In addition, co-stimulation resulted in significantly higher production of IL-1 β , IL-12 β and IL-10, as compared to control or LTA alone. LTA at 10 $\mu\text{g}/\text{mL}$ had no effect but with LPS at 1 $\mu\text{g}/\text{mL}$ had synergistic effect in IL-6 and IL-1 β production. Costimulation with PAMPs failed to induce IFN- γ (Fig. 6).

Nitric oxide production

There was no significant difference in NO production between the treated samples and controls (data not shown).

Discussion

Alveolar macrophages are the primary airway cells responsible for innate immune defenses, including phagocytosis of respiratory pathogens. LPS triggers inflammatory responses by inducing inflammatory cytokine production and thus influences the rate of phagocytosis of macrophages in an autocrine manner (Sriskandan and Cohen 1999). In this study, first we focused on the role of a various concentrations of LPS in regulating phagocytosis of fluorescein-labeled *Escherichia coli* (K-12 strain) bioparticles by AMs. We found that macrophages exhibited a significant enhancement in their ability to phagocytise bacteria when exposed to as little as 0.1 µg/mL of LPS. In addition, we observed that macrophages exhibited a slight increase of phagocytosis with the other concentrations of LPS used. It is well known that production of cytokines by LPS-stimulated immune cells is time and dose dependent manner (Islam et al. 2012a; Islam et al. 2012b; Vandesompele et al. 2002). Recently, we reported that TNF- α , a key pro-inflammatory factor that can be released by porcine AMs immediately at 1 h after LPS stimulation (Islam et al. 2012a). A recent study reported that LPS-mediated induction of TNF- α inhibited the phagocytic ability of macrophages (Sriskandan and Cohen 1999). Hence, from the findings of the present study, we may speculate that the lack of significant phagocytosis enhancement after stimulation with higher LPS concentrations can be attributed to LPS-mediated early induction of TNF- α . The finding of our study is also consistent with the observations that phagocytic activities of macrophages are augmented following LPS treatment (Vogel et al. 1979; Wu et al. 2009). Exposure of AMs to LPS induced an early release of TNF- α and IL-8 (Islam et al. 2012b). Thus, the LPS-caused induction of inflammatory cytokine production provides another possible mechanism for the stimulation of phagocytic activities by this endotoxin. Phagocytic action of macrophages requires energy (Lammas et al. 1997). Thus, LPS may promote mitochondrial ATP synthesis that enhances the phagocytic activities of macrophages.

In this study, one time point i.e. 24 h was observed following LPS and LTA treatment according to previously published work (Gao et al. 2010). Moreover, we are aware that all the results reported here correspond to this unique time point i.e. 24 hours after stimulation. It has been reported that time points earlier than 24 hours are more relevant to decipher the onset of the response to stimulus as shown in kinetics studies in cow (Hirschfeld et al. 1999), pig (Jaekal et al. 2007), mouse (Morath et al. 2002) or human (Loike and Silverstein, 1983). Moreover, kinetics studies have revealed that many genes return to their basal expression level by 48 hours of stimulation, suggesting that homeostasis is restored at that time (Hirschfeld et al. 1999; Jaekal et al. 2007). Therefore, in this paper, we were interested in studying the AMs stimulation at the time when cytokines released in the medium are efficiently measured. It should be noted here that there was no difference in AMs viability at 24 h between cells incubated with LPS and LTA or controls. Although this assay does not directly measure apoptosis, it indicates that cell death was not a significant issue when using LPS, LTA or LPS plus LTA with these macrophages. Notable, the house keeping genes used in this study, *PPIA* and *B2M* are stably expressed in porcine AM cells (Cinar et al. 2012). These two reference genes did not show any significant variation for TLRs and the down stream genes expression at the different concentrations of PAMP.

Taking into account that AM cells play a crucial role in the inflammatory process in the pulmonary system, we investigated the effects of PAMP on the expression of TLRs and down-stream genes from porcine AMs. The findings of the present study have shown that AMs responded to the presence of both LPS and LTA by up-regulating CD-14 and MyD-88 in a dose-dependent manner. We also demonstrated that LTA was unable to stimulate TLR-4 and MD-2, whereas LPS induced an increased expression of TLR-4, TLR-2 and MD-2, regardless of the concentrations used. In accordance with our results, it has been shown that LTA activates immune cells via TLR-2, whereas TLR-4 and MD-2 are not involved (Su et al. 2006). Consistent with an earlier report we conclude that innate immune recognition of LTA via CD-14 and TLR-2 may represents an important mechanism in the pathogenesis of infection through MyD-88 dependent pathway (Schroder et al. 2003). Moreover, the effects of LPS and LTA on the up-regulation of the IRAK-4 and TRAF-6 gene expression on cells may play a role in further augmentation of innate immune responses.

The measurement of cytokine production is the easiest assessment for cell responsiveness, which is used for different *in vitro* models (Jaekal et al. 2007). Our study shows differences in cytokine release *in vitro*, in response to LPS compared with LTA, regardless of the concentrations used. Overall, LTA appears to be a less potent cytokine-inducer than LPS. In this study, the lowest concentration of LPS was relatively more potent to induce cytokines IL-12 β , TNF- α and IL-8 compared to the highest concentration of LTA. LTA resulted IL-10 secretion, but lesser extent than LPS and was influenced by their doses. However, the production of inflammatory cytokines is reported to be affected by the concentration of PAMP in swine (Sorensen et al. 2011). By contrast, the lack of detectable IL-1 β , IL6 and IFN- γ in response to LTA may be related to the relative insensitivity to cell type in pigs. This finding may speculate the low sensitivity of lung and pulmonary cells to Gram-positive bacterial stimuli via the MyD88-dependent pathway. We detected IFN- γ by LPS-induced AM cell which is somewhat consistent with the previously published work (Underhill and Ozinsky 2002). There is one contradictory data that IFN- γ was not evident in LPS-induced porcine AMs (Hou et al. 1999). This might explain in the present study that AMs contaminated with PMN cells and lymphocytes may lead to the alteration of cytokine secretion.

