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Innate Immune Mechanisms Involved in Airway Inflammation
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Annerose Berndt

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INNATE IMMUNE MECHANISMS INVOLVED IN AIRWAY INFLAMMATION IN EQUINE RECURRENT AIRWAY OBSTRUCTION

By

Annerose Berndt

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ABSTRACT

INNATE IMMUNE MECHANISMS INVOLVED IN AIRWAY INFLAMMATION IN EQUINE RECURRENT AIRWAY OBSTRUCTION

By

Annerose Berndt

Introduction: Recurrent airway obstruction (RAO) is characterized by neutrophilic airway inflammation and obstruction, and stabling of susceptible horses triggers acute disease exacerbations. Stable dust is rich in endotoxin and fungi, such as *Faenia rectivirgula*, *Thermoactinomyces vulgaris* and *Aspergillus fumigatus.* Toll-like receptors (TLRs) are pathogen recognition receptors that are transmembrane proteins on a variety of airway cells (e.g. epithelial cells). Toll-like receptor 4 is the principal recognition receptor for lipopolysaccharide (LPS), and TLR2 is the principal recognition receptor for fungal products. In human bronchial epithelium, TLR4 and TLR2 stimulation leads to increased production of interleukin (IL)-8, a potent neutrophil attractant. The zinc finger protein A20 negatively regulates these pathways. ***Hypotheses:*** We hypothesized i) that horses in stables are exposed to higher endotoxin concentrations than on pasture; ii) that during stabling TLR4, TLR2, and IL-8 mRNA expression are increased in RAO-affected horses compared to controls, and that this would be paralleled by elevated neutrophil counts in bronchoalveolar lavage fluid (BAL); and iii) that A20 mRNA expression is not increased in RAO-affected horses during stabling compared to controls. ***Materials and Methods:*** We determined endotoxin concentrations in the breathing zone of six horses. Furthermore, we measured the maximal change in pleural pressure (ΔPpl_{max}),

determined inflammatory cell counts in BAL, and quantified TLR4, TLR2, IL-8, and A20 mRNA in bronchial epithelium by quantitative real-time polymerase chain reaction (qRT-PCR). For these experiments, we studied six age-matched horse pairs, each pair consisting of one RAO-affected horse and one control horse. Each pair was studied when the RAO-affected horse had airway obstruction induced by stabling, and after 7, 14 and 28 days on pasture. ***Results:*** Endotoxin concentrations in the breathing zone of stabled horses were significantly higher than in the breathing zone of these horses on pasture. While stabling increased BAL neutophils and ΔPpl_{max} as well as TLR4 and IL-8 mRNA expression significantly, TLR2 and A20 were unaffected. TLR4, but not TLR2, correlated with IL-8 and IL-8 was significantly correlated with BAL neutrophils and A20. When data were pooled, TLR2 and A20 were significantly correlated and A20 was negatively associated with inflammatory cells. ***Conclusions:*** In our stables, horses are exposed to an at least 8-fold higher concentration of endotoxin than on pastures. Elevated TLR4 expression and lack of A20 upregulation in bronchial epithelial cells from RAO-affected horses may contribute to elevated IL-8 production, leading to exaggerated neutrophilic airway inflammation in response to inhalation of stable dust. Stable dust exposure does not lead to an increase in TLR2 mRNA expression. The localization or the amount of the TLR2 receptor complex rather than the amount of TLR2 mRNA may be important in fungal-induced airway inflammation. Equine epithelial-derived A20 may protect against airway inflammation by decreasing the number of bronchoalveolar lavage inflammatory cells, and is involved in modulation of airway inflammation.

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This work is dedicated to the people, who, each with their own example, inspired me to search enthusiastically for new knowledge. Foremost to my parents **Jutta and Dietmar Berndt** for their true love and guidance that strengthened my steps towards developing an independent mind and strong personality, and whose values will always last in me and my work. In addition, to my sister, **Antje Berndt**, and my brother-in-law, **Eric A. Stone**, for their never-ending support, advice, and patience when facing my need for endless scientific conversations. They are the best shoulders one can fall back onto. Last, but not least, to **Tawnya L. Weaver** for her unconditional friendship and her living example of balancing an intensive work load.

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I would like to express my deepest thanks to my major advisor **Dr. Frederik J. Derksen** who, in every instance, supported my wish and carefully guided my path to become an independent researcher. I am not only thankful for all the scientific conversations, during which I learned how to integrate detailed scientific work into a complex biological and medical system, but also for his example of a truth-searching and humane scientist. I also wish to express my thanks to my guidance committee members: **Dr. N. Edward Robinson** for the many inspirational conversations and his never-ending support and trust in my research, **Dr. Susan Ewart** for her advice and support regarding research and gradate school, and for the many times she inspired me with her example as an outstanding researcher, **Dr. Vilma Yuzbasiyan-Gurkan** for her vision and everlasting energy to establish the amazing graduate program of Comparative Medicine and Integrative Biology (CMIB) in the veterinary college at Michigan State University, and **Norbert Kaminski** for establishing opportunities and his support and advice regarding my research. Furthermore, I am grateful for the chance to have met and worked with many other amazing people. In particular, I would like to thank **Dr. Annette Thelen** for her ability to put the sometimes stressful and draining bench work when in molecular biology into a colorful new and exciting world. One of my deepest Thanks goes to **Dr. Patrick J. Venta** for his patience in answering many of my burning questions about our current understanding in genetic and genomic research with a never-ending source of knowledge. In addition, I would like to express my thanks to **Lisa R. Bartner** for her friendship and her talented drawings that became part of this dissertation (Figure 2-1) and

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papers. Furthermore, I would like to acknowledge **Victoria Hoelzer-Maddox** for her help in preparing manuscripts, presentations, and this dissertation. Last, but not least, I express my gratitude to all the people I have had the chance to work with in the Pulmonary Laboratory, especially **Heather deFeijter-Rupp**, **Susan Eberhart** and **Cathy Berney,** without whom I would not have been able to carry through these intense research years. My stay at MSU would not have been possible without the experiences at the Department of Internal Medicine at the Veterinary Faculty at University of Leipzig, Germany, under the guidance of **Dr. Gerald F. Schusser**. Finally, I would like to acknowledge the funding sources that have made my research possible: foremost the Matilda Wilson Endowment, the 2003-04 German Academic Exchange Service (DAAD) Award, the 2005-06 grant from College of Veterinary Medicine's Genetic Research Fund, the 2005 Fellowship Award for PhD Training of Veterinarians from the College of Veterinary Medicine and the 2007 Graduate School Dissertation Completion Fellowship Award from Michigan State University.

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IL –
IL-R
IRAK
IRF
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I-κB
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LBP
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LIST OF ABBREVIATIONS

%Lym - percentage of lymphocytes in BAL
%Mac - percentage of macrophages in BAL
%Neu - percentage of neutrophils in BAL
A20 – zinc finger protein A20
AF - aspergillus fumigatus
Arab – arabian horse
BAL - bronchoalveolar lavage fluid
BB - bronchial brushing sample
C5a - complement protein 5a
CB – clenbuterol
CXCL - CXC chemokine ligand
DNA - deoxyribonucleic aci
EMSA - electromobility shift assay
EU – endotoxin unit
FC - fold change
fMLP - formyl-methionylleucylphenylalanine
G-CSF - granulocyte-colony stimulating factor
GM-CSF - granulocyte macrophage-colony-stimulating factor
GPCR - G-protein-coupled receptor
Grd - grade horse
GRK - GPCR kinase
H&E - Hematoxylin and Eosin
hBD - human β-defensin
HDS - hay dust suspension
HMGB - high-mobility group box
HSP -heat shock protein
ICAM - intracellular adhesion molecule
IFNR - IFN receptor
IFN-γ - interferon gamma
Ig - immunoglobulin
IKK complex - inhibitor of nuclear factor-κB (IκB)-kinase complex
IL – interleukin
IL-R - interleukin receptor
IRAK - IL-1 receptor associated kinase
IRF - interferon regulatory factor
ISH - *in-situ* hybridization
I-κB - inhibitor of NF-κB
kDa – kilo Dalton
LBP - LPS-binding protein
logLym - log10 transformed total BAL lymphocyte count
logMac - log10 transformed total BAL macrophage count
logNeu - log10 transformed total BAL neutrophil count
logTCC - log10 transformed total BAL cell count

LPS - lipopolysaccharide
LRR - leucine-rich repeat
LTB_4 - leukotrien B_4
Lym - total BAL lymphocyte count
Mac - total BAL macrophage count
MAC - macrophage antigen
MAL - MyD88 adaptor-like
MAP - mitogen-activated protein
MBL - mannan-binding lectin
MCP - monocyte chemotactic protein
MD2 - macrophage-derived molecule 2
MIP - macrophage inflammatory protein
mRNA – messenger RNA
MyD88 - myleloid differentiation primary response protein 88
n.m. - not measured
n/a - not applicable
Neu - total BAL neutrophil count
NF-κB - nuclear factor kappa B
NOD – nucleotide-binding oligomerization domain
PAF - platelet-activating factor
PAR – protein-activated
PBS - phosphate buffered saline
poly[I;C] - polyinosinic-polycytidylic acid
PRR - pattern recognition receptor
QH - quarter horse
qRT-PCR - quantitative real-time polymerase chain reaction
RNA – ribonucleic acid
S.D. - standard deviation
S.E.M. – standard error of the mean
SB - standard bred
SCC - spearman correlation coefficient
SP-A - surfactant protein A
SPAOPD - summer pasture associated obstructive pulmonary disease
STAT - signal transducer and activator of transcription
TAB - TAK1-binding protein
TAK - transforming-growth-factor-β-activated kinase
TB – thoroughbred horse
TCC - total BAL cell count
TICAM - TIR-containing adaptor molecule
TIR - toll/interleukin-1 receptor
TIRAP - TIR domain-containing adaptor protein
TLR – toll-like receptor
TNFR – TNF receptor
TNF-α - tumor necrosis factor alpha
TRAF6 - TNF receptor-associated factor 6
TRAM - TRIF-related adaptor molecule

TRA
TRI
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TRAM - TRIF-related adaptor molecule
TRIF - TIR domain-containing adaptor inducing IFN-β
UBC13 - ubiquitin-conjugating enzyme 13
UEV1A - ubiquitin-conjugating enzyme E2 variant 1
ΔPpl_{max} – maximal change in pleural pressure

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INTRODUCTION

Airborne pathogens, such as bacterial products (e.g., lipopolysaccharide [LPS]), fungal (e.g., spores, mycelium fragments, glucan), and yeast (e.g., zymosan) elements, plant pollens, and dust mites are inhaled and subsequently deposited on the airway mucosa. Different innate immune mechanisms involved in removing and defending the airways against these pathogens are present in the lung. Initially, inhaled pathogens become embedded in mucosal fluid and are removed by active transportation due to the ciliary movement of the airway epithelium. In addition, airborne particles are recognized by soluble factors, which are released into the mucosal surface fluid. Finally, pathogen recognition receptors are expressed on cell membranes of a variety of airway cells, such as alveolar macrophages, dendritic cells, epithelial cells, and airway smooth muscle cells. These innate receptors belong to different families, such as the toll-like receptor (TLR) family, the nucleotide-binding oligomerization domain (NOD) receptor family, and the protein-activated receptor (PAR) family. *In vivo* animal studies and *in vitro* studies using monocytes, alveolar macrophages, and airway epithelial cell cultures have been used to show the release of cytokines and chemokines (preferentially interleukin (IL-)1β, tumor necrosis factor (TNF-) α, IL-6, IL-8 etc.) upon stimulation with LPS, fungal and yeast products, dust mites, and pollen. The signaling cascades of recognition receptors are under feed-forward and feed-back regulatory mechanisms (e.g., feed-back mechanism through the zinc finger protein A20 of the TLR4 and TLR2 signaling pathways).

Recurrent airway obstruction (RAO) is a common airway disorder in horses characterized by recurrent neutrophilic airway inflammation and airway obstruction due

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to exposure to stable dust. Originally, it had been suggested that RAO is an allergic condition – especially, a type I hypersensitivity reaction has been suggested by different investigators – in response to inhalation of allergens found in hay dust. However, in the recent literature there is also evidence that the pathogenesis of RAO may involve aberrant innate immune mechanisms. Stable dust is a mixture of organic (e.g., bacterial, fungal, and viral) products and, therefore, could potentially stimulate signaling pathways, including those of TLRs. Toll-like receptors are the major cell membrane receptors that are stimulated by the recognition of conserved structures found in microorganisms (e.g., LPS, peptidoglycan) and endogenous products produced during acute inflammation. In the recent literature, inhalation of endotoxin from gram-positive bacteria and inhalation of fungi, such as *Faenia rectivirgula*, *Thermoactinomyces vulgaris,* and *Aspergillus fumigatus,* have been shown to trigger RAO in susceptible horses. Furthermore, the neutrophilic chemokine IL-8 is elevated in RAO-affected horses during acute exacerbations compared to control animals or to RAO-affected horses during remission. Although there were some attempts to study TLR4, the principal recognition receptor for LPS, and TLR2, the principal recognition receptor for fungal products, in equine airways, the exact mechanisms involved in pathogen recognition and subsequent signaling are still incompletely understood.

For my dissertation, I developed a pathway model (Figure 1-1) including molecules and receptors involved in TLR signaling pathways that could potentially be altered in RAO-affected horses and, therefore, contribute to the development of airway inflammation and obstruction during stabling. The overall organization of my dissertation follows the flow of this pathway model. Starting with the determination of endotoxin

concentrations in the breathing zone of horses while stabled or maintained on pasture, I subsequently quantified and compared gene expressions in bronchial brushing samples (BBs) at the messenger ribonucleic acid (mRNA) level for TLR4, TLR2, IL-8, and A20 between RAO-affected and control horses during and at three different time points after stabling. Finally, I adjusted the hypothetical pathway model for RAO to develop a pathway that is potentially involved in airway inflammation in the general horse population. The following is an overview of the topics covered in this dissertation:

1. The determination of endotoxin concentrations in the breathing zone of stabled horses compared to horses on pasture. The mean concentrations within stables were at least 8-fold higher than the inhaled endotoxin concentration on pasture (chapter 2);
2. The quantification of mRNA expression of TLR4 and IL-8 in BBs and their correlation with each other. TLR4 and IL-8 mRNA expression were increased during stabling compared to periods on pasture and correlated with each other (chapter 4);
3. The quantification of mRNA expression of TLR2 in BBs and its relation to the IL-8 expression. TLR2 mRNA expression was not effected by stabling nor correlated with IL-8 mRNA expression (chapter 5);
4. The quantification of mRNA expression of A20 in BBs and its relation to inflammatory cell counts in bronchoalveolar lavage fluid (BAL). A20 mRNA expression was not different between diagnostic groups and time points. However, when data from both diagnostic groups were pooled, A20 in BBs and inflammatory cells in BAL correlated negatively (chapter 6);

5. A statistical pathway analysis discussing airway inflammation in RAO-affected horses and the general horse population (chapter 7);
6. An outlook for potential future research developments in studying immune mechanisms involved in RAO's pathogenesis (chapter 8).

My dissertation is divided into the following chapters. Chapter 1 provides the current knowledge in the literature about the studied scientific field. Chapters 2, 4, 5, and 6 are written in the form of scientific peer-review papers, including abstract, introduction, materials and methods, results, and discussion. Their modified versions have been published or have been submitted for publication. Whereas these chapters can be read independently of each other, chapters 3, 7, and 8 either provide background information or draw conclusions about the research results obtained in chapters 2, 4, 5, and 6.

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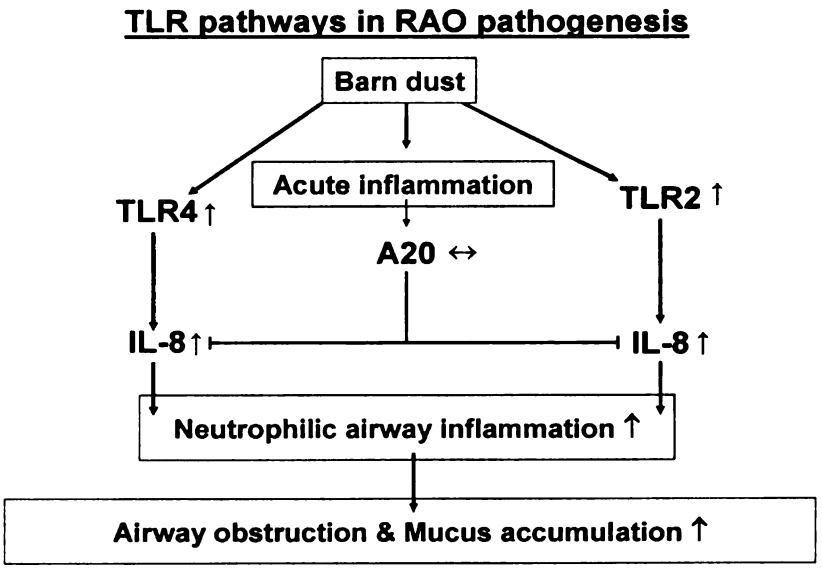
Figure 1-1: Pathway model of Toll-like receptors that are potentially involved in RAO pathogenesis.



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CHAPTER 1

LITERATURE REVIEW

Recurrent Airway Obstruction – Definition and Pathogenesis

Recurrent airway obstruction (RAO), also known as heaves, is a common pulmonary disorder in horses. Acute exacerbations are characterized by neutrophilic airway inflammation, mucus accumulation, and bronchospasm, which are triggered by exposure of susceptible horses to stable and hay dust. Recurrent airway obstruction is a diffuse airway disease involving large conducting airways down to the terminal bronchioles. Periods of acute exacerbation are interspersed by periods of remission, which can be achieved by returning RAO-affected horses from stables to pasture (151).

The mechanisms responsible for airway inflammation in RAO-affected horses are not well understood. Originally, it was suggested that RAO is an allergic condition (151). Elevated immunoglobulin (Ig) E levels in bronchoalveolar lavage (BAL) of affected animals support the assumption of a type-I hypersensitivity reaction (63, 162). Increased expression of T-helper cell (Th) type 2 (Th-2) specific cytokines, such as IL-4 and IL-5, as well as decreased expression of Th-1 specific cytokines, such as interferon gamma (IFN-γ), further support the suggestion that adaptive immune mechanisms are involved in the pathogenesis of RAO (18, 29, 59, 99). Interleukin-4 is important for the differentiation of uncommitted T-cells into the Th-2 subset and, therefore, an increase in Th-2-type cytokine production. Furthermore, IL-4 is required for B-cell isotype switching to IgE antibody production, which might contribute to the local IgE production in the lungs of RAO-affected horses during acute exacerbations (63, 162). Interleukin-5 is a

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cytokine involved in proliferation of granulocyte progenitors, especially eosinophils, in the bone marrow (119). The increase in IL-5 in stabled RAO-affected horses (29, 99) is supported by an increase in IL-5 mRNA expression in sensitized ponies after ovalbumin inhalation challenge (18). However, horses affected by summer pasture associated obstructive pulmonary disease (SPAOPD) did not exhibit a difference in IL-5 production (77). Since RAO is characterized by a neutrophilic rather than an eosinophilic influx into the airway lumen, the reported increase of IL-5 in BAL lymphocytes needs to be the subject of further studies. Interestingly, there was an increased expression of the IL-5 receptor (IL-5R) on peripheral neutrophils from RAO-susceptible horses compared to controls on pasture and compared to RAO-affected horses after stabling (34). Thus, it could potentially be interpreted that IL-5 might have a role in neutrophil recruitment into the lung. On the other hand, mRNA expression of IL-17 – a cytokine, which is secreted by activated T-cells and which is indirectly involved in maturation, chemotaxis and activation of neutrophils – has been shown to be higher in the BAL of RAO-affected horses than in control horses after stabling (4, 31). Cytokine expression profiles in RAO-affected horses have been studied by different investigators and are inconsistent among their studies (3, 29, 59, 99). For example, while Lavoie et al. showed an increase in IL-4 and IL-5 mRNA expression and a decrease in IFN-γ expression in RAO-affected horses during stabling (29, 99), Ainsworth et al. did not find changes in IL-4 and IL-5 mRNA expression but rather showed an increase in IFN-γ mRNA expression (3, 4). The latter is supported by Giguere et al., who also showed an increase in IFN-γ (3, 59). One should keep in mind that the different results might be due to either different protocol designs or the use of different analytical methods (e.g., *in situ* hybridization vs. q-PCR etc.). Finally,

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the finding that allergic skin reactions to common barn allergens lack significant differences between affected and control horses (41, 115) supports the assumption that adaptive immune mechanisms may only partially explain the RAO disease pathogenesis.

Beside adaptive immune mechanisms, innate immunity seems to play a crucial role as well (142). Hay dust is a mixture of organic (e.g., bacterial, fungal, and viral products) and non-organic (e.g., metals, particles) materials. Inhalation of endotoxin-depleted hay dust significantly attenuates airway neutrophilia in affected horses (144). The inflammatory response can be re-established by adding endotoxin back to the endotoxin-depleted hay dust (141). These observations confirm that attenuation of airway inflammation is due at least partially to endotoxin. Furthermore, horse stables contain airborne fungi (128), such as *Faenia rectivirgula* and *Aspergillus fumigatus*, that when inhaled can induce neutrophilic airway inflammation and deterioration of lung function in susceptible horses (33, 116, 143, 162). Finally, pro-inflammatory cytokines and chemokines, such as IL-1β, TNF-α, and IL-8 have been shown to be increased in either BAL cells or airway epithelial cells in RAO-affected horses during acute exacerbations (4, 13, 51, 59).

Endotoxin-activated Immune Mechanisms in the Lung

In conventional horse stables, airborne endotoxin concentrations exceed those on pasture (117) and those that can induce airway inflammation in human subjects (171, 172). In our own stables, we observed that mean endotoxin concentrations in the breathing zone of stabled horses are at least 8-fold higher than concentrations on pasture (chapter 3). While endotoxin is present in barn dust in high concentrations, barn dust does

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not cause exaggerated airway inflammation in stabled control horses. Marti et al. showed that there is a greater risk of RAO in the offspring from two affected parents than if only one or no parent were affected (112). This suggests that RAO is a multifactorial equine pulmonary disorder, in which environmental and genetic factors combined cause the phenotypical outcome.

In many stables airborne endotoxin concentrations exceed those that can induce pulmonary inflammation and bronchial hyperresponsiveness in human subjects (occupational asthma), and those that can cause bronchospasm in humans with pre-existing pulmonary conditions (25, 26, 171, 172). It has been reported that respiratory symptoms of humans increase when the air endotoxin concentration is greater than 250 Endotoxin Units (EU)/m^3 (1 ng/m^3 ~ 10 EU). Therefore, the 'Dutch Expert Committee on Occupational Standards' has recommended a health-based occupational exposure limit of 50 EU/m^3 over an eight-hour period of endotoxin exposure. However, endotoxin levels in stables greatly exceed these limits (177), and may be as high as 35,000 EU/m^3 within the breathing zone of stabled horses (chapter 3).

Pathophysiology: Inhalation of endotoxin has been linked to increased amount of neutrophils in BAL (129, 140, 142-144, 169). This may be either due to an elevated migration of neutrophils from the pulmonary vasculature into the airway lumen or due to delayed neutrophil apoptosis and clearance from the air lumen by alveolar macrophages (20). Endotoxin stimulates neutrophil migration on different levels. In other animal models and in *in vitro* studies, potent CXC-chemokines (e.g., IL-8 and macrophage inflammatory protein 2 [MIP 2]), chemoattractants (e.g., platelet-activating factor (PAF), leukotrien B_4 (LTB_4), complement protein 5a [C5a]), and pro-inflammatory cytokines

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(e.g., TNF, IL-1 and IFN-γ) that are involved in modulation of adhesion molecules (e.g., an L-selectin ligand on endothelial cells (176), P-selectin (114), E-selectin (88, 163), and intracellular adhesion molecule 1 [ICAM-1]; (80)) are elevated due to endotoxin exposure (189, 190). During acute exacerbations due to stabling, RAO-affected horses exhibit elevated amounts of IL-8 (4, 13, 50, 51, 59), MIP-2 (51), and ICAM-1 (21).

Sources of endotoxin in the environment: Endotoxin is found at high concentrations in agricultural dust, such as dust from grain (164), poultry (35), swine (36), dairy cow (94), and horse housings (117, 145) as well as in cotton mills (27) and potato plants (76). Furthermore, endotoxin exposure is also an important contaminant in water-mist generating industries, such as the paper (158), metal (1, 150), and fiber-glass (120-122) industry. It has also been suggested that the *sick building syndrome* results at least partially from the endotoxin loads found in office buildings (182). Humidifiers are linked to an increased endotoxin concentration, whereas dehumidifiers decrease it. The presence of a dog in family homes has been shown to be the strongest predictor for endotoxin levels (120). Cigarette smokers are associated with an increased exposure to endotoxin than non-smokers (64).

Measurement of endotoxin – Endotoxin concentrations are commonly detected by the *Limulus amebocyte lysate* assay (LAL assay) (180). Amebocytes, which are nucleated, granular cells that aggregate and clot at the site of an injury, from the hemolymph of the horseshoe crab (*Limulus*) are used for this test. Aggregation of the amebocytes in the limulus is caused by the endotoxin originating from the blue-green algae. The content from the intracytoplasmic granules from the amebocytes is used in the LAL assay. Enzyme activation can either be determined by clot formation (clot end-

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point) or by color changes over time using a dye that reacts with the enzyme (kinetic chromogenic). The latter is frequently used to determine endotoxin in environmental samples (160). Different endotoxin collecting and analytical methods (e.g., extraction, dilution, storage, commercially available lysates) contribute to varying results (up to 100-fold) among laboratories and, therefore, contribute to difficulties in establishing safety regulations for endotoxin exposure (28, 177).

Recruitment of Inflammatory Cells into the Lung

Neutrophil Emigration Mechanisms that are Important during Lung Inflammation - Neutrophil migration from blood to the lung lumen can be seen as a series of sequential steps, including 1) capture and rolling of neutrophils on endothelial cells (ECs) in postcapillary venules; 2) firm adhesion, which is mediated by integrins; and 3) transmigration.

Capture and rolling is directed by adhesion molecules, called selectins. E-selectin and P-selectins are expressed on endothelium and L-selectins on neutrophils. Whereas L-selectin is important for neutrophil rolling on ECs, P-selectin is crucial for their capture. Both, L- and P-selectin work in combination to initiate migration processes during inflammation (30). Selectins are known to be autoproteolyzed in response to LPS and TNF-α (190). On the other hand, *in vitro* studies showed induction of L-selectin ligand expression after LPS or cytokine exposure of ECs (176). Endothelium-bound selectins (P- and E-selectin) are membrane-expressed only when appropriate inflammatory stimuli are present. P-selectin is stored in intracellular vesicles (Weibel-Palade bodies) and is mobilized to the cell surface within minutes after exposure (114). E-selectins are

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transcribed upon stimulation with inflammatory cytokines, so that their membrane expression is highest after 4 to 6 hours of stimulation (88, 163). The earliest accumulation of neutrophils within the airways of RAO-affected horses was reported after 5 hours stabling (20).

During the processes of firm adhesion, neutrophils bind to ECs, which is mediated by CD18-containing integrins (e.g., CD11a/CD18 and CD11b/CD18). The integrin CD11b/CD18 (also termed macrophage antigen-1 [MAC-1]) plays a crucial role in neutrophil migration during inflammatory processes (107). It is intracellularly stored and, upon stimulation (e.g., LPS, TNF-α, etc.), it is translocated to the cell membrane (8). An endothelial ligand for CD11b/CD18 is ICAM-1, which in general is expressed at low levels. However, due to stimulation of ECs by cytokines, ICAM-1 becomes strongly up-regulated (80, 88, 163). In addition, LPS and IL-8 stimulate the production of integrin-modulating factor-1 on neutrophils, which is responsible for augmenting CD18-ligand binding (89). ICAM-1 (21) and IL-8 (4, 13, 50, 51, 59) are elevated in lungs from RAO-affected horses during acute exacerbations.

The final step of recruitment of neutrophils into the lung is called transmigration. Migratory processes are accompanied by release of neutrophil-derived proteases (32). In RAO-affected horses it has been shown that equine neutrophil elastase A2 is increased in bronchoalveolar lavage during stabling than on pasture (20).

Soluble Mediators of Migration – There are two classes of promigratory stimuli, i) nonchemotactic cytokines and ii) chemoattractants.

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Cytokines: Inflammatory cytokines, for example TNF-α and IL-1β, increase expression of adhesion molecules on neutrophils and ECs, but they do not exhibit chemotactic functions for neutrophils. Within the lung, TNF-α and IL-1β are primarily released by alveaolar macrophages due to stimulation with inflammatory stimulants, such as LPS. Laan et al. showed that after 6 and 24 hours of stabling, production of TNF-α and IL-1β by alveolar macrophages from RAO-affected horses was higher than in control horses (95). TNF-α and IL-1β activate neutrophils and ECs by stimulating the expression of adhesion molecules (e.g., ICAM-1) and chemoattractants (e.g., IL-8).

Chemoattractants: Beside the increase of adhesion molecule expression, chemoattractants, such as IL-8, LTB_4, C5a, and PAF primarily direct migration of neutrophils through tissue. They are released by macrophages, neutrophils, lymphocytes, parenchymal cells, endothelial and epithelial cells, etc. The initiation of neutrophilic transendothelial and tissue migration is initiated by binding of chemoattractants to receptors on the neutrophil surface. Specific receptors for chemoattractants on neutrophils have been shown for chemokines, such as IL-8, for C5a, LTB_4 and bacterial products (e.g., endotoxin or formyl-methionylleucylphenylalanine [fMLP]).

Chemokines are small proteins (6 to 15 kDa) and function as the primary stimulus for neutrophilic migration. Interleukin-8 is the most studied chemokine in this group and has been shown to be produced by neutrophils themselves as well as endothelium, epithelium, and a variety of parenchymal cells (139). Interleukin-8 belongs to the CXC-chemokines. Chemokines are divided into two classes due to the sequence of their two most amino-terminal cysteines. The CXC chemokines (also termed α-chemokines) contain an amino acid (X) between the cysteines and attract most strongly neutrophils,

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whereas the cysteines in CC chemokines (also termed β–chemokines) are located next to each other and elicit the stronglest chemoattractive effects for mononuclear cells. Although IL-8 is the most potent stimulus for neutrophil migration in humans and most of other mammals, rodents (e.g., mouse, rat) do not have IL-8. However, they have proteins similar to growth-related oncogenes called cytokine-induced neutrophil chemoattractants (CINCs), for example CINC3, also called macrophage-inflammatory protein-2 (MIP-2) (166). LPS-stimulation of rat macrophages leads to elevated release of CINCs (166). It has been shown that CINCs are involved in neutrophilic response in the lung due to bacterial and inhalation challenges (38, 52, 53).

Interleukin-8 has been studied in RAO-affected horses by several research groups. Franchini et al. showed for the first time that a high dust exposure was paralleled by an increased chemotactic activity for neutrophils in BAL from RAO-affected versus control horses. Isolated macrophages showed the secretion of two chemoattractants, which were subsequently identified as IL-8 and MIP-2 (51). In addition, IL-8 was increased in parallel with the percentage of neutrophils in BAL (50). Later, Giguere et al. showed that IL-8 protein concentrations and mRNA expression were significantly higher in BAL from heaves-affected horses than from controls (59). Recently, Ainsworth et al. reported that IL-8 mRNA expression did not differ in bronchial epithelium and BAL neutrophils between RAO-affected horses and controls during remission and after one day of stabling. However, after 14, 35, and 49 days of stabling, RAO-affected horses exhibited an up-regulation in IL-8 mRNA in epithelial cells (3.3-fold, 8.5-fold, and 10.3-fold, respectively). After 1, 14, 35, and 49 days IL-8 was higher in BAL neutrophils (3.7-fold, 9.5-fold, 7.6-fold, and 14.8-fold, respectively) due to its down-regulation in controls,

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followed by an up-regulation in RAO-affected horses. The mRNA expression was paralleled by increased staining of IL-8 protein in bronchial biopsy samples (4). Beside IL-8, other chemokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte CSF (G-CSF), and growth-related oncogene α (CXCL1) have also been investigated in airway epithelial cells; however, their gene expression was not altered between affected and control horses (4).

Leukotriene B_4 (LTB_4) is a product of the arachidonic acid pathway and is released from stimulated monocytes and neutrophils. It functions in neutrophilic chemotaxis and supports the attachment of neutrophils to the endothelial cells in the vasculature (131). Our laboratory reported previously that stabling is associated with an increase in LTB_4 in RAO-affected horses and that this increase was correlated with the migration of neutrophils into the airway lumen (103).

Complement protein C5a is a cleavage product of the complement protein C5 in the classical complement as well as the alternative complement pathways. In the airways, C5 is produced by alveolar macrophages and type II pneumocytes (178). Therefore, neutrophils migrate along the C5a gradient, with their highest concentration within the airway lumen and declining concentrations towards vasculature.

Another chemoattractant is *PAF,* which is produced by endothelial cells, platelets, neutrophils, and alveolar macrophages. It consists of an acetylated phosphoglycerolipid, which originates from cell membranes. Beside chemotaxis, PAF also modulates neutrophilic adhesion to endothelial cells (15, 93, 202). Platelet-activating factor has also been studied in RAO-affected horses in the last decade. Marr et al. showed that administration of a PAF receptor antagonist (WEB 2086) did not suppress the

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neutrophilic migration from the vasculature into the airway lumen after seven hours of stabling. Therefore, it was suggested that PAF may not play a central role in the pathogenesis of neutrophilic airway inflammation in RAO-affected horses (110). On the other hand, Fairbain and colleagues showed that intravenous administration of PAF led to an immediate but transient increase of radiolabeled neutrophils in the lung of healthy and asymptomatic RAO-susceptible horses. This response could be inhibited by the PAF receptor antagonist. Therefore, PAF might be involved in the chemotaxis of neutrophils into the lung due to inflammatory stimuli in the general horse population (42). In addition, phosphodiesterase isoenzymes (PDE) have been shown to inhibit PAF release (109). However, the PAF-induced adhesion of equine neutrophils obtained from RAO-affected horses to fibronectin-coated plastic could not be modulated with the PDE4 inhibitor *rolipram* (149).

Pathways of Innate Immune Mechanisms within the Lung

Introduction: Our understanding of the innate immune system originated with the recognition of leukocytes in inflamed tissue in 1779, and Metchnikov's discovery of mobile ameboid cells (phagocytes) in sea anemones in 1882 (168). Our current understanding of innate immunity includes studies about microbial binding proteins and microbial-recognizing receptors. Throughout evolution, strategies have evolved that enable the differentiation between airborne pathogenic and nonpathogenic particles that come in contact with the pulmonary epithelium. In higher vertebrates, two interactive defense mechanisms, the innate and the adaptive immunity, have been developed. The evolutionary older system, the **innate immune system**, consists of i) soluble factors,

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released into the airway mucosa by a variety of cells, that bind microbial products and, therefore, make them available for clearance by phagocytic cells; ii) innate receptors on phagocytic cells (e.g., macrophages, neutrophils, dendritic monocytes), that bind microbial products and initiate other defense mechanisms (e.g., production of chemokines); and iii) innate receptors on airway epithelial cells (e.g., toll-like-receptors). The innate immunity is constantly ready to recognize and eliminate microbial products and by recognizing common microbial patterns, the innate immune system exhibits broad specificity.

In contrast, the **adaptive immune system** consists of lymphocytes that specifically recognize peptide sequences that are presented on specialized antigen-presenting cells (APCs). Upon recognition, these lymphocytes respond with the production of high-affinity antibodies that are capable of opsonizing microbes that express the same specific peptide sequences. Leukocytes (e.g., macrophages, neutrophils) clear opsonized microbial products within the airways or adjacent lymph nodes. The adaptive immunity is further characterized by its memory (i.e., specialized lymphocytes initiate a faster response upon recognition of microbes, which were encountered at an earlier time point).

Innate Immunity of the Airways: The respiratory system is the largest interface between host and environment. For humans, it has been estimated that the alveolar epithelium alone equals about the size of a tennis court (199). At rest humans breathe about 10,000 liters (11) of air daily and it has been established that the average horse breathes at least 70,000 liters of air daily. Therefore, the lung is constantly exposed to a large number of airborne pathogens. However, infectious airway diseases are relatively

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rare and this is evidence for a highly efficient defense mechanism located within the airway mucosa. The innate immunity of the airways is a combination of humoral and cellular components. The humoral component includes antimicrobial products released into the epithelial lining fluid, such as collectins, defensins, lysozyme, lactoferrin, cathelicidins, complement, and immunoglobulins (e.g., IgG) (199). The cellular component consists of leukocytes, such as neutrophils, alveolar macrophages, dendritic monocytes, cytotoxic T cells, natural killer cells, and airway epithelial cells. Although different innate receptor families, such as TLRs, NODs, and PARs, can be found on leukocytes and epithelial cells, TLRs are the major cell surface receptors for recognition of microbial and toxic endogenous products (199).

Recognition of respiratory pathogens by the airway epithelium: As a response to inhaled pathogens, airway epithelial cells activate defense mechanisms, such as the release of microbicidial products into the airway lumen as well as the release of cytokines and chemokines into the submucosa. Chemokines and cytokines play a crucial role in recruiting phagocytes for clearance of airways and airway wall of airborne pathogens that could not be removed by the movement of the mucosal cilia. Additionally, dendritic cells and lymphocytes are also attracted by mechanisms of epithelial cells. Therefore, airway epithelium plays a central role in stimulating and modulating the immune response in the respiratory system.

The response of epithelial cells to inhaled pathogenic molecules, such as lipopolysaccharides and leipoteichoic acid, has been studied over the last decades. However, detailed mechanisms and molecules involved in the recognition process were still incompletely understood. Airway epithelium uses pattern recognition receptors

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(PRRs) for recognition of conserved molecular patterns presented on inhaled pathogens. These receptors can be found as transmembrane receptors on epithelial cells (e.g., TLRs) or can be released into the epithelial lining fluid or the circulation in soluble forms (e.g., mannan-binding lectins [MBLs]). TLRs are recognized in almost all cells that have been studied. Their function lies not only in initiating innate immune processes, but also in modulating the cross-talk between the innate and the adaptive immunity. For example, TLRs have been shown to be distributed widely on dendritic cells, where they modulate the adaptive immune response by influencing dendritic cells in the way they interact with T-cells. In airway epithelial cells, as in many other cell types, recognition of microbial products by TLRs leads to the production of cytokines and chemokines, as well as antimicrobial peptides. Additionally, TLRs have also been reported to modulate the function of surfactant protein A. For that reason, TLRs play a crucial role in chronic fungal and allergic asthma and inflammatory lung diseases, as well as allograft rejection after lung tranplantation.

Toll-like receptors (TLRs) are highly conserved across the animal kingdom and were first recognized as proteins involved in the dorsal-ventral development in *Drosophila melanogaster*. Later they were found to be important in antifungal defenses. Medzhitov et al. recognized that the intracellular domain of one of these proteins, termed *Drosophila Toll*, was highly homologous with the human interleukin (IL)-1 receptor (12). Therefore, TLRs belong to the Type I transmembrane receptor family (i.e. TLR/IL-1 R superfamily), which bears a leucine-rich repeat (LRR) domain at the extracellular amino (NH_2)-terminus and a toll/interleukin-1 receptor (TIR) domain at the intracellular carboxy (COOH)-terminus, which is critical for adaptor protein binding. Currently,

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thirteen mammalian TLRs have been identified. In mice, TLR1-9 and TLR11-13 have been discovered. Table 1-1 gives an overview of human and mouse TLRs and their ligands. Among the TLRs, TLR4 and TLR2 have been the most intensively studied TLRs in the recent literature. Their most important function is the detection and response to pathogen associated molecules, such as LPS (TLR4) or lepoteichoic acid (TLR2) (71, 124, 146, 147). However, endogenous factors that are released during inflammation and cell damage can also activate TLRs. For example, the transcription factor high-mobility group box 1 (HMGB1) can activate TLR4 and TLR2 when it is released extracellularly during inflammation (138). In addition, TLR4 responds to tissue break-down products, such as hyaluronan oligosaccharides (181, 183), and to proteins that are common at inflammatory sites, such as fibrinogen (173), surfactant protein A (SP-A) that activates macrophages by binding to TLR4, and β-defensin-2, which activates dendritic cells by binding to TLR4. However, activation of TLR4 by heat shock protein 60 and 70 (HSP60 and 70) is potentially caused by LPS contamination of commercially available preparations (54, 55) as it was the case with TLR2 activation by LPS-contaminated preparations (71). Toll-like receptors have been shown to be expressed in a variety of lung cells, such as alveolar macrophages, dendritic cells (183, 188), epithelial cells (61, 123), endothelial cells (10, 181), and airway smooth muscle cells (125).

Toll-like receptor 4 is best known for its response to LPS. In 1998-99, research groups identified the gene responsible for LPS resistance in two inbred strains of mice (C3H/HeJ and C57BL/10ScCr) (78, 146). Whereas C3H/HeJ mice have a single point mutation in the cytosolic TIR domain (see below), C57BL/10ScCr mice do not express TLR4 mRNA. In C3H/HeJ mice a point mutation in the 712^{th} amino acid of TLR4

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replaces the proline residue with a histidine residue, which renders TLR4, and therefore, the animals LPS unresponsive (84). However, mutations observed in the equine TLR4 sequence could not be related to an altered LPS response in this species (193). Mutations in the signaling domain of TLR4 in these LPS-hyporesponsive mice initiated a search for polymorphisms in the human homolog. Subsequently, polymorphisms in the human TLR4 gene were associated with diseases (e.g., gram-negative bacterial infections in patients in intensive care units) (66). Inhaled LPS is bound to LPS-binding protein (LBP), which is an acute phase protein, produced not only by liver cells but also by airway epithelium. Lipopolysaccharide, bound to LBP, is transferred to GPI-anchored or soluble protein CD14. Beside CD14 the LPS recognition complex also consists of the LRR-domain of TLR4 and the macrophage-derived molecule 2 (MD2). In addition to TLR4, genomic sequence alterations of CD14 and MD2 have been shown to be associated with altered responses to LPS in mice and humans. No alterations could be found in equine CD14 and MD2, however (193). The LPS recognition complex (TLR4/CD14/MD2) initiates an intracellular signaling cascade by recruiting adaptor molecules, such as the myleloid differentiation primary response protein 88 (MyD88), to the intracellular domain of TLR4 (details of the signaling cascade are discussed below).