Differences in our study between LPS- and LTA-activated cytokines *in vitro* concur with previous findings. Our finding is similar to what is observed in humans and mice with lower concentrations of LPS being required to stimulate pro-inflammatory cytokine production compared to LTA and PG (Finney et al. 2011; Nakagawa et al. 2002). Similarly, in a range of human and murine macrophage cells, LTA was consistently less potent than LPS in causing cytokine and or chemokine release (Jones et al., 2005; Kimbrell et al., 2008). LPS is a more potent inducer of TNF- α , IL1, IL-6 and IL-8 than other PAMP from equine and feline whole blood (Declue et al. 2011; Stich and DeClue 2011). Furthermore, IL-1, IL-6 and IL-18 levels have been reported as significantly higher in Gram-negative bacterial infections compared to Gram-positive bacteria (Feezor et al. 2003).

Next, we determined the potential synergistic production of inflammatory cytokines provoked by combination of LPS and LTA. Simultaneous stimulation of TLR-2 and TLR-4 results in amplified cytokine signaling during polymicrobial infections in humans and leads to poor outcomes and an increased risk of septic shock and mortality (Lin et al. 2009; Sato

et al. 2000). In the present study, co-stimulation of LTA with LPS led to the synergistic activation of TLR-2, CD-14 and MyD-88 genes. Additionally, combined effects significantly release IL-6, TNF- α and IL-8 than neither LPS nor LTA alone at 24 h post-stimulation. Although co-stimulation resulted in greater IL-6 production, there was no significant production when stimulated with LTA alone. Collectively, our data suggest that during pulmonary infections, an exacerbated inflammatory response may occur inducing the enhanced release of pro-inflammatory cytokines and chemokines, which may lead to septic shock and death. The mechanism underlying the synergistic up-regulation of TLRs-associated genes and inflammatory cytokines by PAMP combinations is of interest. Co-stimulation with TLR ligands accelerated gene expression and synergistically induced pro-inflammatory cytokines which are primarily associated with cellular immune function (Tross et al. 2009). Previous studies of cytokine protein production by ELISA and mRNA levels of TLR-associated molecules by qRT-PCR confirmed the synergistic activation of certain PAMP (Napolitani et al. 2005; Zhu et al. 2008) and are consistent with our study. Notably, pure LTA appears to be a less potent cytokine-inducer than LPS and the ability of LTA to induce higher concentrations of cytokines has been attributed to its synergistic interactions with other bacterial components such as muramyl dipeptide (Yang et al. 2001), PGN or host macromolecules such as glycosphingolipids (Meron-Sudai et al. 2008). Our findings support the hypothesis of Yang et al. (Yang et al. 2001) that bacterial cell surface components in various combinations might exert more powerful activities on host cells than the expected levels based on the activities of individual components. The pathophysiology of an infectious disease may be caused not only by the pathogen involved but also by excessive inflammatory responses, which are sometimes more damaging to the host than the virulence activity of the pathogen that elicited them. Thus, the understanding of the response of AMs to co-stimulation will provide important insights into some effect of Gram-negative and Gram-positive bacteria in the pulmonary infection.

Nitric oxide is a very important molecule involved in a wide range of physiologic and pathologic processes in mammalian systems and its production by macrophages is fundamental for immune defense (MacMicking et al. 1997). In the present study, at the tested concentrations, LPS and LTA failed to stimulate NO production from porcine AMs as assessed by the supernatant Griess reaction. Our results confirmed the previous findings,

who also failed to induce NO production from porcine AMs, Kupffer cells and peripheral blood mononuclear cells upon stimulation with LPS, LTA and IFN- γ (Akunda et al. 2001; Declue et al. 2011; Pampusch et al. 1998; Zelnickova et al. 2008). On the other hand, NO production was induced in LPS-activated rat and bovine AMs (Zelnickova et al. 2008). However, the fundamental differences among species in the abilities of macrophages to produce NO are not clear.

The current study has several limitations. Firstly, we studied a relatively small number of animals that may be not reflecting the real scenario of a big sized animal population. Secondly, we took a limited concentration range of LPS and LTA, single concentration for combined stimulation with one time point for inflammatory response of cells. Hence, using a wide range of PAMP concentrations, particularly when evaluating the synergistic effects of PAMPs, may have yielded different results. The form of LPS and LTA used in this study also may influence the immuno-stimulatory effect on cells. In this respect, it should be noted that our study does not provide direct evidence for a mechanism underlying the effect of PAMPs on AM cells.

Conclusion

To our knowledge, the present study is the first to provide insight into the *in vitro* effects of LPS, LTA and their co-stimulation in innate immune responses of AM cells in pigs. Additionally, the present study showed that LPS-activated macrophages led to the increased phagocytic activity. Taken together, LTA is a potent mediator of innate immune response, but compared to LPS, it was a relatively weak inducer in immuno-stimulating response and induces less cytokine secretion. In addition, our findings suggest that the mimic of Gram-negative bacteria synergizes with the mimic of Gram-positive bacteria in macrophages inducing pro-inflammatory cytokines. These obtained data could be helpful in understanding the molecular mechanisms involved in the pathogenesis of infections caused by Gram-negative and Gram-positive bacteria, and their co-infections, and may be potentially use in developing novel intervention strategies. However, further experiments are clearly awaited to clarify the mechanisms underlying the enhanced responsiveness of AM cells upon co-stimulation of Gram-negative and Gram-positive bacterial components.

Acknowledgement

The authors are indebted to Mr. Ludger Buschen in research station “Frankenforst” at University of Bonn for providing pigs. Authors are also thankful to Ms. Birgit Koch-Fabritius for her technical assistance during the experiments.