Toll-like receptor 2 recognizes a wide variety of microbial products from bacteria and fungi. Like TLR4, it is expressed in epithelial cells. However, whereas TLR4 seems to be localized more basolaterally, TLR2 is generally recognized on the apical cell membrane (65). Although TLR2 is the principal receptor involved in the recognition of leipoteichoic acid from gram-positive bacteria, TLR2 also detects structural variants of LPS, e.g., from leptospira spp. Additionally, TLR2 is involved in the recognition of

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mycobacterial lipoarabinomannan. It has been suggested that TLR2 might have a role in defense against atypical mycobacterial infections and *M. tuberculosis*. There is evidence that TLR2 is important in fungal pneumonias, as TLR2 knock-out mice have a decreased survival when challenged intra-nasally with *Cryptococcus neoformans* when compared with wild-type controls and TLR4 knock-outs (198). However, the survival of MyD88 knock-out mice in this study was worse in comparison to the TLR2 knock-outs, outlining the complexity and redundancy of components involved the innate immunity (198). Most interestingly, the pathogen involved in the most common cause of gram-positive pneumonia (*Streptococcus. pneumoniae*), does not exert its pathology through TLR2 signaling (90). Although, alveolar macrophages need TLR2 to response to *S. pneumoniae in vitro*, TLR2 knock-outs and control mice do not respond differently when stimulated with *S. pneumoniae* intranasally. Other immune mechanisms might be involved in the defense against this pathogen. Toll-like receptor 2 recognizes airborne pathogens, such as products from gram-positive bacteria, mycobacteria, and fungi by heterodimerization with TLR1 or TLR6. For example, alterations in the expression or availability of one of the TLR2 co-receptor (e.g., TLR1 or TLR6) have been shown to be important in contributing to disease processes. Indeed, recently it has been reported that a defect of TLR2/1 signaling may be due to decreased TLR1 surface expression, while TLR2 expression was unchanged (186).

Toll-like receptor 3 knock-out mice experiments revealed that TLR3 recognizes double stranded (ds) ribonucleic acid (RNA), which is released during viral infection (7). A role for TLR3 in epithelial cells has been suggested in experiments using rhinovirus, rhinovirus dsRNA, and synthetic dsRNA (polyinosinic-polycytidylic acid; poly[I;C]),

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leading to increased expression of chemokines as well as defensins (human β-defensins [hBD-2 and hBD-3]) (39, 58).

Toll-like receptor 5 recognizes bacterial flagellins (67). Interestingly, human and mouse TLR5 can recognize different flagellins due to an amino acid variation in the TLR5 residue 268 within the extracellular domain (9). In addition, mutational studies in conserved surface domains proved the importance of the residues D295 and D367 for the flagellin recognition (9, 174). There is evidence that TLR5 is involved in important inflammatory mechanisms against invading pathogens (126, 148).

Toll-like receptor 9 recognizes bacterial deoxyribonucleic acid (DNA), resulting in increased expression of IL-8 in colonic epithelial cells (5).

Activation of TLRs leads primarily to an increase in cytokine and chemokine production. However, it has also been shown that expression of other antimicrobial proteins (e.g., hBD-2) can also be stimulated through TLR signaling. Finally, TLR activation may result in increased expression of TLRs themselves. For example *Haemophilus influenzae* stimulates TLR2, resulting in an increased TLR2 expression on bronchial epithelial cells (81, 167). In addition, stimulation of TLR4 with LPS results in TLR2 expression (43). Furthermore, cytokines, such as INF-γ, can also lead to an increased expression of selected TLRs (195). Finally, TLR4 expression is up-regulated in airway epithelial cells due to stimulation with respiratory syncytial virus (123).

How can a specific response to the variety of microbes develop from 'just' 13 different TLRs? Some of the individual TLRs may have their own signaling pathways; others may share pathways, but different cell types may only express a subset of TLRs. Simultaneous activation of different TLRs creates the unique response that is

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characteristic for the cell type and the microorganism. In recent years, the intracellular signaling cascades of TLR pathways have been studied in detail. The common intracellular TIR domain of TLRs plays a central role in TLR signaling. TIR is about 200 residues long and contains the TIR consensus motif including 3 consensus boxes (6). The consensus box 2 reveals a proline residue, which is critical for the interaction between TIR domains, and therefore, for the interaction between TLRs and adaptor proteins. Like most of the TLR proteins, TLR4 is involved in the activation of a signaling cascade, which involves MyD88 (MyD88 dependent pathway). MyD88 itself contains a carboxy-terminal TIR domain, through which it interacts with the TIR domain of TLRs. Stimulation of TLR4 with LPS triggers MyD88 to recruit IRAK4 (IL-1 receptor associated kinase 4) allowing the association and phosphorylation of IRAK1. The TNF receptor-associated factor-6 (TRAF6) is also recruited to the receptor-complex, by association with phosphorylated IRAK1. Phosphorylated IRAK1 and TRAF6 then dissociate from the receptor to form a complex with TAK1 (transforming-growth-factor-β-activated kinase 1), TAB1 (TAK1-binding protein 1) and TAB2 at the plasma membrane, which induces the phosphorylation of TAB2 and TAK1. IRAK1 is degraded at the plasma membrane, leading to the translocation of the remaining complex (consisting of TRAF6, TAK1, TAB1 and TAB2) to the cytosol, where it associates with the ubiquitin ligases UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1). Ubiquitination of TRAF6 induces the activation of TAK1, which in turn phosphorylates both mitogen-activated protein (MAP) kinases and the IKK complex (inhibitor of nuclear factor-κB (IκB)-kinase complex). The latter consists of IKK-α, IKK-β and IKK-γ and phosphorylates IκB, which leads to its

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ubiquitination and subsequent degradation. Therefore, stimulation of the TLR4 signaling cascade allows nuclear factor kappa B (NF-κB) to translocate to the nucleus and induce the expression of its target genes (6). This *MyD88-dependent* pathway contributes primarily to the activation of NF-κB and the production of pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, and IL-8 (*early-phase NF-κB response*). A second *MyD88-independent/TRIF (TIR-containing adaptor inducing interferon (INF)-β)-dependent* pathway leads to the activation of the transcription factor IRF-3 (interferon regulatory factor 3), which controls IFN-β production (72, 197). IFN-β binding to IFN receptor (IFNR) initiates signaling through STATs 1 and 2 (signal transducers and activators of transcription 1 and 2), activating additional genes. The TRIF-dependent pathway of TLR4 also collaborates with the MyD88-dependent pathway by activating NF-κB (*late-phase NF-κB response*) and various MAPKs pathways, promoting pro-inflammatory cytokine production (6). During the discovery of the MyD88-independent pathway additional adaptor molecules have been found: MyD88 adaptor-like (MAL; also known as TIR domain-containing adaptor protein [TIRAP]; TIR domain-containing adaptor inducing IFN-β (TRIF; also known as TIR-containing adaptor molecule-1 [TICAM-1]); TRIF-related adaptor molecule (TRAM) and α- and HEAT-Armadillo motifs. Like MyD88, these adaptor molecules have the TIR domain, but they show structural difference to MyD88 (132). This might be the reason for the different down-stream signaling due to different transducer binding. The advantage of using different adaptor molecules is the response specificity in the defense against different microorganisms. As a matter of fact, it is tempting to speculate that these adaptor molecules are future

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therapeutic targets, for example in fighting pro-inflammatory cascades initiated through TLRs, while maintaining TLR-mediated protective responses.

Beside the production of the potent chemotactic factors for neutrophils, such as IL-8, the TLR4 signaling pathway also cross-talks with the G-protein-coupled receptor (GPCR) signaling pathway in neutrophils, which is used by chemokine receptors (44). Endotoxin-activated signaling through TLR4 transcriptionally down-regulates the expression of GRK 2 and 5 (GPCR kinases), which are cytosolic proteins that desensitize the GPCR signaling cascade by binding to activated GPCRs, uncoupling them from their cognate G-proteins. The reduced expression of GRKs lowers the chemokine receptor desensitization (i.e., preserves GPCR expression) and markedly augments the neutrophil migratory response (44).

Negative regulation of TLR signaling: Negative feedback mechanisms are known to be important for the regulation of TLR initiated signaling, and, therefore also play an important role in protecting the lung against excessive responses to airborne pathogens (6, 133). A20, a zinc finger binding protein, is encoded by an immediate early response gene (133). In primary cultured human AECs A20 expression is significantly increased by the binding of LPS to TLR4 (60). Expression of A20 is regulated by NF-κB, which is mediated by two adjacent NF-κB-binding sites in the A20 promoter (92). A20 exhibits ubiquitin ligase activity and, therefore, targets proteins for degradation (194). Due to the poly-ubiquitination of the receptor interacting protein (RIP), an essential mediator of the proximal TNF receptor 1 (TNFR1) signaling complex, TNF-α-dependent NF-κB activation is attenuated. Beside RIP, other proteins such as TRAF6 (69) and IκK-γ (187), which are important signaling molecules along the TLR4 pathway, can also bind

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to A20, therefore, clearly identifying anti-inflammatory characteristics of A20. It has been demonstrated that $A20^{-/-}$ mice are highly susceptible to chronic inflammation and cell death (100) and that A20 in human airway epithelial cells inhibits NF-κB-dependent TNF-α- and IL-1β- expression (82). Also, A20 is able to inhibit TLR4 and TLR2-dependent production of IL-8 (60). In that study it was shown in detail that dominant-negative myeloid differentiation protein (MyD88) and a mutant form of IκB-α attenuates the LPS activation of NF-κB and IL-8 and that over-expression of A20 inhibits activation of NF-κB and the IL-8 promoter by LPS in a similar way. These results suggest that A20 acts in different ways as a negative regulator of the TLR4/NF-κB/IL-8 axis. Failure to down-regulate NF-κB transcriptional activity results in chronic inflammation and cell death (100).

Summary of Literature Review and Hypotheses

Recurrent airway obstruction in horses is characterized by neutrophilic airway inflammation and obstruction, and stabling of susceptible horses triggers acute disease exacerbations. Stable dust is rich in endotoxin from gram-negative bacteria and fungi, such as *Faenia rectivirgula*, *Thermoactinomyces vulgaris,* and *Aspergillus fumigatus.* Toll-like receptors (TLRs) are pathogen recognition receptors that are trans-membrane proteins on a variety of airway cells, such as alveolar macrophages, dendritic cells, epithelial cells, and airway smooth muscle cells. Toll-like receptor 4 is the principal recognition receptor for LPS, and TLR2 is the principal recognition receptor for fungal products. In human bronchial epithelium, TLR4 and TLR2 stimulation leads to increased production of interleukin (IL)-8, a potent neutrophil attractant, through activation of the

transcription factor NF-κB. During stabling, RAO-affected horses exhibit an elevated NF-κB activity and IL-8 production compared to control animals and RAO-affected horses on pasture. In laboratory animal models and cell culture studies, the zinc finger protein A20 negatively regulates the TLR4 or TLR2/NF-κB/IL-8 axis.

Therefore, I hypothesized i) that horses in stables are exposed to higher endotoxin concentrations than on pasture; ii) that during stabling TLR4, TLR2, and IL-8 mRNA expression are increased in RAO-affected horses compared to controls, and that this would be paralleled by elevated neutrophil counts in bronchoalveolar lavage fluid (BAL); and iii) that A20 mRNA expression is not increased in RAO-affected horses during stabling compared to controls.

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Table 1-1: Human and murine Toll-like receptors and their ligands.

Receptor	Animal Species	Ligands
TLR1	Human, Mouse	Tri-acyl-lipoproteins Associates with TLR2
TLR2	Human, Mouse	Lipoproteins Lipoteichoic acid (gram positive) Lipoarabinomannan (mycobacteria) Peptidoglycan zymosan (yeast) Aspergilus fumigatus conidia Associates with TLR1 and TLR6
TLR3	Human, Mouse	Poly(I-C) ds RNA (viral) Influenza A
TLR4	Human, Mouse	LPS (gram negative) Host heat shock protein 60 and 70 Host hyaluronic acid RSV protein F Taxol Aspergillus fumigatus conidia and hyphae
TLR5	Human, Mouse	Flagellin
TLR6	Human, Mouse	Di-acyl-lipopeptides Zymosan (yeast) Associates with TLR2
TLR7	Human, Mouse	Single-stranded viral RNA, imiquimod, resiquimod 848
TLR8	Human, Mouse	Single-stranded viral RNA, imiquimod, resiquimod 848
TLR9	Human, Mouse	Unmethylated CpG DNA
TLR10	Human	Uncertain
TLR11	Mouse	Uncertain
TLR12	Mouse	Uncertain
TLR13	Mouse	Uncertain

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CHAPTER 2

ENDOTOXIN CONCENTRATIONS WITHIN THE BREATHING ZONE OF HORSES IS HIGHER IN STABLES THAN ON PASTURE

Abstract

Dust in horse stables is rich in respirable, pathogenic material, such as endotoxin. The amount of respirable dust is associated with the concentration of endotoxin in stable dust. Our laboratory has shown previously that the amount of dust in the breathing zone of stabled horses is higher than dust concentrations in stables that were measured with area samplers as well as dust concentrations in the breathing zone of horses on pasture. Hay dust endotoxin causes equine neutrophilic airway inflammation. Endotoxin concentrations in outdoor environments are influenced by ambient temperature and relative humidity. We hypothesized that endotoxin concentrations in the breathing zone of stabled horses are higher than in the breathing zone of horses kept on pasture. We further hypothesized that the endotoxin exposure for horses kept on pasture is associated with changes in ambient temperature and relative humidity. We measured endotoxin concentrations in the breathing zone of six stabled horses and six horses on pasture using the *Limulus Amebocyte Lysate* (LAL) Kinetic Chromogenic Assay and reported the climatological data of ambient temperature and percent relative humidity from the National Climatic Data Center. Mean endotoxin concentrations in the breathing zone of

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stabled horses were significantly higher (about 8-fold) than of horses on pasture. Concentrations of endotoxin on pasture were not associated with changes in ambient temperature or relative humidity. We concluded that stabled horses are exposed to higher endotoxin concentrations than horses on pastures. Differences in individual behavior rather than ambient climatic conditions may contribute to the actual endotoxin exposures of individual horses in stables and on pasture.

Introduction

In the northern hemisphere it is common to keep horses in stables, where they are fed hay. The economical advantage of this practice, however, can be diminished by the impairment of horse's welfare and exercise performance due to exposure to high concentrations of airborne pathogenic/inflammogenic materials. For example, we and others have reported that stabled horses without clinical signs or a history of airway disease experience influx of inflammatory cells into the airway lumen (29, 31, 57, 73, 184). Furthermore, feeding hay from round bales, where horses are assumed to have a higher dust exposure than feeding hay from traditional square bales, exhibit an about 5-fold increased risk for >20% neutrophils in trachea (152). From these data, it can be inferred that hay dust exposure increases the risk of equine inflammatory airway disease (IAD). The prevalence of IAD in Michigan pleasure horses has been reported to be 17.3% (152).

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Airway inflammation has functional consequences as increased mucus accumulation, which is associated with airway inflammation, is a risk factor for poor racing performance of stabled race horses (74). Dust exposure can have even more important consequences in horses with lung disease. For example, in horses with Recurrent Airway Obstruction (RAO) organic substances that are frequently found in equine stables and hay dust can cause airway inflammation. Specifically, endotoxin and fungal products (from e.g., *Faenia rectivirgula, Aspergillus fumigatus*) within stable dust can trigger neutrophilic airway inflammation and obstruction in RAO-susceptible horses (33, 143, 144). A 12 to 50% prevalence of RAO has been reported for horses in North America and Europe (19, 98, 118).

We have previously reported that the respirable airborne dust concentration in the breathing zone of horses in conventional stables (straw bedding and hay feed) are significantly higher than in recommended stables (shavings as beddings and complete pelleted diet) (196). McGorum et al. showed a strong correlation between dust concentrations and airborne endotoxin concentrations (117). In this study, the authors also showed that the content of endotoxin within respirable dust in conventional stables was significantly higher than in respirable dust collected on pasture. Endotoxin in horse stables contributes at least partially to the development of airway inflammation in both healthy and RAO-susceptible horses (144). Beside the amount of dust, endotoxin concentrations are also influenced by seasonal and climatological factors, such as temperature and relative humidity (24).

Endotoxin in horse stables arises from different point sources (e.g., hay, manure etc.). Due to differences in the individual behavior of horses or in stable hygiene, area

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samplers may not be adequate to reflect the actual endotoxin exposure of stabled horses. Indeed, Woods et al. reported that, by using personal samplers, total and respirable dust concentrations in the breathing zone of horses in conventional stables are higher than concentrations measured in stables with area samplers (196).

Therefore, in the present study we determine the endotoxin concentration in the breathing zone of horses by use of a personal sampler device. We hypothesized that the endotoxin concentrations in the breathing zone of stabled horses are higher than of horses on pasture. We also hypothesized that ambient temperature and relative humidity are associated with endotoxin concentrations in the breathing zone of horses on pasture.

Materials and Methods

Animals and study design: Horses were selected from a herd maintained by the Pulmonary Laboratory at Michigan State University. Six horses (three mares, three geldings; 21 ± 6.7 years; two Quarter horses, one Quarter Horse/Arabian mix, one Grade horse, one Thoroughbred horse and one Standardbred horse) were studied.

Endotoxin exposure was measured when horses were stabled in conventional stables and subsequently on pasture. For determination of endotoxin concentrations in the breathing zone of stabled horses, animals were brought into the stable and were allowed to adjust to the environment for one hour before the personal sampler for endotoxin measurement was attached (see below). The stable was cleaned and horses were fed between 7 and 8 AM and fed again in the afternoon between 4 and 5 PM. For the first

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measurement horses were brought into the barn between 8 and 10 AM. After the stable measurement, horses were returned to the pasture. The second measurement (pasture) was performed with all horses on the same pasture. Horses were allowed to move around freely with the personal sampler attached to the halter and surcingle. The study was performed between the 15th and the 26th of September, 2005. The protocol was approved by the All-University Committee for Animal Use and Care of Michigan State University.

Determination of endotoxin concentrations in the breathing zone of horses in stables and on pasture. For the collection of endotoxin in the breathing zone around the horse nostril, personal samplers were attached to each horse (Figure 2-1). The personal sampler consisted of a filter cassette that was attached to the halter, and an *AirCheck 52 Personal Sample Pump* (kindly provided by EMSL Analytical, Inc., Westmont, NJ) that was attached to a surcingle. The filter cassette and the air pump were connected by a rubber hose. Endotoxin was sampled as described previously (191). Briefly, the breathing zone air was sampled at a flow rate of 1.5 liters per minute for an average of 245 minutes (~ 4 hours). Endotoxin that was collected on the air filter was quantified by the IH Laboratory at EMSL Analytical, Inc. (Westmont, NJ) using the *Limulus Amebocyte Lysate* (LAL) Kinetic Chromogenic Assay (104).

Climatological data: Climatological data for Lansing, MI, were obtained from the National Climatic Data Center (http://www7.ncdc.noaa.gov/IPS/). We report four dew point temperature and relative humidity measurements in three-hourly intervals (7:00AM, 10:00 AM, 1:00PM, and 4:00PM) on the days when endotoxin measurements were taken.

Statistical analysis. Raw data of endotoxin concentrations were not normally distributed (Kolmogorov-Smirnov $P \leq 0.05$). Therefore, data were log10-transformed.

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Means of endotoxin concentrations in stables and on pasture were compared using Student's t-test. Data are reported as geometric means (anti-log-transformed) with the 5% and 95% values. Mean values were calculated for the climatological data. Data were analyzed by use of SAS version 8, SAS/STAT Software (SAS Institute Inc., Cary, North Carolina, USA). Differences were considered significant for P-values ≤ 0.05.

Results

Endotoxin concentrations: Endotoxin concentrations (ECs) for each horse are shown in Table 2-1. Mean values of endotoxin concentrations in the breathing zone of stabled horses (EC = 7.08 * 10^3 EU/m^3) were significantly higher than of horses on pasture (EC = 0.85 * 10^3 EU/m^3) (Figure 2-2). The mean difference was more than 8-fold. Endotoxin concentrations for each horse were always less on pasture than in the stable (Figure 2-2).

Climatological data. Data for temperature and percent relative humidity are shown in Table 2-2. The average ambient temperature during stabling (10.4 ± 1.3 °C) was not significantly different from the average ambient temperature on pasture (11.4 ± 1.8°C) (P = 0.35). In addition, there was no significant difference between the relative humidity on days when horses were stabled (60.75 ± 6.66 %) as compared to days when horses were kept on pasture (68 ± 0.24 %) (P = 0.3).

Associations between endotoxin concentrations and climatological data. Endotoxin concentrations did not correlate with the ambient temperature or the relative humidity when horses were kept on pasture (Figure 2-3A or 2-3B, respectively).

Discussion

In this study we showed that the mean endotoxin concentration measured in the breathing zone of stabled horses is more than 8-fold higher than that of horses kept on pasture. Our results are in agreement with a study reported previously by McGorum et al., in which the authors showed an approximately 15-fold higher airborne endotoxin concentration in conventional stables as compared to concentrations on pasture (117). However, in that study average endotoxin concentration in stables and on pasture were higher than in our study (1.67 mg/m^3 (= 139.2 * 10^3 EU/m^3) and 0.11 mg/m^3 (= 9.2 * 10^3 EU/m^3), respectively). While in the present study, we used a pump flow rate of 1.5 l/min over 4 hours to collect endotoxin concentrations in the horse's breathing zone, McGorum et al. collected dust with a rate of 2 l/min over periods of 4 to 10 hours (117). Alternatively, the differences might be due to differences in experimental design. Whereas McGorum et al. obtained measurements by using one pony under three different maintenance situations we attached personal samplers to six individual horses at the different collection occasions. The differences in endotoxin concentrations between the studies may, therefore, be a function of individual, behavioral differences between the horses. This might also explain the wider range in endotoxin concentrations in our data.