References

- Akira S (2009): Pathogen recognition by innate immunity and its signaling: Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 85, 143-156.
- Akira S, Uematsu S, Takeuchi O (2006): Pathogen recognition and innate immunity. Cell 124, 783-801.
- Akunda JK, Ahrens FA, Kramer TT (2001): Evaluation of phagocytosis, bactericidal activity, and production of superoxide anion, nitric oxide, and tumor necrosis factor-alpha in Kupffer cells of neonatal pigs. Am. J. Vet. Res. 62, 1040-1045.
- Bagchi A, Herrup EA, Warren HS, Trigilio J, Shin HS, Valentine C, Hellman J (2007): MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. J. Immunol. 178, 1164-1171.
- Beutler B, Jiang Z, Georgel P, Crozat K, Croker B, Rutschmann S, Du X, Hoebe K (2006): Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. Annu. Rev. Immunol. 24, 353-389.
- Bogdan C (2001): Nitric oxide and the immune response. Nat. Immunol. 2, 907-916.
- Chow JM, Lin HY, Shen SC, Wu MS, Lin CW, Chiu WT, Lin CH, Chen YC (2009): Zinc protoporphyrin inhibition of lipopolysaccharide-, lipoteichoic acid-, and peptidoglycan-induced nitric oxide production through stimulating iNOS protein ubiquitination. Toxicol. Appl. Pharmacol. 237, 357-365.
- Cinar MU, Islam MA, Uddin MJ, Tholen E, Tesfaye D, Looft C, Schellander K (2012): Evaluation of suitable reference genes for gene expression studies in porcine alveolar macrophages in response to LPS and LTA. BMC Res. Notes 5, 107.

- Daly KA, Mailer SL, Digby MR, Lefevre C, Thomson P, Deane E, Nicholas KR, Williamson P (2009): Molecular analysis of tammar (*Macropus eugenii*) mammary epithelial cells stimulated with lipopolysaccharide and lipoteichoic acid. *Vet. Immunol. Immunopathol.* 129, 36-48.
- Declue AE, Johnson PJ, Day JL, Amorim JR, Honaker AR (2011): Pathogen associated molecular pattern motifs from Gram-positive and Gram-negative bacteria induce different inflammatory mediator profiles in equine blood. *Vet. J.* 192, 455-460.
- Dempsey PW, Vaidya SA, Cheng G (2003): The art of war: Innate and adaptive immune responses. *Cell. Mol. Life Sci.* 60, 2604-2621.
- Ebersole JL, Cappelli D (2000): Acute-phase reactants in infections and inflammatory diseases. *Periodontol.* 2000 23, 19-49.
- Feezor RJ, Oberholzer C, Baker HV, Novick D, Rubinstein M, Moldawer LL, Pribble J, Souza S, Dinarello CA, Ertel W, Oberholzer A (2003): Molecular characterization of the acute inflammatory response to infections with gram-negative versus gram-positive bacteria. *Infect. Immun.* 71, 5803-5813.
- Finney SJ, Leaver SK, Evans TW, Burke-Gaffney A (2011): Differences in lipopolysaccharide- and lipoteichoic acid-induced cytokine/chemokine expression. *Intensive Care Med.* 38, 324-332.
- Gao Y, Flori L, Lecardonnel J, Esquerre D, Hu ZL, Teillaud A, Lemonnier G, Lefevre F, Oswald IP, Rogel-Gaillard C (2010): Transcriptome analysis of porcine PBMCs after in vitro stimulation by LPS or PMA/ionomycin using an expression array targeting the pig immune response. *BMC Genom.* 11, 292.
- Ginsburg I (2002): Role of lipoteichoic acid in infection and inflammation. *Lancet Infect. Dis.* 2, 171-179.
- Hirschfeld M, Kirschning CJ, Schwandner R, Wesche H, Weis JH, Wooten RM, Weis JJ (1999): Cutting edge: inflammatory signaling by *Borrelia burgdorferi* lipoproteins is mediated by toll-like receptor 2. *J. Immunol.* 163, 2382-2386.
- Hou YC, Janczuk A, Wang PG (1999): Current trends in the development of nitric oxide donors. *Curr. pharm. Des.* 5, 417-441.

- Islam MA, Cinar MU, Uddin MJ, Tholen E, Tesfaye D, Looft C, Schellander K (2012a): Expression of toll-like receptors and downstream genes in lipopolysaccharide-induced porcine alveolar macrophages. *Vet. Immunol. Immunopathol.* 146, 62-73.
- Islam MA, Uddin MJ, Tholen E, Tesfaye D, Looft C, Schellander K Cinar MU (2012b): Age-related changes in phagocytic activity and production of pro-inflammatory cytokines by lipopolysaccharide stimulated porcine alveolar macrophages. *Cytokine* 60, 707-717.
- Jaekal J, Abraham E, Azam T, Netea MG, Dinarello CA, Lim JS, Yang Y, Yoon DY, Kim SH (2007): Individual LPS responsiveness depends on the variation of toll-like receptor (TLR) expression level. *J. Microbiol. Biotechnol.* 17, 1862-1867.
- Jones KJ, Perris AD, Vernallis AB, Worthington T, Lambert PA, Elliott TS (2005): Induction of inflammatory cytokines and nitric oxide in J774.2 cells and murine macrophages by lipoteichoic acid and related cell wall antigens from *Staphylococcus epidermidis*. *J. Med. Microbiol.* 54, 315-321.
- Kimbrell MR, Warshakoon H, Cromer JR, Malladi S, Hood JD, Balakrishna R, Scholdberg TA, David SA (2008): Comparison of the immunostimulatory and proinflammatory activities of candidate Gram-positive endotoxins, lipoteichoic acid, peptidoglycan, and lipopeptides, in murine and human cells. *Immunol. Lett.* 118, 132-141.
- Krishnan J, Selvarajoo K, Tsuchiya M, Lee G, Choi S (2007): Toll-like receptor signal transduction. *Exp. Mol. Med.* 39, 421-438.
- Lammas DA, Stober C, Harvey CJ, Kendrick N, Panchalingam S, Kumararatne DS (1997): ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z(P2X7) receptors. *Immunity* 7, 433-444.
- Laterre PF, Garber G, Levy H, Wunderink R, Kinasewitz GT, Sollet JP, Maki DG, Bates B, Yan SC, Dhainaut JF (2005): Severe community-acquired pneumonia as a cause of severe sepsis: data from the PROWESS study. *Crit. Care Med.* 33, 952-961.
- Lin JN, Tsai YS, Lai CH, Chen YH, Tsai SS, Lin HL, Huang CK, Lin HH (2009): Risk factors for mortality of bacteremic patients in the emergency department. *Acad. Emerg. Med.* 16, 749-755.

- Loike JD, Silverstein SC (1983): A fluorescence quenching technique using trypan blue to differentiate between attached and ingested glutaraldehyde-fixed red blood cells in phagocytosing murine macrophages. *J. Immunol. Methods* 57, 373-379.
- MacMicking J, Xie QW, Nathan C (1997): Nitric oxide and macrophage function. *Annu. Rev. Immunol.* 15, 323-350.
- Medzhitov R (2007): Recognition of microorganisms and activation of the immune response. *Nature* 449, 819-826.
- Meron-Sudai S, Matityahou A, Keisari Y, Cox KH, Hasty DL, Ofek I (2008): Lipoteichoic acid synergizes with glycosphingolipids to potently stimulate secretion of interleukin-6 from human blood cells. *Clin. Vaccine Immunol.* 15, 1309-1315.
- Morath S, Stadelmaier A, Geyer A, Schmidt RR, Hartung T (2002): Synthetic lipoteichoic acid from *Staphylococcus aureus* is a potent stimulus of cytokine release. *J. Exp. Med.* 195, 1635-1640.
- Nakagawa Y, Maeda H, Murai T (2002): Evaluation of the in vitro pyrogen test system based on proinflammatory cytokine release from human monocytes: comparison with a human whole blood culture test system and with the rabbit pyrogen test. *Clin. Diagn. Lab. Immunol.* 9, 588-597.
- Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A (2005): Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat. Immunol.* 6, 769-776.
- O'Garra A, Murphy KM (2009): From IL-10 to IL-12: how pathogens and their products stimulate APCs to induce T(H)1 development. *Nat. Immunol.* 10, 929-932.
- Pampusch MS, Bennaars AM, Harsch S, Murtaugh MP (1998): Inducible nitric oxide synthase expression in porcine immune cells. *Vet. Immunol. Immunopathol.* 61, 279-289.
- Pulendran B, Ahmed R (2006): Translating innate immunity into immunological memory: implications for vaccine development. *Cell* 124, 849-863.
- Ramjeet M, Deslandes V, St Michael F, Cox AD, Kobisch M, Gottschalk M, Jacques M (2005): Truncation of the lipopolysaccharide outer core affects susceptibility to antimicrobial peptides and virulence of *Actinobacillus pleuropneumoniae* serotype 1. *J. Biol. Chem.* 280, 39104-39114.

- Sato S, Nomura F, Kawai T, Takeuchi O, Muhlradt PF, Takeda K, Akira S (2000): Synergy and cross-tolerance between toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways. *J. Immunol.* 165, 7096-7101.
- Schroder NW, Morath S, Alexander C, Hamann L, Hartung T, Zahringer U, Gobel UB, Weber JR, Schumann RR (2003): Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J. Biol. Chem.* 278, 15587-15594.
- Sorensen NS, Skovgaard K, Heegaard PM (2011): Porcine blood mononuclear cell cytokine responses to PAMP molecules: comparison of mRNA and protein production. *Vet. Immunol. Immunopathol.* 139, 296-302.
- Sriskandan S, Cohen J (1999): Gram-positive sepsis. Mechanisms and differences from gram-negative sepsis. *Infect Dis Clin. North. Am.* 13, 397-412.
- Stich AN, DeClue AE (2011): Pathogen associated molecular pattern-induced TNF, IL-1beta, IL-6 and CXCL-8 production from feline whole blood culture. *Res. Vet. Sci.* 90, 59-63.
- Su SC, Hua KF, Lee H, Chao LK, Tan SK, Yang SF, Hsu HY (2006): LTA and LPS mediated activation of protein kinases in the regulation of inflammatory cytokines expression in macrophages. *Clin. Chim. Acta.* 374, 106-115.
- Takada H, Galanos C (1987): Enhancement of endotoxin lethality and generation of anaphylactoid reactions by lipopolysaccharides in muramyl-dipeptide-treated mice. *Infect. Immun.* 55, 409-413.
- Takeda K, Kaisho T, Akira S (2003): Toll-like receptors. *Annu. Rev. Immunol.* 21, 335-376.
- Trinchieri G, Sher A (2007): Cooperation of Toll-like receptor signals in innate immune defence. *Nat. Rev. Immunol.* 7, 179-190.
- Tross D, Petrenko L, Klaschik S, Zhu Q, Klinman DM (2009): Global changes in gene expression and synergistic interactions induced by TLR9 and TLR3. *Mol. Immunol.* 46, 2557-2564.
- Twigg HL (2004): Macrophages in innate and acquired immunity, 3rd. *Semin. Respir. Crit. Care Med.* 25, 21-31.

- Underhill DM, Ozinsky A (2002): Phagocytosis of microbes: complexity in action. *Annu. Rev. Immunol.* 20, 825-852.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002): Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome. biology.* 3, RESEARCH0034.
- Verfaillie T, Cox E, To LT, Vanrompay D, Bouchaut H, Buys N, Goddeeris BM (2001): Comparative analysis of porcine cytokine production by mRNA and protein detection. *Vet. Immunol. Immunopathol.* 81, 97-112.
- Vogel SN, Marshall ST, Rosenstreich DL (1979): Analysis of the effects of lipopolysaccharide on macrophages: differential phagocytic responses of C3H/HeN and C3H/HeJ macrophages in vitro. *Infect. Immun.* 25, 328-336.
- Wray GM, Foster SJ, Hinds CJ, Thiemermann C (2001): A cell wall component from pathogenic and non-pathogenic gram-positive bacteria (peptidoglycan) synergises with endotoxin to cause the release of tumour necrosis factor-alpha, nitric oxide production, shock, and multiple organ injury/dysfunction in the rat. *Shock* 15, 135-142.
- Wu TT, Chen TL, Chen RM (2009): Lipopolysaccharide triggers macrophage activation of inflammatory cytokine expression, chemotaxis, phagocytosis, and oxidative ability via a toll-like receptor 4-dependent pathway: validated by RNA interference. *Toxicol. Lett.* 191, 195-202.
- Yancy H, Ayers SL, Farrell DE, Day A, Myers MJ (2001): Differential cytokine mRNA expression in swine whole blood and peripheral blood mononuclear cell cultures. *Vet. Immunol. Immunopathol.* 79, 41-52.
- Yang S, Tamai R, Akashi S, Takeuchi O, Akira S, Sugawara S, Takada H (2001): Synergistic effect of muramyl dipeptide with lipopolysaccharide or lipoteichoic acid to induce inflammatory cytokines in human monocytic cells in culture. *Infect. Immun.* 69, 2045-2053.
- ZDS (2003): Richtlinie für die Stationsprüfung auf Mastleistung, Schlachtkörperwert und Fleischbeschaffenheit beim Schwein, 10.12.2003.