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Outdoor endotoxin concentrations, when measured with area samplers, are positively correlated with temperature and negatively with percent of relative humidity (24). Here we reported that the endotoxin concentration in the breathing zone of horses on pastures, which was measured with personal samplers, does not correlate with the ambient temperature or the percent of relative humidity. This suggests that endotoxin concentrations in the breathing zone of horses are dependent on the immediate environment in which the horse keeps its nose, rather than on environmental conditions, such as temperature or humidity, which might contribute to the production of endotoxin due to bacterial growth. Therefore, differences in the individual behavior (e.g., head positioning, sniffing, frequency of moving) of the animals is likely to result in variations in endotoxin exposures.

In summary, we reported here that the use of personal samplers in a population of horses–stabled or on pasture–allows for detection of endotoxin exposure of the individual horse. On average, stabled horses are exposed to at least 8-fold higher concentrations of endotoxin than horses maintained on pastures. Differences in individual behavior rather than ambient climatological conditions, such as temperature or humidity, may contribute to variations in endotoxin exposures between individual horses.

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Table 2-1: Endotoxin concentrations in the breathing zone of stabled horses and horses kept on pasture. (*) indicates significant different ($P \leq 0.05$) from endotoxin concentration measured on pasture.

	Endotoxin concentration (10^3 x EU/m^3)	
	Stable	**Pasture**
Horse 1	5.94	1.13
Horse 2	6.48	0.45
Horse 3	6.8	0.74
Horse 4	35.9	0.71
Horse 5	2.82	2.58
Horse 6	4.98	0.56
Geometric mean (5%, 95%)	7.08 (2.82; 36.3)*	0.85 (0.45; 2.57)

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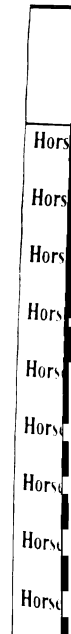


Table 2-2: Climatological data (ambient temperature and percent relative humidity) at days of endotoxin measurments obtained from the *National Climatic Data Center*. E = Environment, in which horses were maintained, S = Stable, P = Pasture, QH = Quarter Horse, Grd = Grade Horse, TB = Thoroughbred, SB = Standard bred, Arab = Arabian

	E	Age (yrs.)	Breed	Date	Time	Temperature (°C)	Relative Humidity (%)
Horse 1	S	19	QH	9/15/05	8:40-12:45	9.4	56.5
Horse 2	S	28	Grd	9/15/05	8:40-12:45	9.4	56.5
Horse 3	S	21	TB	9/16/05	10:15-2:15	12	80.75
Horse 4	S	28	QH	9/16/05	10:15-2:15	12	80.75
Horse 5	S	20	SB	9/21/05	8:30-12:35	9.7	45
Horse 6	S	10	Arab/QH	9/21/05	8:30-12:35	9.7	45
Horse 1	P	19	QH	9/23/05	9:10-1:15	9.2	67.5
Horse 2	P	28	Grd	9/23/05	9:00-1:05	9.2	67.5
Horse 3	P	21	TB	9/17/05	11:10-3:15	13	68.75
Horse 4	P	28	QH	9/17/05	11:50-3:55	13	68.75
Horse 5	P	20	SB	9/24/05	10:30-2:35	12	67.75
Horse 6	P	10	Arab/QH	9/24/05	10:35-2:40	12	67.75

Figure 2-1: Personal sampler device for endotoxin measurements in the breathing zone around the horse nostril. The personal sampler consisted of a filter cassette that was attached to the halter, and an *AirCheck 52 Personal Sample Pump* (kindly provided by EMSL Analytical, Inc., Westmont, NJ) that was attached to a surcingle. The filter cassette and the air pump were connected through a rubber hose. Endotoxin was sampled at a flow rate of 1.5 liters per minute for an average of 245 minutes (~ four hours)

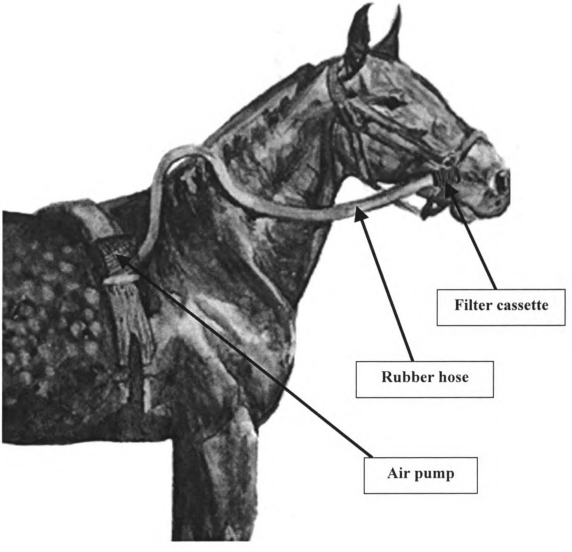


Figure 2-2: Endotoxin concentrations (10^3 x EU/m^3) in the breathing zone of stabled horses and horses on pasture. Individual diamonds represent individual measurements. Horizontal bars represent the geometric means. (*) indicates significantly different ($P \leq 0.05$) from endotoxin concentration measured on pasture.

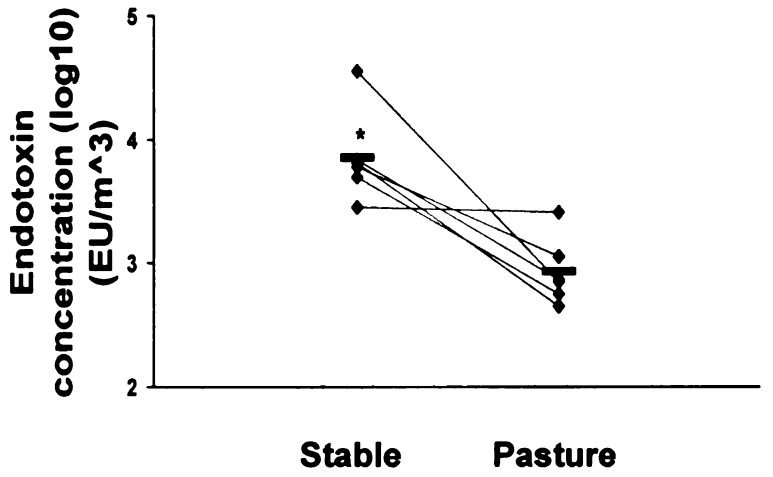
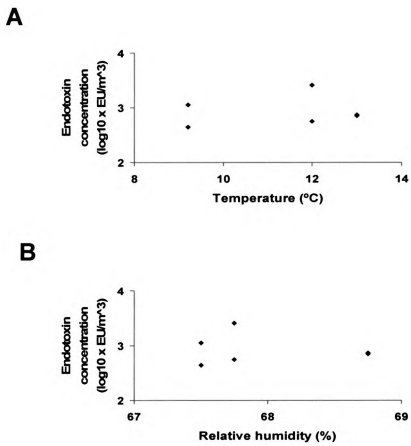


Figure 2-3: Associations between endotoxin concentrations (10^3 x EU/m^3) in the breathing zone of horses maintained on pasture and ambient temperature (°C) (A) and relative humidity (%) (B). Individual diamonds represent individual measurements.



CHAPTER 3

PRIMER DESIGN AND QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (qRT-PCR)

The Quantitative Real Time Polymerase Chain Reaction Assay

Introduction: In the following, I describe the thought processes that led me to choose the standard curve method of relative quantitative real-time polymerase-chain reaction (RT-PCR) using SYBR Green chemistry for gene expression analysis in the research described in the following chapters of this dissertation.

RT-PCR versus end-point PCR: In comparison to the end-point PCR, where data are collected only at the end of the PCR cycle, RT-PCR allows measurement of the accumulation of amplification products, also termed amplicons, throughout the PCR cycle (i.e., in real time (RT)). During RT-PCR, the amount of amplification products is measured at the time of first fluorescence detection rather than after a fixed number of cycles. Therefore, the higher the copy numbers within a sample, the sooner its detection.

SYBR Green I dye versus Taqman chemistry: For the detection of PCR products two different types of chemistry are commercially available: TaqMan chemistry and SYBR Green I dye chemistry. For the detection of these chemistries *Sequence Detection System (SDS)* instruments have been developed by Applied Biosystems (e.g. SDS 7500, 7700, 7900). Whereas TaqMan chemistry uses fluorogenic probes for the detection of

specific PCR products, SYBR Green chemistry uses SYBR Green I dye for the detection of double-stranded DNA. The PCR-products (i.e., amplicons) are detected by binding of SYBR Green I dye to each amplified target sequence as they are produced by DNA polymerase amplification during the PCR cycles. The result is an increased fluorescence signal that is proportionate to the number of amplicons produced. SYBR Green chemistry can be used to detect the amplification of any double-stranded DNA sequence and, in comparison to TaqMan chemistry, it does not require the labor and cost intensive generation of specific probes. However, SYBR Green chemistry is error-prone, especially for false-positive signals, due to non-specific double-stranded DNA sequences within samples. Therefore, when using SYBR Green chemistry additional optimization experiments become necessary.

In quantitative RT-PCR, concentrations of nucleic acid targets (i.e., RNA, cDNA/DNA) that are amplified during each cycle are measured. In our studies, we measured the amount of transcribed RNA of the genes of interest (i.e., TLR4, TLR2, IL-8, A20, and 18S rRNA) after its reverse transcription into complementary DNA (cDNA). We assumed a 100% efficacy of the reverse transcription. The SDS 7900 instrument by Applied Biosystems was used to detect the increase of fluorescence signal during each cycle and results were shown in amplification plots (i.e., plot of fluorescence signal versus cycle number). During the initial cycles, usually between the 5^{th} and the 15^{th} cycle number, only minor changes in the fluorescence signal could be detected (baseline). In our studies, we adjusted the baseline cycles as follows: for 18S rRNA: from the 5^{th} to the 10^{th} cycle number; for TLR4: from the 3^{rd} to the 15^{th} cycle number; for TLR2: from the 11^{th} to the 17^{th} cycle number; for IL-8: from the 2^{nd} to the 15^{th} cycle number; and for

A20: from the 5th to the 25th cycle number. The detection of a fluorescence signal above the baseline specifies accumulated PCR products. A specific cycle (threshold cycle or C_T) is usually defined within the linear part of the S-shaped amplification plot to compare quantities of an amplicon within (e.g., standard curve) or between samples (e.g., test samples).

Relative versus absolute quantification: There are two types of PCR product quantification. Whereas the absolute quantification measures the amounts of unknown PCR products by interpolating their amount using a standard curve with known concentrations, relative quantification compares the amounts of PCR products in a sample of interest (e.g., bronchial brushing sample from RAO-affected horses) in relation to a reference sample (e.g., bronchial brushing sample from control horses).

Standard curve versus comparative C_T method: The calculation of relative quantification is either performed using the standard curve method or the comparative C_T method. In the present research, we decided to use the standard curve method for relative quantification, because i) we only detected five different genes and, therefore, the labor and cost intensity were low enough to run the target genes (TLR4, TLR2, IL-8, and A20) and the reference gene (18S rRNA) in separate tubes, and ii) we did not test for amplification efficacies of each target and the reference gene over all samples. For each target and the reference gene, standard curves (10-fold serial dilution) were designed using the bronchial brushing samples with the lowest Ct value (highest amount of cDNA). Each standard curve sample was measured in triplicate. The following standard curves were obtained during our studies:

TLR4: $y = 26.1 - 3.2x$ ($R^2 = 0.99$),

TLR2: $y = 29.3 - 3.6x$ ($R^2 = 0.99$),

IL-8: $y = 28.7 - 3.7x$ ($R^2 = 0.99$),

A20: $y = 38.1 - 3.8x$ ($R^2 = 0.99$) and

18s rRNA: $y = 17 - 3.1x$ ($R^2 = 0.99$).

Primer design: Primer pairs were designed using Primer Express software from Applied Biosystems. This software automatically uses a set of parameters to select primer and probe sets. Therefore, it can be used for the design of primer pairs used in both TaqMan and SYBR Green chemistry assays. Although no probe is needed in the SYBR Green chemistry assay it is advantageous to automatically design a probe, in case verification experiments using a TaqMan assay are required to a later time point. Furthermore, the default setting in Primer Express meets all required criteria for sequence detection when using SYBR Green chemistry (i.e., amplicon size: 50 to 150 base pairs, G/C content: 20 to 80%, avoids runs of identical nucleotides, especially four and more Gs in a row, melting temperature: 58 to 60°C). By using Primer Express software we also controlled for possible primer-dimer formation.

Beside for 18S rRNA, primer pairs used in our studies were designed using nucleotide sequences available online. The Genbank accession numbers of the sequences used for primer pair design using Primer Express software are shown in Table 3-1. Complete mRNA sequences for TLR4, TLR2, and IL-8 were available for *Equus caballus*. The primer set for A20 was designed by aligning the known human and murine cDNA sequences and choosing regions of high sequence identity. Care was taken that this primer pair would span known intron-exon borders.

Verification of amplification product for primer pairs: *18S rRNA, TLR4, TLR2, IL-8:* Amplified equine genomic DNA, using the primer pairs of 18S rRNA, TLR4, TLR2, and IL-8, yielded a single band of approximately the amplification product sizes reported in Table 3-1 in agarose gel electrophoresis (Figure 3-1). Therefore, primer pairs for these genes did not span intro-exon borders. Further verification steps were necessary to show that we indeed amplified the transcription products of the genes of interest. Therefore, we subsequently performed automated (18S rRNA, TLR4) or manual (TLR2) sequencing of the PCR products from genomic DNA. The primer pair for **18S rRNA** was presented in another dissertation previously by the Pulmonary Laboratory (56). Automatic sequencing resulted in the following nucleotide sequence:

ttagtccctgccctttgtacacaccgcccgtcgctactaccgattggatggtttagtgag.

A BLAST (basic local alignment search tool) analysis at the NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST/) showed that this sequence aligned with many different species. We did not find a horse-specific sequence in the online database for this 18S rRNA sequence. Automatic sequencing of the **TLR4** amplified PCR product from genomic DNA resulted in the following sequence:

tcctgctggactccacggtttaccatccagcaaggctttcctgagtcgtctcc.

This sequence matched the published equine TLR4 sequence in the nr database exactly. By using BLAT at the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgBlat) this sequence detected the TLR4 sequence in the human genome. BLAT analysis on DNA finds sequences of 95% or more similarity. The amplified PCR product when using **TLR2** primer set was manually sequenced and the sequence (Figure 3-2) yielded the

highest BLAST hit to the equine TLR2. In BLAT this sequencing product detected the TLR2 sequence in human genome.

A20: No sequence has been published for equine A20; therefore, the primer pair was designed by using the published A20 human mRNA sequence (Genbank accession number for human A20: NM_006290) in a region of high identity to the published murine mRNA sequence (Genbank accession number for mouse A20: NM_009397). Recently, the unassembled whole equine genome sequence became available with over 31 x 10^6 traces published at the Trace Archive at NCBI (about 8-fold coverage). A cross-species MegaBLAST of the human A20 against the whole equine genome showed high sequence identity in the region used for the A20 primer pair. One nucleotide mismatch for the forward primer and one for the reverse primer were found (Figure 3-3). Single nucleotide mismatches are known to have little impact on amplification of targeted sequences and, therefore, our A20 primer set was specific for detecting equine A20 mRNA (79).

During sample analysis we added additional verification steps. A dissociation curve for each amplicon in each sample was generated to verify specificity of primer pairs. Finally, each RNA sample was analyzed for genomic contamination by testing RT-negative samples (use of RNAse free H_2O instead of Omniscript Reverse Transcriptase in RT reaction) for the reference gene.

Table 3-1: The oligonucleotide primer pair sequences used for amplification of 18S rRNA, TLR4, TLR2, IL-8 and A20 transcripts in bronchial brushing samples. Amplicon length of the primer pairs and Genbank accession numbers of sequences used during primer design or sequence source are shown.

	Forward primer	Reverse primer	Amplicon length	Genbank accession number
18S rRNA	**GCA ATT ATT CCC CAT GAA CG**	**GGC CTC ACT AAA CCA TCC AA**	**123**	See (56)
TLR4	**TCT GGA GAC GAC TCA GGA AAG C**	**GCA AGA AGC ACC TCA GGA GTT T**	**91**	AY005808
TLR2	**AGC TGT AGC AGT TGG CTT AGT TCA T**	**GAC ACA TCC GGC TGA AAT CTA TT**	**66**	AY429602
IL-8	**CAG CAT CTC GTC TGA ACA TGA CT**	**AGA GCT GCA GAA AGC AGG AAG A**	**73**	AY184956
A20	**CCT CAT TGA GCA GTC CAT GCT**	**TCC ACA CTC ACC CAC CAG TTC**	**69**	Human: NM_006290 Mouse: NM_009397

Figure 3-1: Agarose gel electrophoreses of 3µl Lambda DNA – *HindIII* Digest (lane 1), 3µl 18S rRNA PCR product (lane 2), 3µl TLR4 PCR product (lane 3), 3µl TLR2 PCR product (lane 4) and 3µl IL-8 PCR product (lane 5). 18S rRNA, TLR4, TLR2 and IL-8 were amplified using equine genomic DNA.

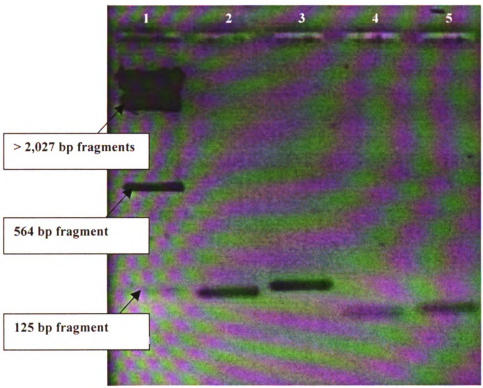


Figure 3-2: Manually sequenced PCR products using the TLR2 forward (A) and reverse (B) primer.

A)

5'...ACATCAACACCATAGAGGAAGACTCTTTTTCCTCCCTGAGGAGTCTTGAA
CATTTGGACTGTCAATAATCACTTATCTAATTTATCGTCCTCCTGGTTCAGGCC
CCTTTCTTCTTTAAAATTCTTAAACTTATGGGAAGCACTTACAAAACACTCGG
GGAAACATCTCTTTTTTTCTCATCTCACATTGCGATCTGAAGTGGAATATCAC
TCACTGAGATCAGGAAGATTGCTGGCCATCTGAGACTGGATG...3'

B)

5'...TAGCATCAATCTCAAGTTCTTCAAGGAAAGTGAGCCCAGCAAAATCCTTT
CCCTGAATCTCAGTGAAGTGAATATTTCCCACTTTCAGGATTCGCAAATTGGT
GAGATGAGAAAAAAGAGATGTTTCCCGAGTGTTTTGTAAGTGCTTCCCAGTA
AG...3'

Figure 3-3: Cross-species MegaBLAST of human A20 mRNA against horse whole genome sequences in Trace Database.