- Zelnickova P, Matiasovic J, Pavlova B, Kudlackova H, Kovaru F, Faldyna M (2008): Quantitative nitric oxide production by rat, bovine and porcine macrophages. *Nitric Oxide* 19, 36-41.
- Zhu Q, Egelston C, Vivekanandhan A, Uematsu S, Akira S, Klinman DM, Belyakov IM, Berzofsky JA (2008): Toll-like receptor ligands synergize through distinct dendritic cell pathways to induce T cell responses: implications for vaccines. *Proc. Natl. Acad. Sci. U S A.* 105, 16260-16265.

Table 1. Product sizes and nucleotide sequence details of primers used for real time quantitative PCR analysis of mRNA expression of porcine TLRs, associated molecules and two reference genes.

Gene name	GenBank accession number	Primer sequence	Annealing temp. (°C)	Amplicon size (bp)
TLR-2	NM_213761.1	F: CAGTCCGGAGGTTGCATATT R: ATGCTGTGAAAGGGAACAGG	58	137
TLR-4	NM_001113039	F: TGGAACAGGTATCCCAGAGG R: CAGAATCCTGAGGGAGTGGA	58	125
CD-14	NM_001097445.2	F: TGCCAAATAGACGACGAAGA R: ACGACACATTACGGAGTCTGA	59	174
MD-2	NM_001104956.1	F: TGCAATTCCTCTGATGCAAG R: CCACCATATTCTCGGCAAAT	59	226
MyD-88	NM_001099923.1	F: CCAGTTTGTGCAGGAGATGA R: TCACATTCCCTTGCTTTGCAG	54	185
IRAK-4	NM_001112693.1	F: CTTTGATGAGCGACCCGTT R: CCACTGCTGCAAGCTTCTTC	58	184
TRAF-6	NM_001105286.1	F: GGAGCTAACTGCGAAAATGG R: ACGCATGCACAGTTTGTAGC	58	234
PPIA	NM_214353.1	F: CACAAACGGTTCCCAGTTT R: TGTCCACAGTCAGCAATGGT	58	171
B2M	NM_213978.1	F: ACTTTTCACACCGCTCCAGT R: CGGATGGAACCCAGATACAT	58	180

F: Forward primer sequence, R: Reverse primer sequence, temp.: temperature.

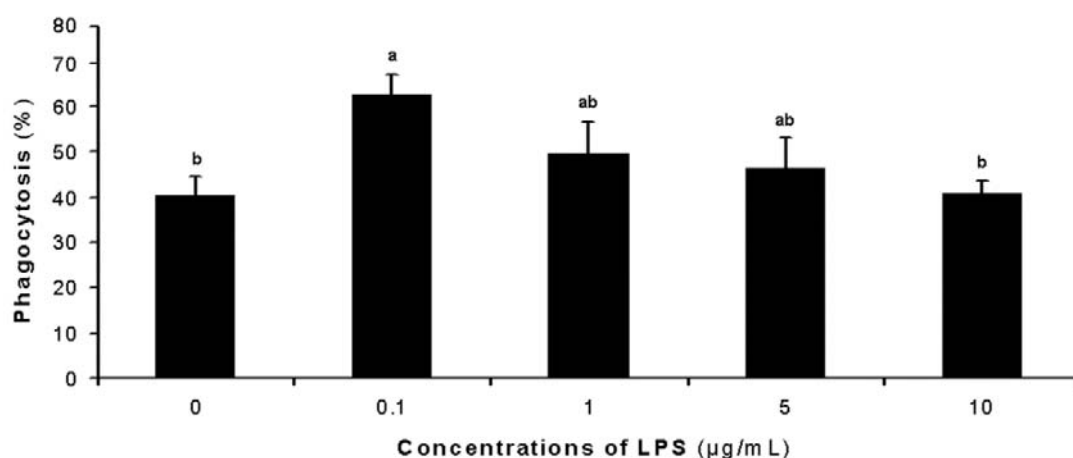


Figure 1. Effect of LPS-induced phagocytosis on porcine alveolar macrophages *in vitro*. Macrophages were incubated with FITC-labeled *E. coli* with LPS in the indicated concentrations at 37°C with 5% CO₂. Fluorescence as a result of bacteria adherent to the outside of the cells was quenched with trypan blue. Cell phagocytic capacity was determined by fluorescence microplate reader. The amount of fluorescent bacteria that has been engulfed by cells was measured as mean fluorescence intensity. The number of cells that were significantly phagocytic is expressed as a percentage of the whole population. Results represent the mean values for three pigs ± SEM. Five determinations were performed in each concentration of LPS per animal. Values with different letters denote a statistical difference among LPS concentrations ($P < 0.05$).

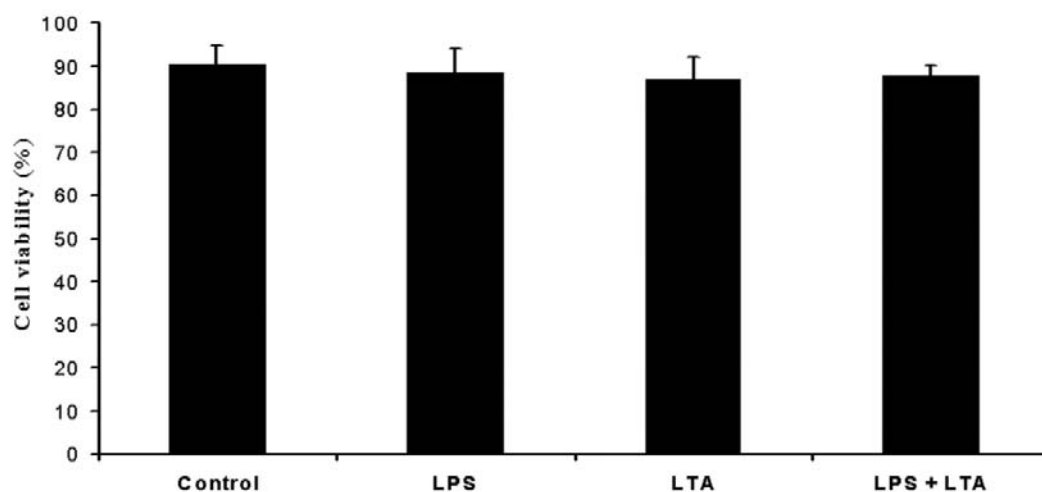


Figure 2. Effects of LPS, LTA and their combined stimulation on alveolar macrophage cell viability. Macrophages were stimulated with LPS at 1 $\mu\text{g}/\text{mL}$, LTA at 10 $\mu\text{g}/\text{mL}$ and with LPS (at 1 $\mu\text{g}/\text{mL}$) plus LTA (at 10 $\mu\text{g}/\text{mL}$) for 24 h. Cell viability was measured by Trypan blue exclusion. The results are expressed as the mean \pm SEM ($n = 3$) percent cell viability.

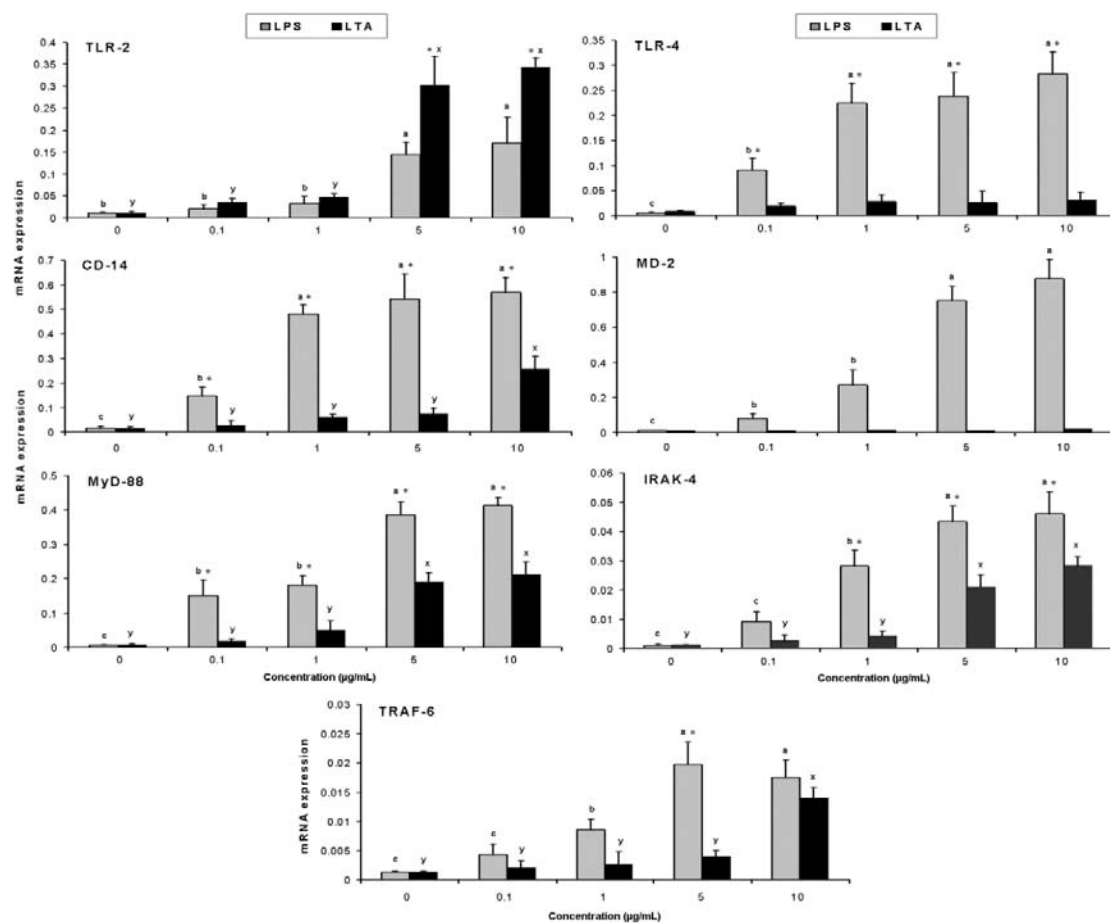


Figure 3. Comparative dose-dependent mRNA expression of TLRs and their associated pathway molecules in PAMP-induced porcine alveolar macrophages. Macrophages (2×10^6 cells/mL/well) from 40 days old piglets were incubated with the indicated concentrations LPS and LTA in triplicate. After stimulation for 24 h, the cells were harvested for quantification of mRNA expression by qRT-PCR. Graph panel are presented as the mean of pigs ($n = 3$) \pm SEM. The X-axis signifies the different concentrations of LPS and LTA ($\mu\text{g/mL}$). The Y-axis indicates the mRNA expression level following stimulation of cells with various doses of LPS and LTA vs. controls. Alphabetic characters a, b, c and x, y represent LPS (■) and LTA (■), respectively. Values with different letters denote a significant expression difference of mRNAs among various concentrations of PAMPs within stimulator group ($P < 0.05$) were analysed by Tukey test. Asterisk indicate significant expression differences between LPS and LTA at each concentration ($*P < 0.05$).