A20 forward primer shown underlined
CCTCATTGAGCAGTCCATGCT

Lineups show human sequence on top, equine sequence below

>gnl|ti|1233324679 G836P67577RA9.T0
Length=893
Get TBLASTX alignments

Score = 404 bits (210), Expect = 1e-108
Identities = 222/228 (97%), Gaps = 0/228 (0%)
Strand=Plus/Minus

```
Query  475  AAAACGCCTGTCTAGGGGCATCTCCCACGCCAGCTCCAGCATTGTTTCCCTGGCCCGGTC  534
            |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||
Sbjct  873  AAAACGCCTGTCTAGGGGCATCTCCCACGCCAGTTCCAGCATTGTTTCCCTGGCCCGGTC  814

Query  535  CCATGTCTCCTCCAATGGT          AGCAATGAGCACCCCCTGGAAATGCCCAT  594
            |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||
Sbjct  813  CCATGTCTCCTCCAATGGTGGGGGTGGGGGGAGCAGTGAGCACCCCCTGGAAATGCCCAT  754

Query  595  CTGTGCCTTCCAGCTTCCAGATCTCACTGTATACAATGAAGACTTCCGCAGCTTCATAGA  654
            |||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||
Sbjct  753  CTGTGCCTTCCAGCTTCCAGATCTCACTGTGTACAATGAAGACTTCCGCAGCTTCATAGA  694

Query  655  GAGAGACCTCATTGAGCAGTCCATGCTGGTTGCCTTGGAACAGGCAGG  702
            ||||||||||||||| |||||||||||||| || ||||||||||||||
Sbjct  693  GAGAGACCTCATTGAACAGTCCATGCTGGTCGCTTTGGAACAGGCAGG  646
```