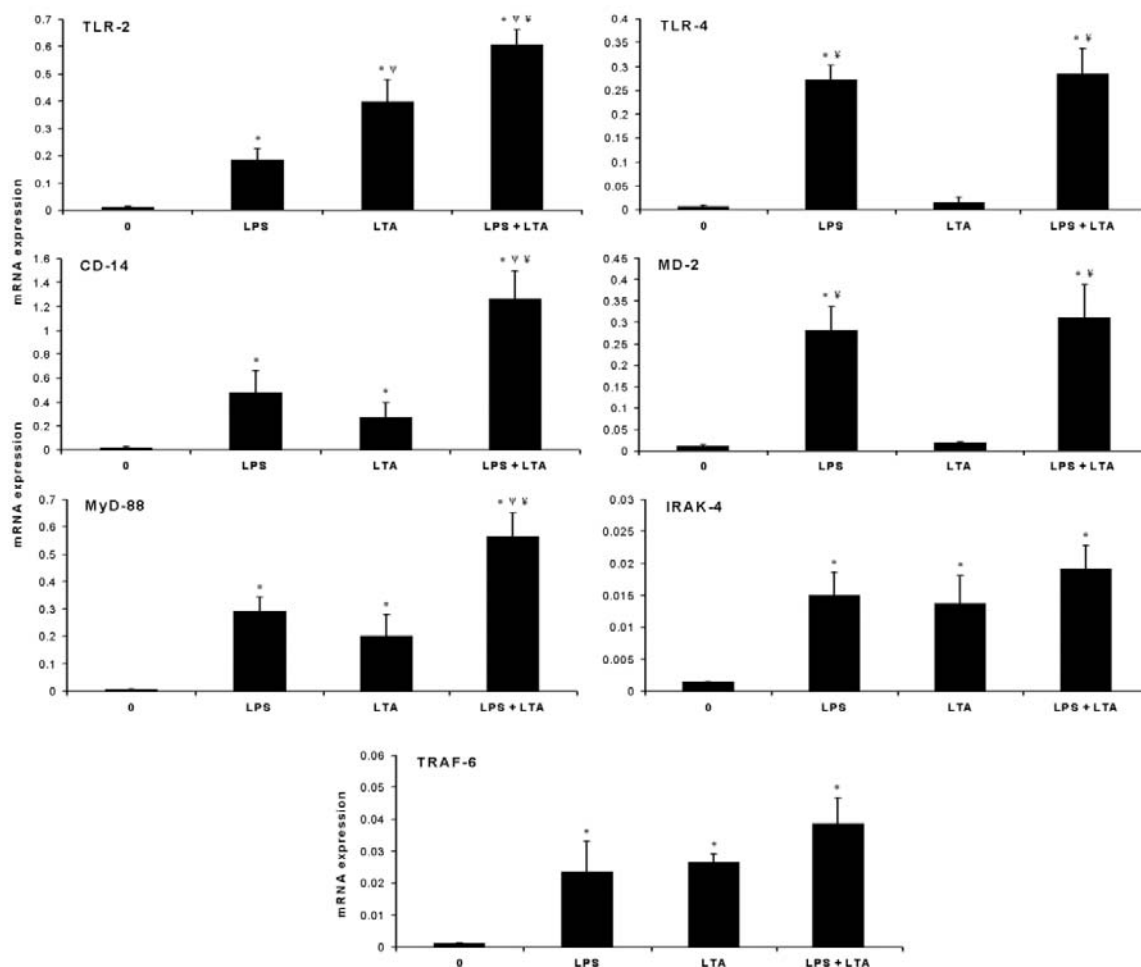


Figure 4. Expression levels of TLRs and their associated signalling molecules by PAMP-stimulated porcine alveolar macrophages. Macrophages (2×10^6 cells/mL/well) from 40 days old piglets were incubated *in vitro* for 24 h with LPS ($1 \mu\text{g/mL}$), LTA ($10 \mu\text{g/mL}$), or co-stimulation of LPS ($1 \mu\text{g/mL}$) and LTA ($10 \mu\text{g/mL}$). After the designated time course, total RNA was extracted from cells, and mRNA expressions were quantified by qRT-PCR. Graph panel are illustrated as mean of three animals \pm SEM. Asterisk indicate significant expression differences between the control and treated group ($*P < 0.05$). Symbols Ψ and ¥ indicate a significant expression difference from LPS and LTA, respectively ($P < 0.05$).

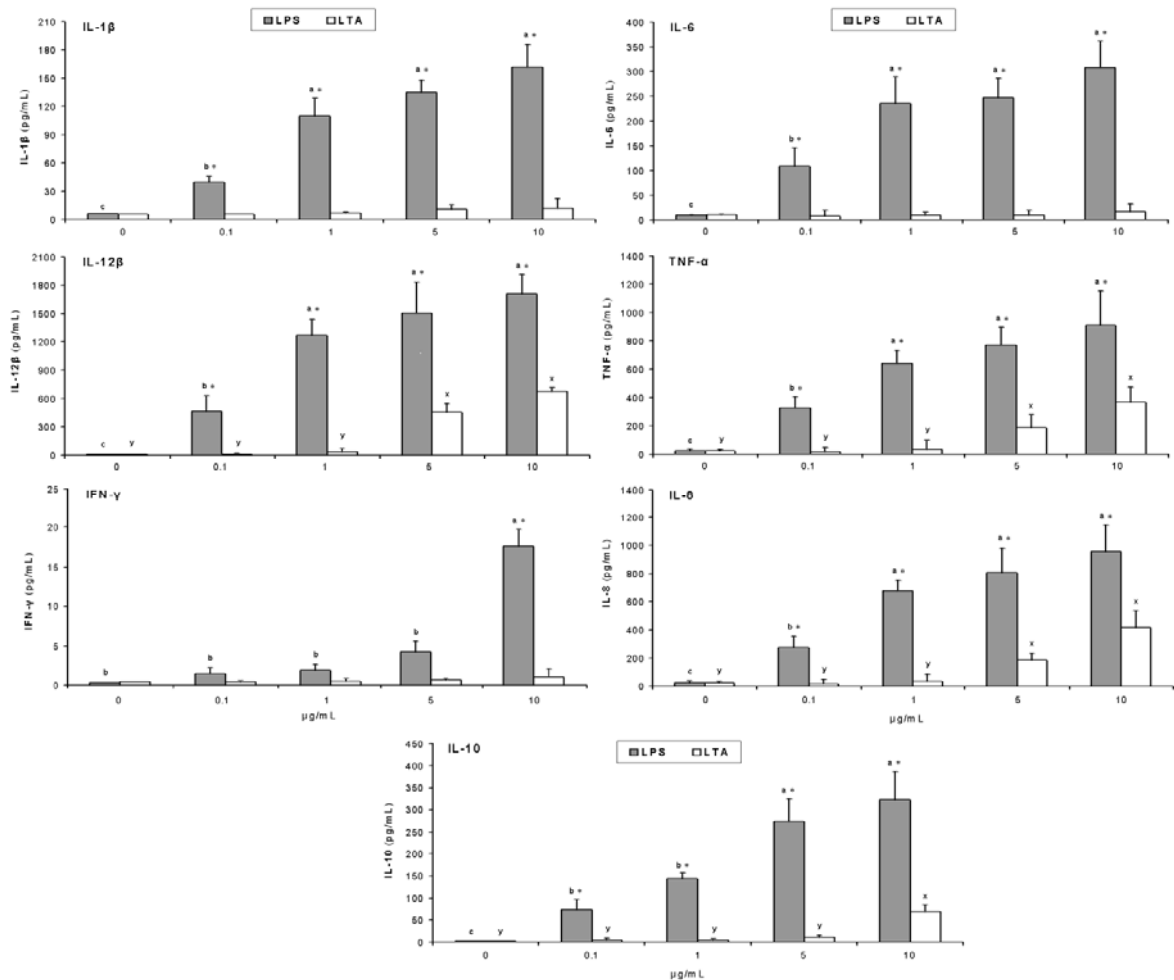


Figure 5. Comparative cytokine production by PAMP-induced alveolar macrophages in a dose-dependent manner. Cells were stimulated for 24 h as described in Fig. 3. The protein concentrations in the supernatants were measured by ELISA. Graph panel are presented as mean of three pigs \pm SEM. The X-axis signifies the different concentrations of LPS and LTA (μ g/mL). The Y-axis indicates the protein concentrations following stimulation of cells with various doses of LPS and LTA vs. controls. Alphabetic characters a, b, c and x, y represent LPS (■) and LTA (□), respectively. Values with different letters denote a significant production difference of cytokines among various concentrations of PAMPs within stimulator group ($P < 0.05$), analysed by Tukey test. Asterisk indicate significant expression differences between LPS and LTA at each concentration ($*P < 0.05$).

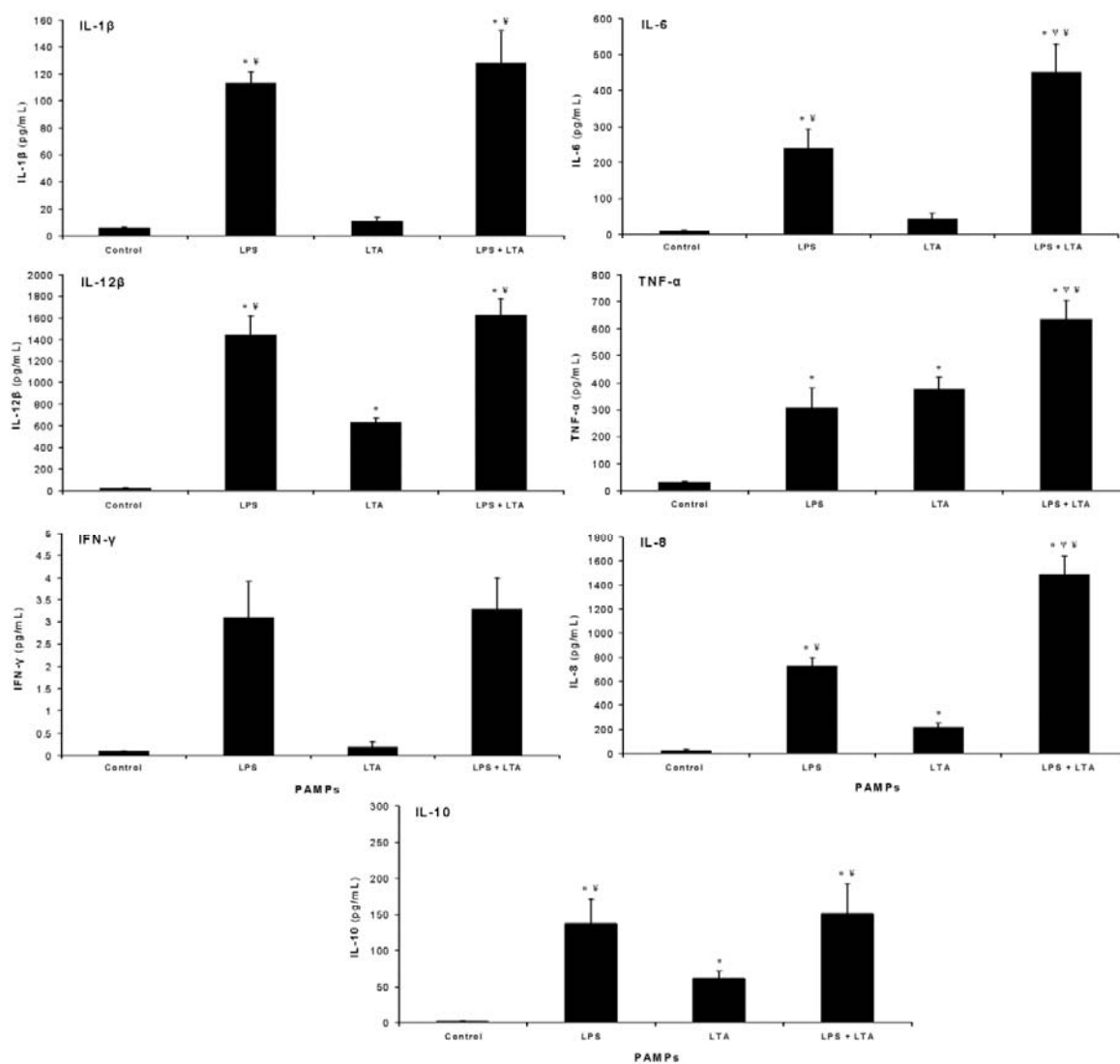


Figure 6. Synergistic effect of LTA with LPS on induction of cytokines in alveolar macrophages. Cells were stimulated for 24 h as described in Fig. 4. Cytokine concentrations from culture supernatants were measured by ELISA. Graph panel are illustrated as mean of three animals \pm SEM. Significant differences ($P < 0.05$) between the control and treated group are indicated with asterisk (*). Symbols Ψ and ¥ indicate a significant production difference from LPS and LTA, respectively ($P < 0.05$).