A20 reverse primer
TCCACACTCACCCACCAGTTC

GAACTGGTGGGTGAGTGTGGA – reverse complement shown underlined

>gnl|ti|1248973705 G836P611230RI10.T0
Length=933
Get TBLASTX alignments

Score = 167 bits (87), Expect = 2e-37
Identities = 99/105 (94%), Gaps = 0/105 (0%)
Strand=Plus/Plus

```
Query  700  AGGGCGTTTGAACTGGTGGGTGAGTGTGGATCCCACCTCTCAGAGGCTGCTTCCTTTGGC  759
            |||||||||||||||||||| ||||||||||||||| ||||||| ||||||||||||||
Sbjct  401  AGGGCGTTTGAACTGGTGGGTAAGTGTGGATCCCACCTGTCAGAGGTTGCTTCCTTTGGC  460

Query  760  AACTACTGGAGATGGGAACTGCCTCCTGCATGCAGCCTCCCTTGG  804
            ||| |||||||||||| ||||||||||||||||||||||| |||||
Sbjct  461  AACAACTGGAGATGGAAACTGCCTCCTGCATGCAGCCTCTCTTGG  505
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CHAPTER 4

ELEVATED TOLL-LIKE RECEPTOR 4 mRNA LEVELS IN BRONCHIAL EPITHELIAL CELLS IS ASSOCIATED WITH AIRWAY INFLAMMATION IN HORSES WITH RECURRENT AIRWAY OBSTRUCTION

Abstract

Recurrent airway obstruction (RAO) is characterized by neutrophilic airway inflammation and obstruction, and stabling of susceptible horses triggers acute disease exacerbations. Stable dust is rich in endotoxin, which is recognized by toll-like receptor (TLR) 4. In human bronchial epithelium, TLR4 stimulation leads to elevation of interleukin (IL)-8 mRNA expression. IL-8 is a potent neutrophil chemoattractant, known to increase BAL neutrophils within the lung. We hypothesized that TLR4 and IL-8 mRNA, and neutrophil numbers are elevated in RAOs during stabling compared to controls and compared to RAOs on pasture. We measured the maximal change in pleural pressure (ΔPpl_{max}), determined inflammatory cell counts in bronchoalveolar lavage fluid (BAL), and quantified TLR4 and IL-8 mRNA in bronchial epithelium by qRT-PCR. We studied six horse pairs, each pair consisting of one RAO and one control horse. Each pair was studied when the RAO-affected horse had airway obstruction induced by stabling, and after 7, 14, and 28 days on pasture. Stabling increased BAL neutophils, ΔPpl_{max} and TLR4 (4.14-fold change) significantly. TLR4 correlated with IL-8 ($R^2 = 0.75$). During

stabling, IL-8 was elevated in all horses and positively correlated with BAL neutrophils (R^2 = 0.43), however, only in RAO-affected horses. Elevated TLR4 expression in bronchial epithelial cells from RAO-affected horses may contribute to elevated IL-8 production, leading to exaggerated neutrophilic airway inflammation in response to inhalation of stable dust.

Introduction

Recurrent airway obstruction (RAO), also known as heaves, is a common pulmonary disorder in horses. Acute exacerbations are characterized by neutrophilic airway inflammation, mucus accumulation, and bronchospasm, which are triggered by exposure of susceptible horses to stable and hay dust. RAO is a diffuse airway disease involving large conducting airways down to the terminal bronchioles. Periods of acute exacerbation are interspersed by periods of remission, which can be achieved by returning RAO-affected horses from stables to pasture (151).

Although adaptive immune mechanisms were shown to contribute to the pathogenesis of RAO (2, 29), innate immune mechanisms are also important (142). Hay dust is rich in microbial products such as endotoxin (144, 145), and inhalation of endotoxin-depleted hay dust significantly attenuates airway neutrophilia in affected horses (144). The inflammatory response can be re-established by adding endotoxin back to the endotoxin-depleted hay dust (141). These observations confirm that attenuation of airway inflammation is due specifically to endotoxin. In conventional horse stables,

airborne endotoxin concentrations exceed those on pasture (117) and those that can induce airway inflammation in human subjects (171, 172). In our own stables, we observed that the endotoxin concentrations in the breathing zone of stabled horses are at least 8-fold higher than concentrations on pasture (chapter 2).

Microbial-derived products, such as endotoxin, have been shown to play an important role in human lung diseases such as asthma, acute respiratory distress syndrome, and chronic obstructive pulmonary disease (159). Pathogen-associated molecules are recognized by pattern recognition receptors commonly referred to as toll-like receptors (TLRs). Toll-like receptor 4 (TLR4) is crucial for the recognition of endotoxin, in particular lipopolysaccharide (LPS) (159). TLR4 is expressed in a variety of cell types within the lung, including pulmonary epithelial cells (4, 68, 123), alveolar macrophages (46), endothelial cells (10, 192), and airway smooth muscle cells (ASM) (125), and its own expression can be stimulated by LPS itself (4, 127, 170). The TLR4 stimulation leads to production of cytokines, such as interleukin (IL)-8 (60, 123).

Little is known about TLR signaling in horses. Toll-like receptor 4 mRNA can be found in the lung tissue of healthy horses (170). The effect of LPS on TLR4 expression in bronchial epithelial cells of horses is unclear. Whereas, in lung tissue obtained from unaffected horses, LPS exposure increases TLR4 mRNA expression (170), a recent report suggests that hay dust exposure does not change the TLR4 mRNA expression in the bronchial epithelial cells of RAO-affected horses (4). Furthermore, IL-8 mRNA expression and protein concentration measured in bronchoalveolar lavage fluid (BAL) are elevated in RAO-affected horses compared to control animals during stabling and to RAO-affected horses in remission (3, 4, 59).

In the present study, we hypothesized that the amount of TLR4 mRNA is elevated in RAO-affected horses during stabling compared to control horses and to RAO-affected horses on pasture. We further hypothesized that in RAO-affected horses the increased mRNA expression of the receptor would be paralleled by an elevated expression of IL-8 mRNA. Furthermore, we hypothesized that the severity of neutrophilic airway inflammation in RAO-affected horses during stabling is correlated with an increase in IL-8 and TLR4 mRNA expression.

Materials and Methods

Animals. RAO-affected horses were selected from a herd maintained by the Pulmonary Laboratory at Michigan State University. These animals have a history and clinical signs compatible with a diagnosis of RAO. To enter the herd, animals fulfilled the following criteria: a) Horses develop airway obstruction and inflammation when stabled and fed hay; b) Airway obstruction and inflammation are reversed by pasturing, where horses have no exposure to stable dust or hay; c) Airway obstruction is reversible with atropine. Control animals did not develop airway obstruction when stabled and fed hay (151).

Study design. Six RAO-affected horses (2 mares, 4 geldings; 18.3 ± 3.3 years) and six control horses (3 mares, 3 geldings; 16 ± 4.6 years) were studied in age-matched horse pairs. Prior to the study, horses were kept on pasture. Acute airway obstruction was initiated by stabling horses in a barn. Horses were brought into the barn in pairs consisting of one RAO-affected and one control horse and were bedded on straw and fed dusty hay. We defined the beginning of the study as the day the RAO-affected horse of

each pair developed acute airway obstruction (*day 0*). When the RAO-affected horses developed a clinical score of 5 or greater, we measured the total change in pleural pressure during tidal breathing (ΔPpl_{max}). The clinical criterion to define acute exacerbations in RAO-affected horses was a ΔPpl_{max} of 15 cm H_2O or greater. Furthermore, we obtained bronchial brushing samples for gene expression analyses and bronchoalveolar lavage samples for total and differential cell count analyses. Horses were then returned to the pasture. Subsequent measurements were obtained on *days 7, 14* and *28*. The protocol was approved by the All-University Committee for Animal Use and Care of Michigan State University.

Determination of the total clinical score (TCS): A scoring system for the subjective clinical assessment of respiratory effort was used as previously described (157). Nasal flaring and abdominal movement were each scored separately on a scale of 1 (normal) to 4 (severe signs). To determine the TCS, scores for nasal flaring and abdominal movement were summed. Therefore, the TCS could range from 2 (normal) to 8 (severe signs).

Measurement of the maximal change in pleural pressure during tidal breathing (ΔPpl_{max}). The maximal change in pleural pressure was used as an indicator of airway obstruction. Measurements were made in unsedated horses by means of an oesophageal balloon connected via a 240-cm-long polypropylene catheter and a pressure transducer (Validyne Model DP/45-22) to a physiograph (Dash Model 18, Astro-Med, Inc.). The balloon was passed through the nose, and placed into the middle third of the esophagus. Twenty breaths were averaged at each measurement period.

Collection of bronchial brushing samples (BB). Bronchial brushings were made between the 3rd-and 6th-generation bronchi via bronchoscopy. Bronchoscopy was performed with a 3-meter-long endoscope (9-mm-diameter) using a transnasal approach. The brushing was performed by advancing a cytology brush (CytoSoft Cytology Brush, MPO) throughout the biopsy channel of the endoscope into the bronchial lumen. The brush was gently stroked against the airway wall 15 to 20 times. Care was taken to avoid bleeding. The brush was then withdrawn into the biopsy channel and the endoscope was removed from the horse's airways. The cytology brushes were then flushed in 1 ml phosphate-buffered saline (PBS) and stored on ice until further analysis. The procedure was repeated twice in the same lung. The side of the lung chosen for brushing was alternated between the measurement periods. The beginning lung side was chosen randomly for each horse.

Quantification of cells. Total and differential cell counts in BBs were performed manually using a hemacytometer. Cell preparations were made with a cytocentrifuge and stained with Hematoxylin and Eosin (H&E) stain. Differential cell counts were performed by counting 200 cells per slide.

Collection of bronchoalveolar lavage fluid (BAL). Bronchoalveolar lavage was obtained by means of a 3-meter-long endoscope that was passed via the nose and wedged in a peripheral bronchus. Three 100 ml aliquots of PBS were infused into the tube and recovered by suction. The lavaged fluids were pooled and the volume was determined.

Quantification of inflammatory cells. Total and differential cell counts in BAL were performed using a hemacytometer. Cell smears were made by use of a cytocentrifuge and

stained with Wright-Giemsa stain. Differential cell counts were performed by counting 200 cells per slide.

RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was isolated from BBs using a phenol/guanidine isothiocyanate mixture (TRI Reagent, Sigma) and 1-Bromo-3-Chloro-propane (Sigma-Aldrich, St. Louis, MO). Total RNA was treated with DNA-free kit (Ambion). Yields of total RNA were determined using NanoDrop technology (ND-1000 Spectrophotometer) and the integrated software version 3.1.2. The quality of total RNA was assessed using the Agilent 2100 Bioanalyzer and the integrity of the 18S and 28S rRNA was determined visually and by determination of the 18S:28S ratio. Depending on the total RNA yield of each sample, 40 to 200 ng total RNA were used as template for reverse transcription (RT) reaction using the OMNISCRIPT reverse transcriptase kit 200 (Qiagen), according to the manufacturer's protocols. The RT reaction contained 10X RT buffer, 0.5 mM of each dNTP, 10 μM random hexamer primers (Applied Biosystems), 10 U/μl of RNAse inhibitor and 4 U of Omniscript Reverse Transcriptase. The conditions for the RT reaction were 37°C for 60 min, followed by 93°C for 5 min using an *Eppendorf Mastercycler*. Quantitative PCR (qPCR) was performed using the ABI 7900 Sequence Detection System (Applied Biosystems). The qPCR reaction contained 20ng of cDNA as template, QuantiTect SYBR Green PCR Kit (Qiagen), and oligonucleotide primer pairs specific to each mRNA of interest (see Table 3-1). Thermal cycling conditions in the ABI 7900 were: 95°C for 15 min, followed by 40 cycles: 95°C for 15 seconds and 60°C for 60 seconds. A dissociation curve for each amplicon in each sample was generated to verify specificity of primer pairs. In addition, each RNA sample was analyzed for genomic

contamination by testing RT-negative samples (use of RNAse free H_2O instead of Omniscript Reverse Transcriptase in RT reaction) for the reference gene. The oligonucleotide primer pair sequences used for amplification of TLR4, IL-8 and 18S rRNA transcripts, their amplicon length and their Genbank accession number or the source for the sequence used in the study are listed in Table 3-1. 18S rRNA served as the reference gene. Primer pairs were designed with Primer Express Software version 2.0 System (Applied Biosystems).

Statistical analysis. The relative amount of mRNA was calculated using the relative standard curve method. Quantitative means and their standard deviations (S.D.) for TLR4 and IL-8 of each BB sample were standardized to the reference gene (18S rRNA). If standardized quantitative mRNA expression data were not normally distributed (Kolmogorov-Smirnov $P \leq 0.05$), they were log10 transformed. Only BBs with more than 80% epithelial cells were included in the statistical analysis. Standardized data were analyzed by repeated measure ANOVA. Fold changes in mRNA expression were determined by calculating the ratio between RAO-affected and control horses at the different time points or between different time points within each horse group. Associations between single variables were determined using Spearman correlation and regression analysis. Associations were determined within a treatment group (RAO or control group) by taking the measurements of all time points (*day 0, 7, 14, and 28*) into account. Data were analyzed by use of SAS v. 9.1. Differences were considered significant for P-values ≤ 0.05.

Results

Airway function (ΔPpl_{max}). In RAO-affected horses during acute exacerbation (*day 0*) ΔPpl_{max} was significantly elevated compared to control horses and to RAO-affected horses during remission (Figure 4-1A). No statistical significant differences in ΔPpl_{max} were observed between horse groups at *day 7, 14,* and *28* as well as between the time points within the control group.

Cellular composition of bronchoalveolar lavage fluid (BAL). Total and differential cell counts of BAL for the two horse groups at the 4 different time points are shown in Table 4-1. In RAO-affected horses at *day 0*, differential neutrophil numbers were significantly greater (Figure 4-1B) and differential lymphocyte counts were significantly reduced compared to control horses and to RAO-affected horses during remission. No statistically significant differences in the differential neutrophil and lymphocyte numbers were observed between horse groups at *days 7, 14,* and *28* and between the time points within the control group. Macrophage, eosinophil, and mast cell counts did not differ between groups or time points, except for the *day 14* measurement period, where the differential macrophage count in BAL of RAO-affected horses was significantly lower than in control horses.

Quantitative mRNA expression analyses in bronchial brushing samples. *TLR4 mRNA expression:* RAO-affected horses during acute exacerbation showed a significantly greater TLR4 mRNA expression compared to control horses ($P = 0.05$) and to RAO-affected horses at *days 7, 14,* and *28* ($P = 0.01$, $P = 0{,}005$ and $P = 0.002$, respectively) (Figure 4-2A). The fold changes (FCs) in mRNA expression are shown in

Table 4-2. The FC in TLR4 mRNA expression between RAO-affected and control horses at *day 0* was FC = 4.14 (P = 0.05). The TLR4 mRNA expression in control horses was significantly lower at *day 28* than at *day 0* (P=0.03). No statistically significant differences in TLR4 expression were observed between horse groups at *day 7, 14,* and *28* and between other time points within the RAO or the control horse group. *IL-8 mRNA expression:* RAO-affected horses during acute exacerbation had a significantly higher IL-8 mRNA expression compared to remission values at *day 14* and *28* (P = 0.04 and P = 0.04, respectively) (Figure 4-2B). The IL-8 mRNA expression in control horses was significantly lower at *days 7, 14,* and *28* than at *day 0* (P = 0.009, P = 0.05 and P = 0.01, respectively). No statistically significant differences in IL-8 expression were observed between horse groups at any other time points.

Associations among mRNA expression with clinical responses. We observed a highly significant correlation between TLR4 and IL-8 mRNA expression in bronchial brushing samples when data were pooled from RAO-affected and control horses (r = 0.81, P < 0.0001) as well as in RAO-affected and control horses separately (r = 0.77, P = 0.0003 and r = 0.84, P < 0.0001, respectively). The least square regression line between TLR4 and IL-8 mRNA expression shows an r^2-value of about 0.75 when data were pooled from all horses, 0.77 in the RAO group (Figure 4-3A) and 0.78 in the control group (Figure 4-3B). TLR4 and IL-8 mRNA expression were significantly correlated with the percentage of neutrophils within BAL in RAO-affected horses (r = 0.56, P = 0.02 and r = 0.61, P = 0.006, respectively) and their least square regression line showed r^2-values of about 0.32 and 0.43, respectively (Figure 4-3C and 4-3E, respectively). However, in control horses the correlations between TLR4 or IL-8 and percent BAL

neutrophils were not significant ($r = 0.34$, $P = 0.16$ and $r = 0.39$, $P = 0.06$, respectively; Figure 4-3D and 4-3F, respectively).

Discussion

LPS is able to increase expression of TLR4 in inflammatory cells (4, 127) and in lung tissue from healthy horses (170). Our data demonstrate that this may also be true in bronchial epithelial cells from horses, as in our control group the amount of TLR4 mRNA in BBs decreased gradually from *day 0*, when endotoxin exposure was greatest, to *day 7, 14*, and *28* (Figures 4-2A, Table 4-2). When horses were on pasture, there was no significant difference in the amount of epithelial-derived TLR4 mRNA between control and RAO-affected horses, but stable dust exposure increased amounts of TLR4 mRNA in BBs from RAO-affected horses more than in controls (Figure 4-2A, Table 4-2). This observation could explain the exacerbated neutrophilic airway inflammation of RAO-affected horses to airborne endotoxin (141, 144).

During stabling, there was no significant difference in the amount of epithelial-derived IL-8 mRNA between RAO-affected and control horses (Figures 4-2B, Table 4-2). In both horse groups, stable dust exposure was associated with significantly higher amounts of epithelial-derived IL-8 as compared to pasture. We observed a strong and highly significant association between the expressions of TLR4 and IL-8 mRNA in BBs in RAO-affected and control horses (Figures 4-3A and 4-3B, respectively). This suggests a role for TLR4 in the stimulation of IL-8 production in equine bronchial epithelial cells.

Similarly, asthmatic patients also show elevated production of IL-8 in bronchial epithelial cells (113, 179) and in cell cultures of human airway epithelial cells stimulation of TLR4 with LPS leads to increased IL-8 production (60, 123). However, in contrast to IL-8 expression, we found TLR4 expression to be higher in RAO-affected horses compared to control horses at *day 0* (Figure 4-2A, Table 4-2). This suggests that the amount of epithelial-derived TLR4 is most likely not the only factor responsible for IL-8 production in horses' airways. Other factors that may stimulate IL-8 expression include bradykinin (154), TNF-α (137), IL-1β (83), or inhaled air pollution particles (23).

Interleukin-8 is a potent neutrophil chemoattractant in human inflammatory airway diseases (130), and this has also been demonstrated for RAO-affected horses (51). Indeed, in RAO-affected horses there was a significant correlation between the amounts of epithelial cell derived IL-8 mRNA and neutrophils in BAL (Figure 4-3E). However, while there was no significant difference in epithelial-derived IL-8 mRNA between RAO-affected and controls at *day 0*, there were significantly higher numbers of neutrophils in RAO-affected compared to control horses at this time point (Figure 4-1B). Therefore, epithelial-derived IL-8 mRNA is unlikely to be the only source for stimulation of neutrophil migration into the airway lumen in horses. Other cells, such as BAL cells (4), airway smooth muscle cells (136), endothelial cells (85), and monocytes (45), also release IL-8 upon stimulation. Also, other neutrophil chemotactic agents such as leukotriene B_4, TNF-α, GM-CSF, complement activation, and reduced apoptosis (62, 103, 160), may contribute to accumulation of neutrophils in the airways.

In the present study we measured the amount of TLR4 and IL-8 mRNA in bronchial epithelial cells from RAO-affected and control horses following natural

challenge induced by stabling. It has been shown previously that stable dust is rich in endotoxin (38, 39) and that endotoxin is involved in the pathogenesis of RAO (36, 38). Because hay dust contains other pro-inflammatory agents (38, 39) that may interact with endotoxin to cause airway inflammation, we chose this natural challenge model. The disadvantage of this approach is, however, that these other pro-inflammatory agents may have contributed to airway inflammation in a TLR4 signaling independent manner.

In conclusion, we showed that exposure to stable dust leads to increased TLR4 mRNA expression in bronchial epithelial cells from RAO-affected horses and that the amount of epithelial TLR4 mRNA correlates with IL-8 mRNA expression as well as neutrophil accumulation in the airways during acute exacerbations in RAO-affected horses induced by stabling. These data suggest that an increased TLR4 signaling is an important contributor to the pathogenesis of RAO.

Table 4-1: Cellular composition of bronchoalveolar lavage fluid (BAL). Only horses that contained more than 80% epithelial cells within their BB samples were included. Mean ± S.E.M. of total (log10 transformed) and differential cell counts in BAL from RAO-affected and control horses during stabling (*day 0*) and after stabling (*days 7, 14 and 28*). (a) indicates significant difference ($P \leq 0.05$) between the horse groups (control, RAO) within a time point (*day 0, 7, 14, and 28*). (b) indicates significant difference ($P \leq 0.05$) from *day 0*.

logTCC = log10 transformed total BAL cell count; logNeu = log10 transformed total BAL neutrophil count; %Neu = percentage of neutrophils in BAL; logMac = log10 transformed total BAL macrophage count; %Mac = percentage of macrophages in BAL; logLym = log10 transformed total BAL lymphocyte count; %Lym = percentage of lymphocytes in BAL

	Day 0		Day 7		Day 14		Day 28	
	Control	**RAO**	**Control**	**RAO**	**Control**	**RAO**	**Control**	**RAO**
logTCC	6.47 ± 0.21	5.9 ± 0.21	6.61 ± 0.15	6.62 ± 0.33	6.61 ± 0.24	6.34 ± 0.2	6.41 ± 0.32	6.11 ± 0.42
logNeu	5.22 ± 0.33	5.48 ± 0.2	4.96 ± 0.26	4.86 ± 0.43	5.34 ±0.15	5.14 ± 0.36	4.72 ± 0.33	4.43 ± 0.35 [b]
%Neu	13.00 ± 7.83	46 ± 15.31 [a]	2.50 ± 1.24	2.4 ± 0.87 [b]	6.1 ± 3.69	10.08 ± 4.12 [b]	2.08 ± 0.76	3.58 ± 1.78 [b]
logMac	5.58 ± 0.26	4.91 ± 0.29	5.81 ± 0.23	5.71 ± 0.42	6.04 ± 0.23	5.09 ± 0.32	5.63 ± 0.44	5.19 ± 0.49
%Mac	15 ± 3.67	12.5 ± 5.36	21.33 ± 8.11	15.7 ± 4.97	29.8 ± 7.88	7.67 ± 2.19 [a]	17.83 ± 6.48	14.25 ± 3.93
logLym	6.26 ± 0.26	5.42 ± 0.33	6.46 ± 0.14	6.52 ± 0.31 [b]	6.35 ± 0.32	6.23 ± 0.18	6.25 ± 0.3	6 ± 0.41
%Lym	69.17 ± 11.22	40.13 ± 13.41[a]	73.42 ± 7.51	79.1 ± 5.19 [b]	61.9 ± 11.4	80.17 ± 6.71 [b]	58.83 ± 13.16	78.42 ± 4.87[b]

Table 4-2: Fold changes (FC) for the amount of TLR4 and IL-8 mRNA in bronchial brushing samples (BBs) between RAO-affected and control horses (RAO/Control) during stabling (*day 0*) and after stabling (*days 7, 14* and *28*) as well as between time points within the RAO-affected (RAO/RAO) and within the control horse group (Control/Control). Only horses that contained more than 80% epithelial cells within their BBs were included. (*) indicates that the FC is significant different ($P \leq 0.05$) to 1 (no change).

	time	TLR4	IL8
RAO/Control	day 0	**4.14 ***	1.72
	day 7	0.98	3.10
	day 14	1.49	1.24
	day 28	1.85	1.74
RAO/RAO	day 0 – day 7	**7.10 ***	4.40
	day 0 – day 14	**9.29 ***	**6.83 ***
	day 0 – day 28	**9.99 ***	**7.10 ***
	day 7 – day 14	1.31	1.55
	day 7 – day 28	1.41	1.61
	day 14 – day 28	1.07	1.04
Control/Control	day 0 – day 7	1.68	**7.91 ***
	day 0 – day 14	3.33	**4.92 ***
	day 0 – day 28	**4.46 ***	**7.18 ***
	day 7 – day 14	1.99	0.62
	day 7 – day 28	2.66	0.91
	day 14 – day 28	1.34	1.46

Figure 4-1: Clinical parameters in RAO-affected and control horses during stabling and on pasture. Only horses that contained more than 80% epithelial cells within their BB samples were included. A) Lung function (ΔPpl_{max}, [cm H_2O]) and B) percentage of neutrophils in bronchoalveolar lavage fluid (BAL) (Neu) from control (grey bars) and RAO-affected horses (black bars) during stabling (*day 0*) and after stabling (*days 7, 14* and *28*). (a) indicates significant difference ($P \leq 0.05$) between the horse groups (control, RAO) within a time point (*day 0, 7, 14,* and *28*). (b) indicates significant difference ($P \leq 0.05$) from *day 0*.

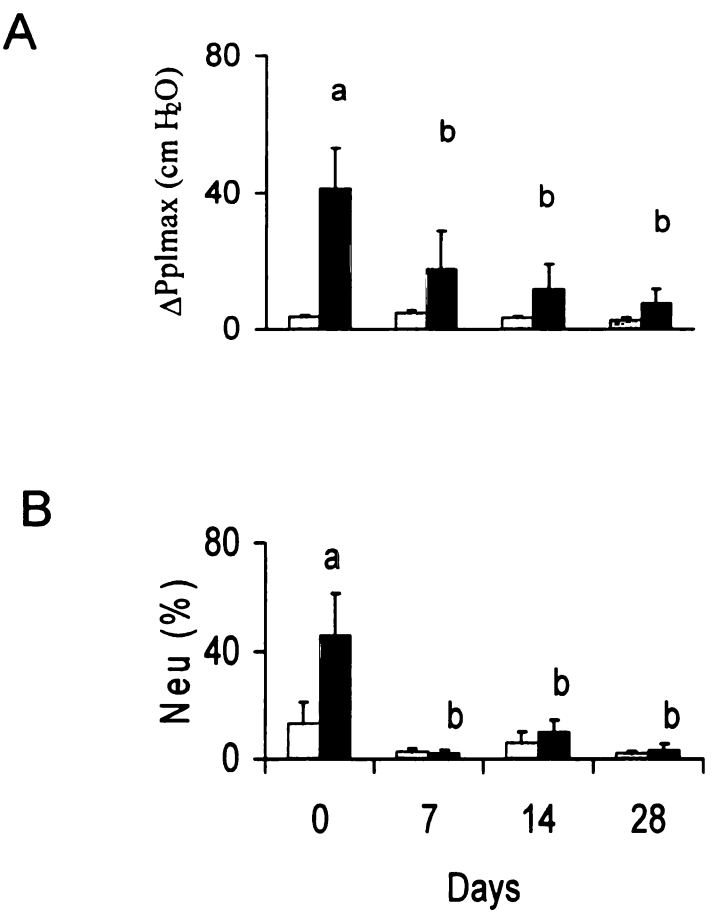


Figure 4-2: Relative mRNA quantification of A) TLR4 and B) IL-8 in bronchial brushing cells from control and RAO-affected horses during stabling (*day 0*) and after stabling (*days 7, 14,* and *28*). Only horses that contained more than 80% epithelial cells within their BB samples were included. Vertical bars show the 5 to 95% range with the mean of the standardized mRNA expression (horizontal bar) (data in antilog transformation). (a) indicates significant difference ($P \leq 0.05$) between the horse groups (control, RAO) within a time point (*day 0, 7, 14,* and *28*). (b) indicates significant difference ($P \leq 0.05$) between time points within a horse group.

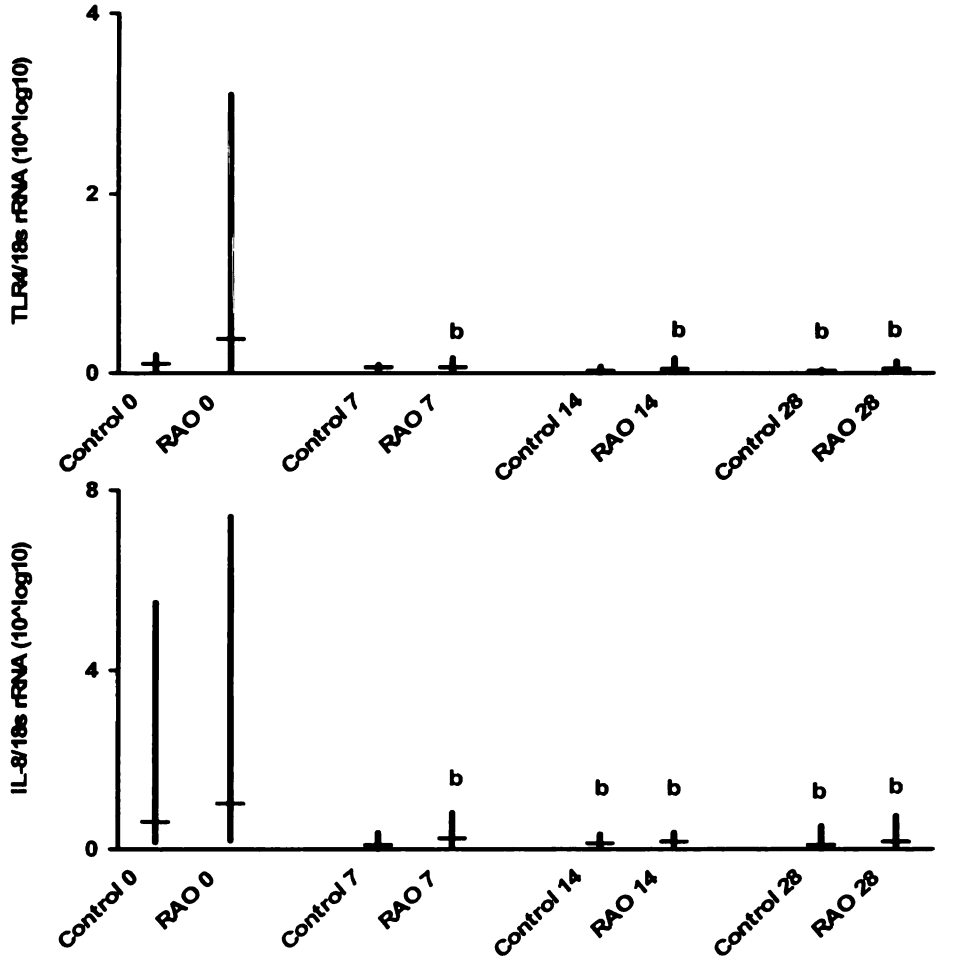
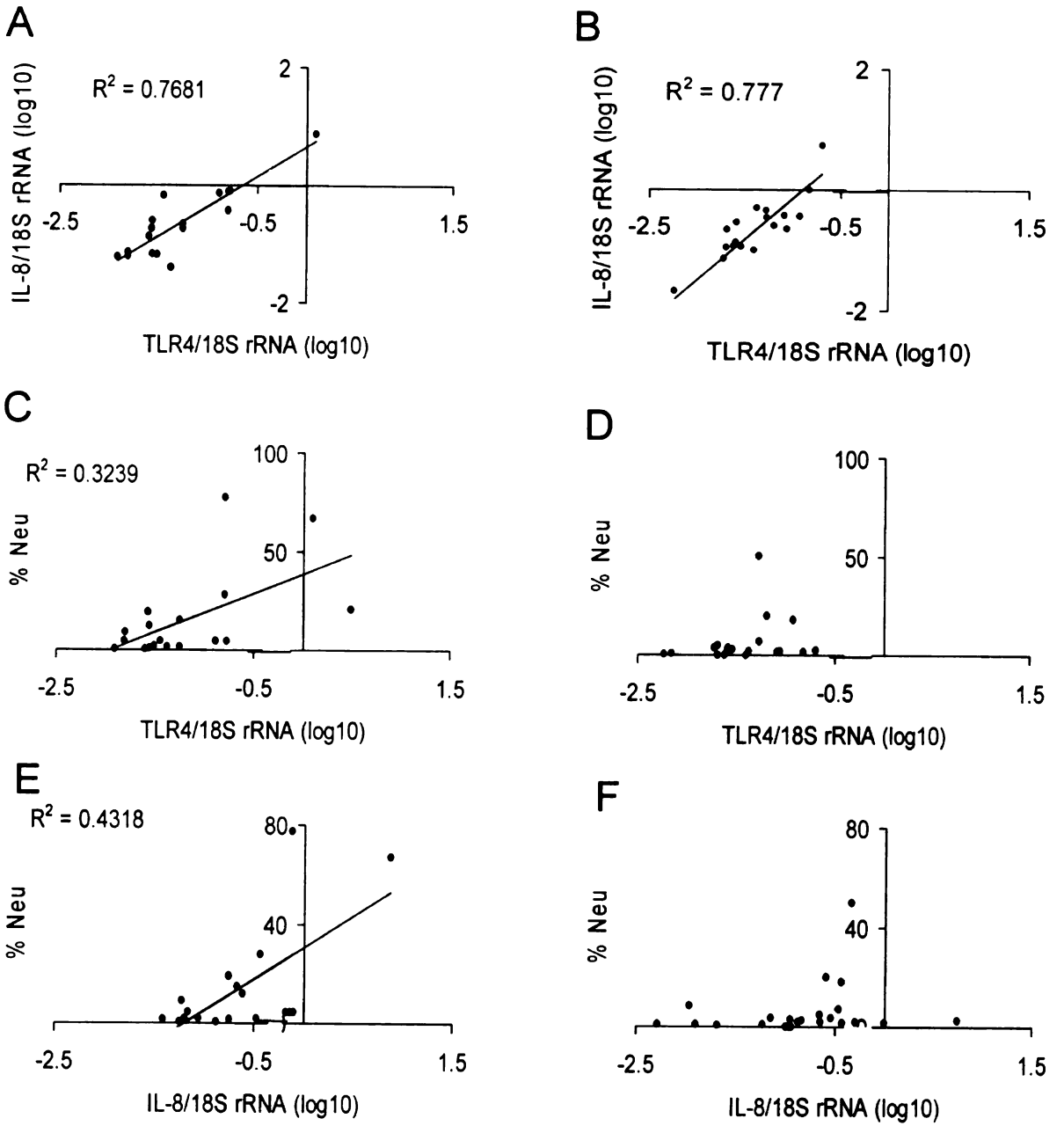


Figure 4-3: Least square regressions between mRNA expressions and clinical responses. Only horses that contained more than 80% epithelial cells within their BB samples were included. Least square regressions between relative amount of TLR4 and IL-8 mRNA in bronchial brushing samples (BB) in (A) RAO-affected and (B) control; between relative amount of TLR4 mRNA in BB and percentage of neutrophils in bronchoalveolar lavage (BAL) (% Neu) in (C) RAO-affected and (D) control horses; and between relative amount of IL-8 mRNA in BB and percentage of neutrophils in BAL in (E) RAO-affected and (F) control horses including measurements at all time points. For least square regressions with $P \leq 0.05$, R^2-values are given in the figures. Curves represent the least square regression line between variables.


A
R² = 0.7681
IL-8/18S rRNA (log10)
2
-2
-2.5
-0.5
1.5
TLR4/18S rRNA (log10)
B
R² = 0.777
IL-8/18S rRNA (log10)
2
-2
-2.5
-0.5
1.5
TLR4/18S rRNA (log10)
C
R² = 0.3239
% Neu
100
50
-2.5
-0.5
1.5
TLR4/18S rRNA (log10)
D
% Neu
100
50
-2.5
-0.5
1.5
TLR4/18S rRNA (log10)
E
R² = 0.4318
% Neu
80
40
-2.5
-0.5
1.5
IL-8/18S rRNA (log10)
F
% Neu
80
40
-2.5
-0.5
1.5
IL-8/18S rRNA (log10)

CHAPTER 5

EXPRESSION OF TOLL-LIKE RECEPTOR 2 mRNA IN BRONCHIAL EPITHELIAL CELLS IS NOT INDUCED BY EXPOSURE OF HORSES TO STABLE DUST

Abstract

Neutrophilic airway inflammation and obstruction are characteristic of recurrent airway obstruction (RAO). Acute disease exacerbations can be triggered by housing in stables. Fungi, such as *Faenia rectivirgula,* commonly found in stable dust, have been reported to initiate RAO in susceptible horses. Fungal products activate innate immune mechanisms by stimulating toll-like receptor (TLR) 2. In normal equine lung tissue TLR2 mRNA expression is increased by LPS exposure. In human airway epithelium, TLR2 stimulation leads to elevation of interleukin (IL)-8, a potent neutrophil attractant. We hypothesized that the amount of TLR2 mRNA in bronchial epithelium is increased when horses are stabled and decreases when they return to pasture. We further hypothesized that TLR2 and IL-8 mRNA expression are correlated with each other and with the numbers of neutrophils in bronchoalveolar lavage fluid (BAL). We measured airway obstruction (maximal change in pleural pressure; ΔPpl_{max}), determined cell counts in BAL, and quantified TLR2 and IL-8 mRNA in bronchial epithelial cells by qRT-PCR. We studied six horse pairs, each consisting of one RAO and one control horse. Each pair was studied when the RAO-affected horses had airway obstruction induced by stabling,

and after 7, 14, and 28 days on pasture. ΔPpl_{max} and neutrophils were increased in RAO-affected horses during stabling. While stabling increased IL-8, TLR2 mRNA expression was unaffected. TLR2 was not associated with numbers of neutrophils in BAL. While TLR2 mRNA is detectable in bronchial epithelial cells from all horses, stable dust does not lead to an increase in its mRNA expression. The localization or the amount of receptor complex rather than the amount of TLR2 mRNA may be more important in fungal-induced airway inflammation.

Introduction

Recurrent airway obstruction (RAO) is a common asthma-like disorder in horses. Susceptible animals develop recurrent neutrophilic airway inflammation and airway obstruction due to exposure to stable dust, and clinical remission can be re-established by returning RAO-affected horses from the disease-initiating stable environment to the pasture. This naturally occurring pulmonary disorder in horses closely resembles occupational asthma in humans (151).

Horse stables contain airborne fungi such as *Faenia rectivirgula* and *Aspergillus fumigatus* (128). Inhaled fungal products induce neutrophilic airway inflammation and changes of lung function in susceptible horses (33, 116, 143, 162). Originally, it had been suggested that RAO is an allergic condition as response to inhalation of, for example, fungal pathogens (151). Elevated IgE levels in bronchoalveolar lavage (BAL) of affected animals support a type-I hypersensitivity reaction (63, 162). Increased expression of T-helper cell (Th) type II (Th-2) specific cytokines, such as interleukin (IL–) 4 and IL-5 mRNA (99), further supports the suggestion that adaptive immune mechanisms are involved in the pathogenesis of RAO. However, allergic skin reactions to common barn allergens do not differ between affected and control horses (41, 115). Furthermore, cytokine expression profiles in RAO-affected horses vary among studies (3, 29, 59). Therefore, adaptive immune mechanisms may only partially explain the disease pathogenesis.

Innate immune mechanisms are also involved in the disease processes of RAO. There is evidence that endotoxin within stable dust can trigger neutrophilic airway inflammation in RAO-susceptible horses (144). Inhalation of endotoxin-depleted hay dust significantly attenuates airway neutrophilia and the inflammatory response can be re-established by adding endotoxin back to the endotoxin-depleted hay dust (144). Our laboratory has recently reported that Toll-like receptor (TLR) 4 mRNA expression in equine airway epithelium is increased during stabling (13). Toll-like receptors are transmembrane receptors that recognize pathogen-derived molecules, such as LPS or lipopeptides, and once stimulated they initiate signaling cascades that culminate in production of pro-inflammatory cytokines (12). TLR4 but not TLR2 mRNA is expressed

in normal horse lung tissue, and LPS exposure induces TLR4 and TLR2 mRNA expression (170). Whereas TLR4 is important for the recognition of LPS, TLR2 is crucial for recognition of *A. fumigatus* (108). In RAO-affected horses, inhalation of particulates isolated from hay dust that mainly contains fungal spores caused an increased influx of neutrophils into the airway lumen (144). Stimulation of TLR2 initiates a signaling cascade that eventually leads to activation of nuclear factor κB (NF-κB) activity and to the production of IL-8 (175), which is a potent neutrophil attractant (51, 130).

In the present study, we tested if the amount of TLR2 mRNA in bronchial epithelial cells of RAO-affected horses is greater than in control animals and we hypothesized that in both horse groups the amount of TLR2 mRNA expression is higher during stabling than on pasture. We also hypothesized that the amounts of TLR2 and IL-8 mRNA expression and the severity of airway inflammation are correlated among each other.

Materials and Methods

Animals. We studied RAO-affected and control horses in the research herd of the Pulmonary Laboratory at Michigan State University. RAO-affected animals were chosen because of a history of airway obstruction and inflammation when stabled and fed hay; because pasturing reversed their airway obstruction and inflammation; and because their airway obstruction was reversible with atropine. Control animals did not develop airway obstruction when stabled and fed hay (151).

Study design. We studied RAO-affected (2 mares, 4 geldings; 18.3 ± 3.3 years) and control horses (3 mares, 3 geldings; 16 ± 4.6 years) in age-matched pairs, with one RAO and one control horse per pair. Prior to the study, horses were kept on pasture. Measurements were made at four different time points. Horses were stabled until clinical signs of acute exacerbation were observed in the RAO-affected horse (*day 0*). When the RAO-affected horses developed a total clinical score of 5 or greater (153), we measured the change in pleural pressure during tidal breathing (ΔPpl_{max}). A ΔPpl_{max} of 15 cm H_2O or greater was used to define acute exacerbation in RAO-affected horses. In addition, we obtained bronchial brushing samples for RNA extraction and gene expression analyses and bronchoalveolar lavage samples for total and differential cell count analyses. Horses were then returned to the pasture. Subsequent measurements were obtained on days 7, 14, and 28. The protocol was approved by the All-University Committee for Animal Use and Care of Michigan State University.

Determination of the total clinical score (TCS)*:* A scoring system for the subjective clinical assessment of respiratory effort was used as previously described (157). Nasal flaring and abdominal movement were each scored separately on a scale of 1 (normal) to 4 (severe signs). To determine the TCS, scores for nasal flaring and abdominal movement were summed. Therefore, the TCS could range from 2 (normal) to 8 (severe signs).

Measurement of the maximal change in pleural pressure during tidal breathing (ΔPpl_{max}). The maximal change in pleural pressure was used as an indicator of airway obstruction. Measurements were made in unsedated horses by means of an oesophageal balloon connected via a 240-cm-long polypropylene catheter and a pressure transducer

(Validyne Model DP/45-22) to a physiograph (Dash Model 18, Astro-Med, Inc.). The balloon was passed through the nose, and placed into the middle third of the esophagus. Twenty breaths were averaged at each measurement period.

Collection of bronchial brushing samples (BB). Bronchial brushings were made between the 3rd- and 6th-generation bronchi via bronchoscopy. Bronchoscopy was performed with a 3-meter long endoscope (9-mm-diameter) using a transnasal approach. The bronchial brushing was performed by advancing a cytology brush (CytoSoft Cytology Brush, Medical Packaging Corporation) throughout the biopsy channel of the endoscope into the bronchial lumen. The brush was gently stroked against the airway wall 15 to 20 times. Care was taken to avoid bleeding. The brush was then withdrawn into the biopsy channel and the endoscope was removed from the horse's airways. The cytology brushes were then flushed in 1 ml phosphate-buffered saline (PBS) and stored on ice until further analysis. The procedure was repeated twice in the same lung. The side of the lung chosen for brushing was alternated between the measurement periods. The beginning lung side was chosen randomly for each horse.

Quantification of cells. Total and differential cell counts in BBs were performed manually using a hemacytometer. Cell preparations were made with a cytocentrifuge and stained with H&E stain. Differential cell counts were performed by counting 200 cells per slide.

Collection of bronchoalveolar lavage fluid (BAL). Bronchoalveolar lavage was obtained by means of a 3-meter long endoscope that was passed via the nose and wedged in a peripheral bronchus. Three 100 ml aliquots of PBS were infused into the tube and recovered by suction. The lavaged fluids were pooled and the volume was determined.

Quantification of inflammatory cells. Total and differential cell counts in BAL were performed using a hemacytometer. Cell smears were made by use of a cytocentrifuge and stained with Wright-Giemsa stain. Differential cell counts were performed by counting 200 cells per slide.

RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was isolated from BBs using a phenol/guanidine isothiocyanate mixture (TRI Reagent, Sigma) and 1-Bromo-3-Chloro-propane (Sigma-Aldrich, St. Louis, MO). Total RNA was treated with DNA-free kit (Ambion). Yields of total RNA were determined using NanoDrop technology (ND-1000 Spectrophotometer) and the integrated software version 3.1.2. The quality of total RNA was assessed using the Agilent 2100 Bioanalyzer and the integrity of the 18S and 28S rRNA was determined visually and by determination of the 18S:28S ratio. Depending on the total RNA yield of each sample, 40 to 200 ng total RNA were used as template for reverse transcription (RT) reaction using OMNISCRIPT reverse transcriptase kit 200 (Qiagen), according to the manufacturer's protocols. The RT reaction contained 10X RT buffer, 0.5 mM of each dNTP, 10 μM random hexamer primers (Applied Biosystems), 10 U/μl of RNAse inhibitor and 4 U of Omniscript Reverse Transcriptase. The conditions for the RT reaction were 37°C for 60 min, followed 93°C for 5 min using an *Eppendorf Mastercycler*. Quantitative PCR (qPCR) was performed using the ABI 7900 Sequence Detection System (Applied Biosystems). The qPCR reaction contained 20 ng of cDNA as template, QuantiTect SYBR Green PCR Kit (Qiagen), and oligonucleotide primer pairs specific to each mRNA of interest (see Table 3-1). Thermal cycling conditions in the ABI 7900 were 95°C for 15 min, followed by 40 cycles 95°C for 15 seconds; and 60°C for 60

seconds. A dissociation curve for each amplicon in each sample was generated to verify specificity of primer pairs. In addition, each RNA sample was analyzed for genomic contamination by testing RT-negative samples (use of RNAse free H_2O instead of Omniscript Reverse Transcriptase in RT reaction) for the reference gene. The oligonucleotide primer pair sequences used for amplification of TLR2, IL-8, and 18S rRNA transcripts, their amplicon length and their Genbank accession numbers or the source for the sequence used in the study are listed in Table 3-1. 18S rRNA served as the reference gene. Primer pairs were designed with Primer Express Software version 2.0 System (Applied Biosystems).

Statistical analysis. The relative amount of mRNA was calculated using the relative standard curve method. Quantitative means and their standard deviations (S.D.) for TLR2 and IL-8 of each BB were standardized to the reference gene (18S rRNA). If standardized quantitative mRNA expression data were not normally distributed (Kolmogorov-Smirnov $P \leq 0.05$), they were log10 transformed. Log-transformed data are reported as geometric means with the 5% and 95% range. Non-log-transformed data are reported as means ± S.E.M. Standardized data were analyzed by repeated measure ANOVA. Associations between single variables were determined using Spearman correlation analysis. Data were analyzed by use of SAS version 8, SAS/STAT Software (SAS Institute Inc., Cary, North Carolina, USA). Differences were considered significant for P-values ≤ 0.05.

Results

Airway function (ΔPpl_{max}). Data are shown in Table 5-1. At *day 0* ΔPpl_{max} was significantly increased in RAO-affected horses compared to control horses and to RAO-affected horses returned to pasture (Figure 5-1). At *days 7, 14,* and *28* no statistically significant differences in ΔPpl_{max} were observed between horse groups.

Cellular composition of bronchoalveolar lavage fluid (BAL). Table 5-1 shows total BAL cell counts for the two horse groups at the four different measurement periods. Total number of neutrophils showed a significant time effect (Figure 5-2). When data from RAO-affected and control horses were pooled, the total numbers of BAL neutrophils were significantly increased at *day 0* as compared to the BAL neutrophil numbers during *days 7, 14,* and *28.*

Quantitative mRNA expression analyses in bronchial brushing samples. We did not observe significant group, time, or group*time effects for the TLR2 expression in BBs (Table 5-1). There were significant group and time effects for IL-8 expression (Figure 5-3A and Figure 5-3B, respectively); however, the interaction between group and time was not significant (Table 5-1). When pooling data from all time points, IL-8 mRNA expression was significantly higher in RAO-affected than control horses (Figure 5-3A). When data from RAO-affected and control horses were pooled, IL-8 mRNA expression was significantly higher during stabling (*day 0*) than on pasture (*day 7, 14, and 28*) (Figure 5-3B).

Correlations between mRNA expression and BAL inflammatory cells. A correlation matrix between TLR2 and IL-8 mRNA expression as well as numbers of BAL

neutrophils is shown in Table 5-2. Neutrophil numbers correlated with IL-8 and TLR2 mRNA expression in RAO-affected horses (Figure 5-4A and 5-4B, respectively) or when data from all horses were pooled. Whereas the correlation between neutrophils and IL-8 (RAO: r = 0.42; pooled: r = 0.29) appeared to be positive, we observed negative correlations between neutrophils and TLR2 (RAO: r = -0.5; pooled: r = -0.36). The correlation of pooled data between neutrophils and IL-8 tended to be significant at P = 0.06).

Discussion

This study demonstrates for the first time that TLR2 mRNA is detectable in equine bronchial epithelial cells, and that this expression is unchanged by stabling. Furthermore, we did not observe a difference in TLR2 mRNA expression in bronchial epithelial cells between RAO-affected and control animals (Table 5-1). It is known that LPS, through stimulation of TLR4, leads to an increase in TLR2 expression (43). Because stable dust is rich in LPS (144, 145), and because we have previously shown an increase in TLR4 expression during stabling (13), we expected an increase in TLR2 expression on *day 0* and a decreasing amount during pasturing. However, a lack of a change in TLR2 mRNA expression does not mean that the TLR2 signaling pathway is unimportant in the pathogenesis of RAO. Altered mRNA stability (156), processes involved in the dynamics of RNA transport and translational regulation (86) or changes in cellular receptor protein localizations (175) could lead to unchanged amounts of

mRNA but increased receptor protein availability for signal transduction. Alternatively, alterations in the expression or availability of one of the TLR2 co-receptor (e.g., TLR1 or TLR6) may be important in contributing to the phenotype of RAO. Indeed, recently it has been reported that a defect of TLR2/1 signaling may be due to decreased TLR1 surface expression, while TLR2 expression was unchanged (186). Interestingly, the amount of intracytoplasmatic TLR1 was not different as compared to control subjects; therefore, suggesting that posttranslational mechanisms control the formation of the membrane receptor complex availability. The authors of this study also suggested that the threshold of TLR2/1 involvement in intracellular signaling is elevated compared to other TLRs due to low baseline TLR1 surface expression. Therefore, in future studies it would be of interest to determine TLR2 co-receptor mRNA and protein expression as well as to determine the cellular localization of TLR2 in relation to its co-receptors in bronchial epithelial cells in RAO-affected horses compared to control animals.

Our current findings are not in agreement with Singh Suri et al., who reported that unstimulated equine lung tissue does not express TLR2 mRNA, and that LPS exposure causes a measurable up-regulation in TLR2 mRNA expression (170). Whereas in the present study we quantified TLR2 mRNA expression in bronchial epithelial cells after natural challenge, Singh Suri et al. used qualitative mRNA expression analysis in whole lung tissue after intravenous LPS exposure (170). Therefore, differences in study design and methodology are likely explanations for these different outcomes.

In human airway epithelial cells, stimulation of the TLR2 signaling cascade leads to an elevation of IL-8 production (175). Clinical evidence suggests that inhalation of fungal products, such as from *F. rectivirgula*, is an important contributor to neutrophilic

airway inflammation in RAO-affected horses during stabling (33). Because IL-8 is a potent neutrophilic attractant in RAO-affected horses (51), we expected a positive association between TLR2 and IL-8 mRNA expression. The lack of correlation between the expression of TLR2 and IL-8 mRNA (Table 5-2) further supports the contention that the quantity of TLR2 protein or a change in the cellular localization of the receptor/co-receptor complex may be more important for TLR2 signal transduction than the amount of TLR2 mRNA.

We observed significantly more epithelial-derived IL-8 mRNA in RAO-affected versus control horses (Figures 5-3A) and a higher IL-8 mRNA expression during stabling than on pasture (Figure 5-3B). However, we observed a significant time effect (Figure 5-2) but no group effect (Table 5-1) for numbers of neutrophils in BAL. This might explain the correlation between IL-8 and neutrophils in RAO-affected horses (Figure 5-4A) and the lack of correlation between those variables in control horses (Figure 5-4B). Our laboratory and others have reported that stabled horses without clinical signs or a history of airway disease experience influx of inflammatory cells into the airway lumen (29, 31, 57, 73, 184). The results we report here suggest that in control horses either epithelial-derived IL-8 mRNA is not the only source for stimulation of neutrophil migration into the airway lumen or that other neutrophil chemotactic agents, such as leukotriene B_4, TNF-α and GM-CSF, complement activation or reduced neutrophilic apoptosis (62, 103, 160) may contribute to accumulation of neutrophils in the airways. Alternatively, regulators of IL-8 production, such as the zinc finger protein A20, may lead to a down-regulation of initially induced IL-8 expression (see below chapter 6).

Clinical evidence demonstrates that fungi, such as *F. rectivirgula*, are important in airway inflammation in RAO-affected horses. In the present study, we reported that while TLR2 mRNA expression is detectable in bronchial epithelial cells from all horses, inhalation of stable dust does not lead to an increase in TLR2 mRNA expression. Further studies are needed to determine whether the quantity of TLR2 protein or a change in the cellular localization of the receptor/co-receptor complex contribute to regulation of inflammation in RAO.

Table 5-1: Clinical parameters, cellular composition of bronchoalveolar lavage fluid (BAL) and TLR2 and IL-8 mRNA expression in bronchial brushing samples (BBs). [$]Mean ± S.E.M. of ΔPpl_{max} (difference between inspiratory and expiratory pleural pressure during tidal breathing) values, [#]geometric means of BAL cell counts and amounts of TLR2 and IL-8 mRNA in BBs from RAO-affected and control horses during stabling (*day 0*) and after stabling (*days 7, 14,* and *28*) as well as P-values for fixed effects (horse group (RAO, control), time, horse group*time) of ΔPpl_{max}, BAL inflammatory cells (neutrophils, macrophages and lymphocytes) as well as TLR2 and IL-8 mRNA expression in BBs. (a) indicates significant difference ($P \leq 0.05$) between the horse groups (control, RAO) within a time point (*day 0, 7, 14*, and *28*). (b) indicates significant difference ($P \leq 0.05$) from day 0.

TCC = Total BAL cell count (/ul); Neu = Total BAL neutrophil count (/ul); Mac = Total BAL macrophage count (/ul); Lym = Total BAL lymphocyte count (/ul); TLR2 = Toll-like receptor 2; IL-8 = interleukin 8.

	Day 0		Day 7		Day 14		Day 28		P-values for fixed effects		
	Control	RAO	Control	RAO	Control	RAO	Control	RAO	Horse group	Time	Horse group*Time
ΔPpl_{max} [$]	3.79 ± 0.34	37.16 ± 7.89[a]	4.73 ± 0.66	15.84 ± 9.2[b]	3.37 ± 0.32	11.74 ± 7.22[b]	2.87 ± 0.74	7.75 ± 3.97[b]	**0.0003**	**0.03**	**0.04**
TCC[#]	29.5 (11; 245)	21.4 (2.09; 200)	40.7 (10.5; 129)	34.7 (3.16; 269)	39.8 (10.5; 166)	21.9 (3.72; 87.1)	25.7 (4.17; 275)	12.9 (0.53; 219)	0.32	0.72	0.97
Neu[#]	1.55 (0.28; 11.5)	9.55 (1.62; 102)	0.91 (0.21; 5.13)	0.87 (0.02; 3.98)	2.19 (0.83; 3.98)	1.38 (0.07; 7.94)	0.53 (0.04; 2.75)	0.27 (0.03; 6.46)	0.79	**0.02**	0.37
Mac[#]	3.8 (0.5; 37.2)	1.74 (0.18; 26.9)	6.46 (1.05; 24.5)	4.27 (0.14; 29.5)	9.55 (1.86; 29.5)	9.55 (1.86; 29.6)	4.27 (0.47; 120)	4.27 (0.47; 120)	0.06	0.77	0.75
Lym[#]	18.2 (2.19; 191)	6.46 (0.28; 74.1)	28.8 (9.12; 110)	26.9 (2.85; 229)	23.4 (2.14; 132)	17 (3.39; 72.4)	17.8 (3.47; 148)	10 (0.41; 186)	0.28	0.47	0.9
TLR2[#]	0.62 (0.05; 2)	0.78 (0.44; 1.58)	0.59 (0.22; 1.48)	0.85 (0.38; 1.95)	0.54 (0.07; 2.45)	0.93 (0.37; 1.91)	0.72 (0.16; 1.48)	0.91 (0.49; 1.95)	0.21	0.97	0.97
IL-8[#]	0.58 (0.15; 5.5)	1.82 (0.18; 17.38)	0.07 (0.01; 0.37)	0.35 (0.04; 2.19)	0.14 (0.02; 0.39)	0.15 (0.06; 0.36)	0.08 (0.01; 0.5)	0.14 (0.06; 0.72)	**0.047**	**0.0013**	0.54

Table 5-2: Correlation matrix of Spearman correlations among total numbers of bronchoalveolar lavage (BAL) neutrophils, and bronchial brushing (BB) TLR2 mRNA and BB IL-8 mRNA in all horses, RAO-affected or control horses.

Neu = Total BAL neutrophil count (/ul); TLR2 = Toll-like receptor 2; IL-8 = interleukin 8; SCC = Spearman correlation coefficient; P = P-value; N = number of samples; n/a = not applicable

			Neu	TLR2	IL-8
Neu	RAO + Control	SCS	1.00		
		P	n/a		
		N	44		
	RAO	SCC	1.00		
		P	n/a		
		N	24		
	Control	SCC	1.00		
		P	n/a		
		N	20		
TLR2	RAO + Control	SCC	-0.36	1.00	
		P	0.02	n/a	
		N	43	47	
	RAO	SCC	-0.50	1.00	
		P	0.01	n/a	
		N	23	23	
	Control	SCC	-0.27	1.00	
		P	0.25	n/a	
		N	20	24	
IL-8	RAO + Control	SCC	0.29	0.13	1.00
		P	0.06	0.41	n/a
		N	42	46	46
	RAO	SCC	0.42	-0.27	1.00
		P	0.05	0.22	n/a
		N	22	22	22
	Control	SCC	-0.01	0.38	1.00
		P	0.95	0.06	n/a
		N	20	24	24

Figure 5-1: Lung function (ΔPpl_{max}, [cm H_2O]) in RAO-affected and control horses during (*day 0*) and after (*day 7, 14,* and *28*) stabling. Grey bars show RAO-affected horses and black bars show control horses. (a) indicates significant difference ($P \leq 0.05$) between the horse groups (control, RAO) within a time point (*day 0, 7, 14*, and *28*). (b) indicates significant difference ($P \leq 0.05$) from *day 0*.

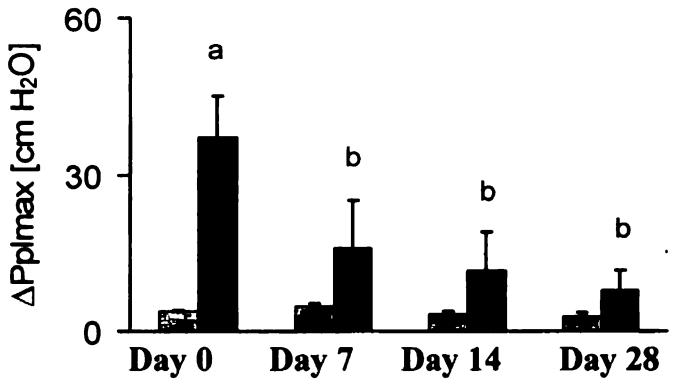


Figure 5-2: Total bronchoalveolar lavage (BAL) neutrophils as a function of time. Data from RAO-affected and control horses are pooled for times during stabling (*day 0*) and after stabling (*days 7, 14*, and *28*). Individual diamonds represent individual measurements. Horizontal bars represent the geometric means of total BAL neutrophils at each time point. (*) indicates significantly ($P \leq 0.05$) higher than on *days 7, 14*, and *28*. Total numbers of BAL neutrophils are $\log_{10}$ transformed.

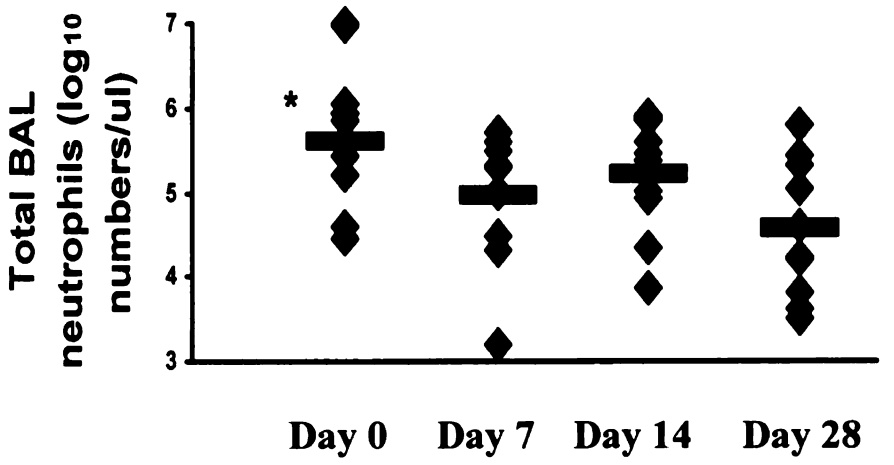
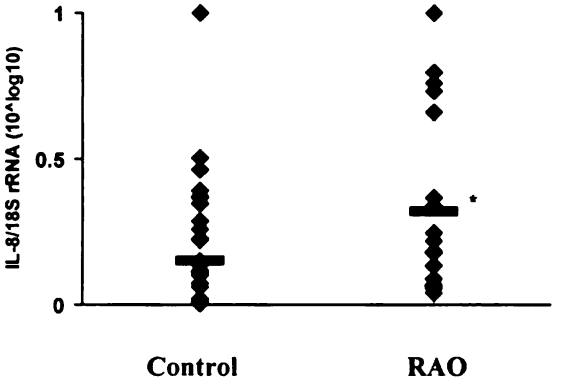


Figure 5-3: Interleukin (IL)-8 mRNA expression in bronchial epithelial cells between the diagnostic groups (RAO, control) and between the time points. A) Data from all time points are pooled for RAO-affected and control horses. Individual diamonds represent individual measurements. Horizontal bars represent the geometric means. (*) indicates a significant difference ($P \leq 0.05$) between the diagnostic groups. B) Data from RAO-affected and control horses are pooled for times during stabling (*day 0*) and after stabling (*days 7, 14*, and *28*). Individual diamonds represent individual measurements. Horizontal bars represent the geometric means of IL-8 mRNA expression at each time point. (*) indicates a significant difference ($P \leq 0.05$) from *days 7, 14*, and *28*. IL-8 mRNA expression is shown in relation to 18S rRNA (reference gene) expression.

A


IL-8/18S rRNA (10^log10)
1
0.5
0
*
Control
RAO

B

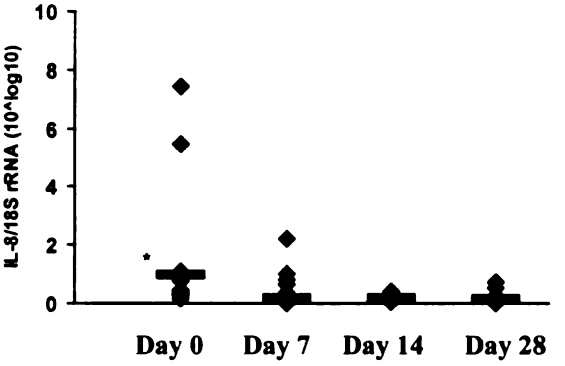
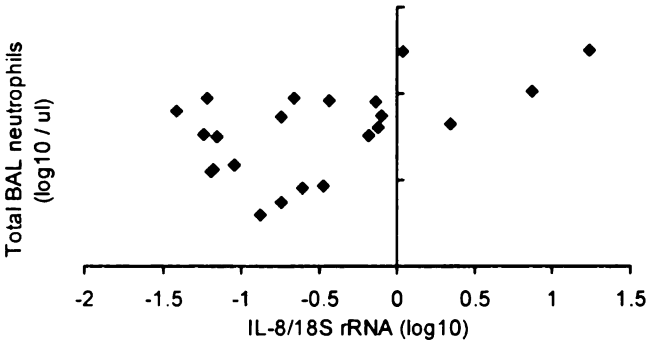
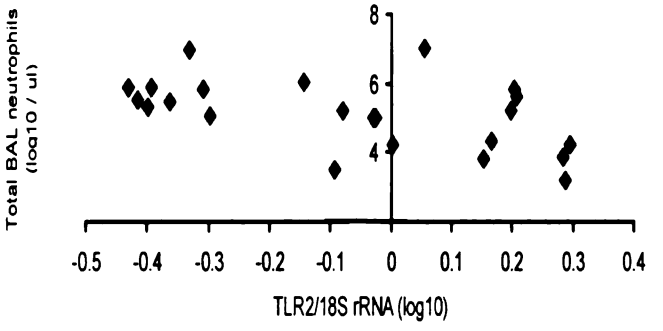

IL-8/18S rRNA (10^log10)
10
8
6
4
2
0
*
Day 0
Day 7
Day 14
Day 28

Figure 5-4: Associations between total bronchoalveolar lavage (BAL) neutrophils and IL-8 (A) or TLR2 (B) mRNA expression in bronchial brushing samples in RAO-affected horses. Total numbers of BAL neutrophils are $\log_{10}$ transformed. IL-8 and TLR2 mRNA expressions are shown in relation to 18S rRNA (reference gene) expressions.

A



B



CHAPTER 6

A20 mRNA EXPRESSION IN EQUINE BRONCHIAL EPITHELIAL CELLS IS NEGATIVELY CORRELATED WITH INFLAMMATORY CELL COUNTS IN BRONCHIAL LAVAGE FLUID

Abstract

Neutrophilic airway inflammation and obstruction are characteristic of recurrent airway obstruction (RAO). In human airway epithelium, TLR4 stimulation leads to elevation of interleukin (IL)-8, a potent neutrophil attractant. We reported previously that in horses IL-8 mRNA expression in bronchial epithelial cells paralleled the expression of toll-like receptor 4 (TLR4) expression (chapter 4). In other species, the TLR4 signaling cascade is negatively regulated by the zinc finger protein A20. Here we hypothesize that in RAO-affected horses the epithelial-derived A20 expression is not elevated compared to control horses. We further hypothesize that A20 is negatively associated with the numbers of inflammatory cells in BAL in control horses, but not in RAO-affected horses. We measured airway obstruction (maximal change in pleural pressure; ΔPpl_{max}), determined cell counts in BAL, and quantified A20 mRNA in bronchial epithelial cells by qRT-PCR. We studied six horse pairs, each consisting of one RAO and one control horse. Each pair was studied when the RAO-affected horses had airway obstruction induced by stabling, and after 7, 14, and 28 days on pasture. ΔPpl_{max} and neutrophils were

increased in RAO-affected horses during stabling. A20 expression did not differ between horse groups or time points. When data from RAO-affected and control horses were pooled, A20 was negatively associated with BAL inflammatory cell numbers and BAL inflammatory cells correlated amongst each other. Equine epithelial-derived A20 protects against airway inflammation by decreasing the number of BAL inflammatory cells. A lack of an increase in A20 expression as compared to control horses may contribute to the exaggerated neutrophilic airway inflammation in RAO-affected horses.

Introduction

Recurrent airway obstruction (RAO) in horses is characterized by recurrent neutrophilic airway inflammation and airway obstruction due to exposure to hay/stable dust. Clinical symptoms become apparent during stabling and are reversed by returning RAO-affected horses to pasture. Regulatory mechanisms involved in recruitment of airway inflammatory cells into the lung of RAO-affected horses have been studied previously. Sandersen et al. showed that nuclear factor kappa B (NF-κB) activity is correlated with the percentage of neutrophils found in bronchoalveolar lavage fluid (BAL) and that NF-κB activity in RAO-affected horses is elevated compared to control animals during and at least 21 days after stabling (161). Nuclear factor κB activation is crucial for production of pro-inflammatory cytokines, such as interleukin (IL-) 8, a potent neutrophil attractant in horses (50, 51). Indeed, IL-8 gene expressions as well as protein amount have been shown to be increased in BAL and airway epithelial cells from RAO-

affected horses compared to control horses (4, 13, 50). Correlations between IL-8 mRNA expression and BAL neutrophils have been reported previously (13, 59) and we have shown that epithelial-derived IL-8 mRNA is paralleled by the expression of toll-like receptor 4 (TLR4) (13).

Regulation of the TLR4 signaling in equine epithelial cells is unknown. The TLR4 signaling cascade is under the influence of both positive and negative feedback regulation. A variety of proteins, such as Tollip (201), suppressor of cytokine-signaling-1 (SOCS1) (87), IL-1R-associated kinase M (IRAK-M) (91), and A20 (60), are involved in the reduction of the TLR signal transduction. The gene of the zinc finger protein A20 was originally characterized as TNF-α-inducible (135). Subsequently, it was shown that it is a NF-κB responsive gene, and as such A20 is inducible by a wide variety of stimuli (92). The A20 transcript is rapidly but transiently induced, reaching its highest level within 1 hour following stimulation (135). A20-deficient mice develop multi-organ inflammation due to LPS stimulation (100). Therefore, A20 has been suggested to be an endogenous regulator of LPS-induced inflammation. Elevated A20 expression functions as a negative feedback loop to block NF-κB-dependent gene expression (14). For example, it has been shown that A20 over-expression leads to inhibition of TLR4-mediated IL-8 synthesis in human airway epithelial cells (60). Also, A20-deficient fibroblasts display prolonged NF-κB activity and are unable to terminate NF-κB activation (100). A20 interferes with the TLR signaling pathway at the level of the tumor necrosis factor receptor (TNFR)-associated factor (TRAF)-6 (16, 70, 105) leading to the inhibition of the phosphorylation of I-κB kinase (IKK), which is necessary for NF-κB activation.

Therefore, in this study we hypothesize that epithelial-derived A20 expression is not elevated compared to control horses. We further hypothesize that A20 is negatively associated with the numbers of inflammatory cells in BAL from control horses, but not from RAO-affected horses.

Materials and Methods

Animals and study design. Animals and study design have been described in detail elsewhere (chapter 5). The protocol was approved by the All-University Committee for Animal Use and Care of Michigan State University.

Determination of the total clinical score (TCS): A scoring system for the subjective clinical assessment of respiratory effort was used as previously described (157). Nasal flaring and abdominal movement were each scored separately on a scale of 1 (normal) to 4 (severe signs). To determine the TCS, scores for nasal flaring and abdominal movement were summed. Therefore, the TCS could range from 2 (normal) to 8 (severe signs).

Measurement of the maximal change in pleural pressure during tidal breathing (ΔPpl_{max}). The maximal change in pleural pressure was used as an indicator of airway obstruction. Measurements were made in unsedated horses by means of an oesophageal balloon connected via a 240-cm-long polypropylene catheter and a pressure transducer (Validyne Model DP/45-22) to a physiograph (Dash Model 18, Astro-Med, Inc.). The

balloon was passed through the nose, and placed into the middle third of the esophagus. Twenty breaths were averaged at each measurement period.

Collection of bronchial brushing samples (BB). Bronchial brushings were made between the 3rd- and 6th-generation bronchi via bronchoscopy. Bronchoscopy was performed with a 3-meter long endoscope (9-mm-diameter) using a transnasal approach. The bronchial brushing was performed by advancing a cytology brush (CytoSoft Cytology Brush, Medical Packaging Corporation) throughout the biopsy channel of the endoscope into the bronchial lumen. The brush was gently stroked against the airway wall 15 to 20 times. Care was taken to avoid bleeding. The brush was then withdrawn into the biopsy channel and the endoscope was removed from the horse's airways. The cytology brushes were then flushed in 1 ml phosphate-buffered saline (PBS) and stored on ice until further analysis. The procedure was repeated twice in the same lung. The side of the lung chosen for brushing was alternated between the measurement periods. The beginning lung side was chosen randomly for each horse.

Quantification of cells. Total and differential cell counts in BBs were performed manually using a hemacytometer. Cell preparations were made with a cytocentrifuge and stained with H&E stain. Differential cell counts were performed by counting 200 cells per slide.

Collection of bronchoalveolar lavage fluid (BAL). Bronchoalveolar lavage was obtained by means of a 3-meter long endoscope that was passed via the nose and wedged in a peripheral bronchus. Three 100 ml aliquots of PBS were infused into the tube and recovered by suction. The lavaged fluids were pooled and the volume was determined.

Quantification of inflammatory cells. Total and differential cell counts in BAL were performed using a hemacytometer. Cell smears were made by use of a cytocentrifuge and stained with Wright-Giemsa stain. Differential cell counts were performed by counting 200 cells per slide.

RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was isolated from BBs using a phenol/guanidine isothiocyanate mixture (TRI Reagent, Sigma) and 1-Bromo-3-Chloro-propane (Sigma-Aldrich, St. Louis, MO). Total RNA was treated with DNA-free kit (Ambion). Yields of total RNA were determined using NanoDrop technology (ND-1000 Spectrophotometer) and the integrated software version 3.1.2. The quality of total RNA was assessed using the Agilent 2100 Bioanalyzer and the integrity of the 18S and 28S ribosomal RNA (rRNA) was determined visually and by determination of the 18S:28S ratio. Depending on the total RNA yield of each sample, 40 to 200 ng total RNA were used as template for reverse transcription (RT) reaction using OMNISCRIPT reverse transcriptase kit 200 (Qiagen), according to manufacturer's protocols. The RT reaction contained 10X RT buffer, 0.5 mM of each dNTP, 10 μM random hexamer primers (Applied Biosystems), 10 U/μl of RNAse inhibitor and 4 U of Omniscript Reverse Transcriptase. The conditions for the RT reaction were 37°C for 60 min, followed 93°C for 5 min using an *Eppendorf Mastercycler*. Quantitative PCR (qPCR) was performed using the ABI 7900 Sequence Detection System (Applied Biosystems). The qPCR reaction contained 20ng of cDNA as template, QuantiTect SYBR Green PCR Kit (Qiagen), and oligonucleotide primer pairs specific to each mRNA of interest (see Table 1). Thermal cycling conditions in the ABI 7900 were 95°C for 15 min, followed by 40 cycles 95°C for 15 seconds; and 60°C for 60

seconds. A dissociation curve for each amplicon in each sample was generated to verify specificity of primer pairs. In addition, each RNA sample was analyzed for genomic contamination by testing RT-negative samples (use of RNAse free H_2O instead of Omniscript Reverse Transcriptase in RT reaction) for the reference gene. The oligonucleotide primer pair sequences used for amplification of A20 and 18S rRNA transcripts, their amplicon length, and their Genbank accession number or the source for the sequence used in the study are listed in Table 3-1. 18S ribosomal RNA (rRNA) served as the reference gene. Primer pairs were designed with Primer Express Software version 2.0 System (Applied Biosystems). The primer pair for A20 was designed by choosing regions of high sequence identity between the human and mouse A20 mRNA sequences.

Statistical analysis. The relative amount of mRNA was calculated using the relative standard curve method. Quantitative mean and their standard deviation (S.D.) for A20 of each BB were standardized to the reference gene (18S rRNA). Because the standardized quantitative mRNA expression data and BAL inflammatory cell counts were not normally distributed (Kolmogorov-Smirnov $P \leq 0.05$), they were $\log_{10}$ transformed. Log_{10}-transformed data are reported as geometric means with the 5% and 95% values. Standardized A20 expression data and inflammatory cell counts were analyzed by repeated measure ANOVA. Associations between single variables were determined using Spearman correlation analysis. Data were analyzed by use of SAS version 8, SAS/STAT Software (SAS Institute Inc., Cary, North Carolina, USA). Differences were considered significant for P-values ≤ 0.05.

Results

Cellular composition of bronchoalveolar lavage fluid (BAL). BAL cell counts for the two horse groups at the four different measurement periods have been reported elsewhere (chapter 5, Table 5-1). Briefly, when data from RAO-affected and control horses were pooled, total numbers of neutrophils were significantly increased at *day 0* as compared to *days 7, 14* and *28* (Figure 5-2).

Quantitative mRNA expression analyses in bronchial brushing samples. There was no statistical significant difference in the A20 mRNA expression in BBs between RAO-affected and control horses or among time points (Figure 6-1).

Correlations among BAL inflammatory cells, and associations between A20 mRNA expression and BAL inflammatory cells. Since A20 mRNA expression was not different between RAO-affected and control horses, data were pooled for correlation analysis. A correlation matrix between A20 mRNA expression and numbers of BAL inflammatory cells is shown in Table 6-1. Neutrophils in bronchoalveolar lavage fluid correlated with BAL macrophage ($r = 0.62$) and lymphocyte numbers ($r = 0.48$) (Figure 6-2A and 6-2B, respectively) and BAL macrophages correlated significantly with BAL lymphocytes ($r = 0.83$) (Figure 6-2C). Epithelial-derived A20 mRNA expression correlated negatively with BAL neutrophils ($r = -0.37$), BAL macrophages ($r = -0.46$) and BAL lymphocytes ($r = -0.42$) (Figure 6-3A, 6-3B and 6-3C, respectively).

Discussion

Here we showed for the first time that equine epithelial-derived A20 may protect against airway inflammation by decreasing the number of BAL inflammatory cells. We observed significant negative correlations between the epithelial-derived A20 mRNA expression and BAL inflammatory cell counts (Figure 6-3). In other species, A20 protects against airway inflammation (134). For example, while $A20^{-/-}$ mice have multiorgan inflammation (100), A20 over-expression leads to an attenuation of NF-κB activity (134), and, therefore, to decreased IL-8 production. In previous studies, we reported a positive correlation between BAL neutrophils and bronchial IL-8 or TLR4 mRNA expression as well as a strong association between IL-8 and TLR4 (chapters 4 and 5). Since stimulation of TLR4 initiates a signaling cascade that eventually leads to activation of NF-κB activity and to the production of IL-8 (175), and because A20 interferes with the TLR signaling pathway at the level of TRAF-6 (16, 70, 105), our results in the present study support the contention that equine A20 protects against neutrophilic airway inflammation by inhibition of NF-κB activation.

Because TNF-α in BAL from RAO-affected horses is greater during stabling than on pasture and compared to control horses (59), and because A20 is a TNF-α response gene, one could assume an up-regulation of A20 mRNA expression in BBs of RAO-affected horses during stabling and compared to controls. Instead we found that A20 expression was unaffected by stabling and did not differ between RAO-affected and control horses (Figure 6-1). This suggests that either the mechanism responsible for A20 up-regulation is defective, or that A20 mRNA expression is down-regulated in RAO-

affected compared to control horses and, therefore, may contribute to the exaggerated airway inflammation in RAO-affected horses during stabling. This is also supported by the finding of a prolonged NF-κB binding activity in RAO-affected versus control horse after stabling (21, 22, 161). Alternatively, other mediators of inflammatory cell migration into airways of RAO-affected horses, such as macrophage inflammatory protein-2 (MIP-2) (51, 110, 111), platelet activating factor and leukotriene B4 (42, 47-49, 110), β_2 integrin (200), and adhesion molecule CD18 (111) that may be differently regulated than IL-8, may play a more important role in modulation of neutrophilic inflammation in RAO-affected horses.

Beside neutrophils, mechanisms that attract macrophages and lymphocytes may also be under the regulation of A20. Our observation that neutrophil, macrophage, and lymphocyte numbers in BAL were correlated with each other (Figure 6-2) leads to the assumption of common migratory patterns for these cells. For example, certain integrins, such as lymphocyte-associated function antigen-1 (LFA-1 or CD11a/CD18) and macrophage antigen-1 (Mac-1 or CD11b/CD18), that are important for transendothelial migration of neutrophils (190, 200), are reported to be important in migration of lymphocytes and monocytes into the lung (101, 102, 190, 200). Furthermore, beside the production of IL-8, production of the monocyte chemoattractant termed CCL2 (monocyte chemotactic protein 1 [MCP-1]) can also be upregulated by stimulation of TLR4 with LPS (125).

Here we reported for the first time that, in horses, epithelial-derived A20 may protect against airway inflammation by decreasing the amount of BAL inflammatory cells, such as neutrophils, lymphocytes, and macrophages. A lack of an increase in A20

expression as compared to control horses may contribute to the exaggerated neutrophilic airway inflammation in RAO-affected horses.

Table 6-1: Correlation matrix of Spearman correlations among total numbers of bronchoalveolar lavage (BAL) inflammatory cells (neutrophils, macrophages, and lymphocytes) and epithelial-derived A20 mRNA after pooling data from RAO-affected and control horses.

Neu = Total BAL neutrophil count (/ul); Mac = Total BAL macrophage count (/ul); Lym = Total BAL lymphocyte count (/ul); A20 = zinc finger protein A20; SCC = Spearman correlation coefficient; P = P-value; N = number of samples; n/a = not applicable

		Neu	Mac	Lym	A-20
Neu	SCC	1.00			
	P	n/a			
	N	44			
Mac	SCC	0.62	1.00		
	P	<0.0001	n/a		
	N	44	47		
Lym	SCC	0.48	0.83	1.00	
	P	0.001	<0.0001	n/a	
	N	44	47	47	
A20	SCC	-0.37	-0.46	-0.42	1.00
	P	0.01	0.002	0.006	n/a
	N	43	42	42	43

Figure 6-1: Relative mRNA quantification of the zinc finger protein A20 in bronchial brushing cells from control and RAO-affected horses during stabling (*day 0*) and after stabling (*days 7, 14* and *28*). Vertical bars show the 5 to 95% range with the mean of the standardized mRNA expression (horizontal bar) (data in antilog transformation).

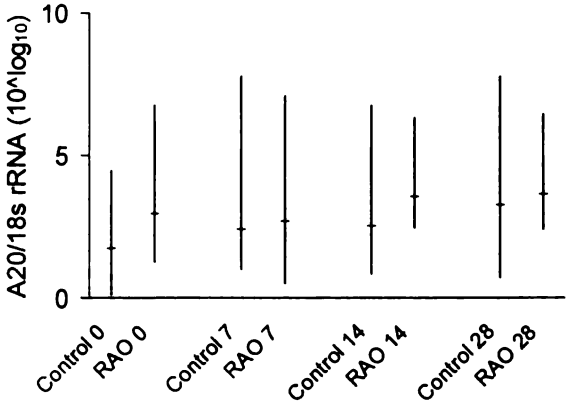


Figure 6-2: Association between total numbers of bronchoalveolar lavage (total BAL) neutrophils and total BAL macrophages (A), total BAL neutrophils and total BAL lymphocytes (B), and total BAL macrophages and total BAL lymphocytes (C).

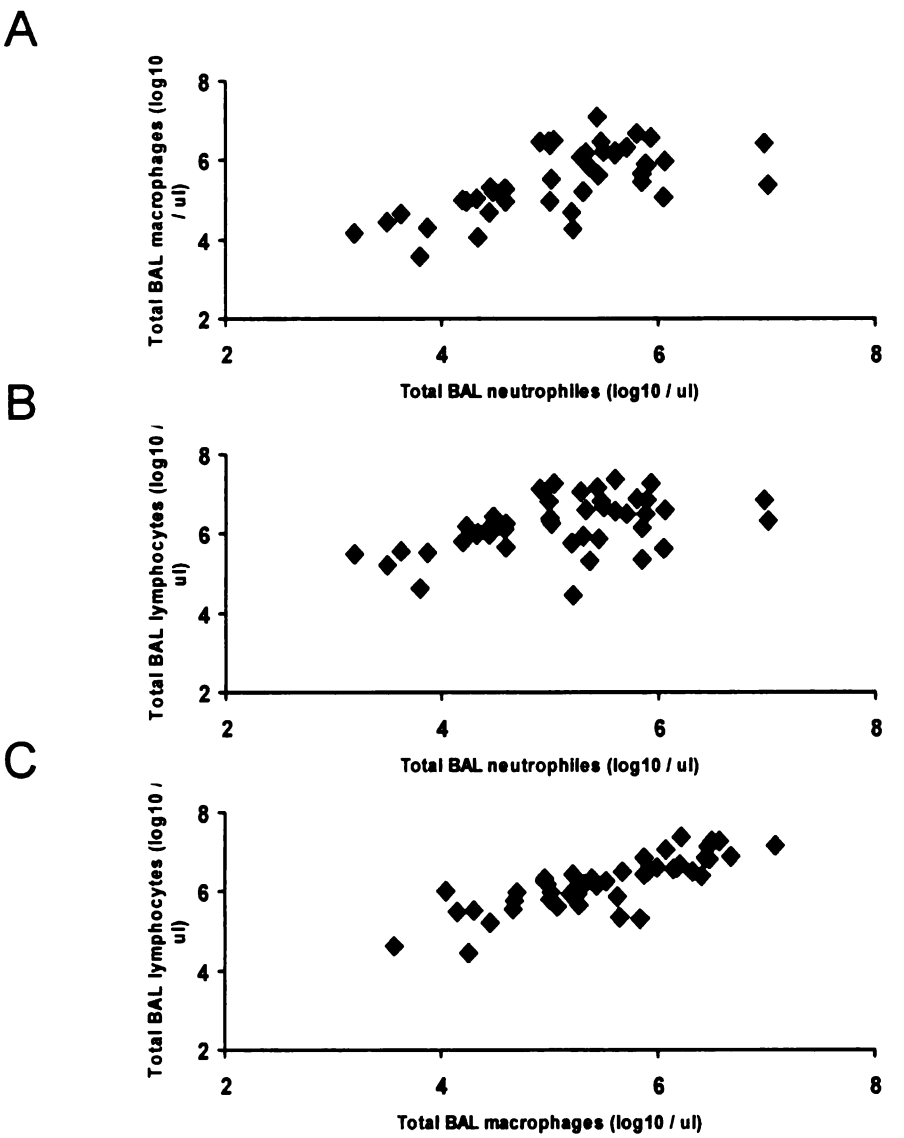
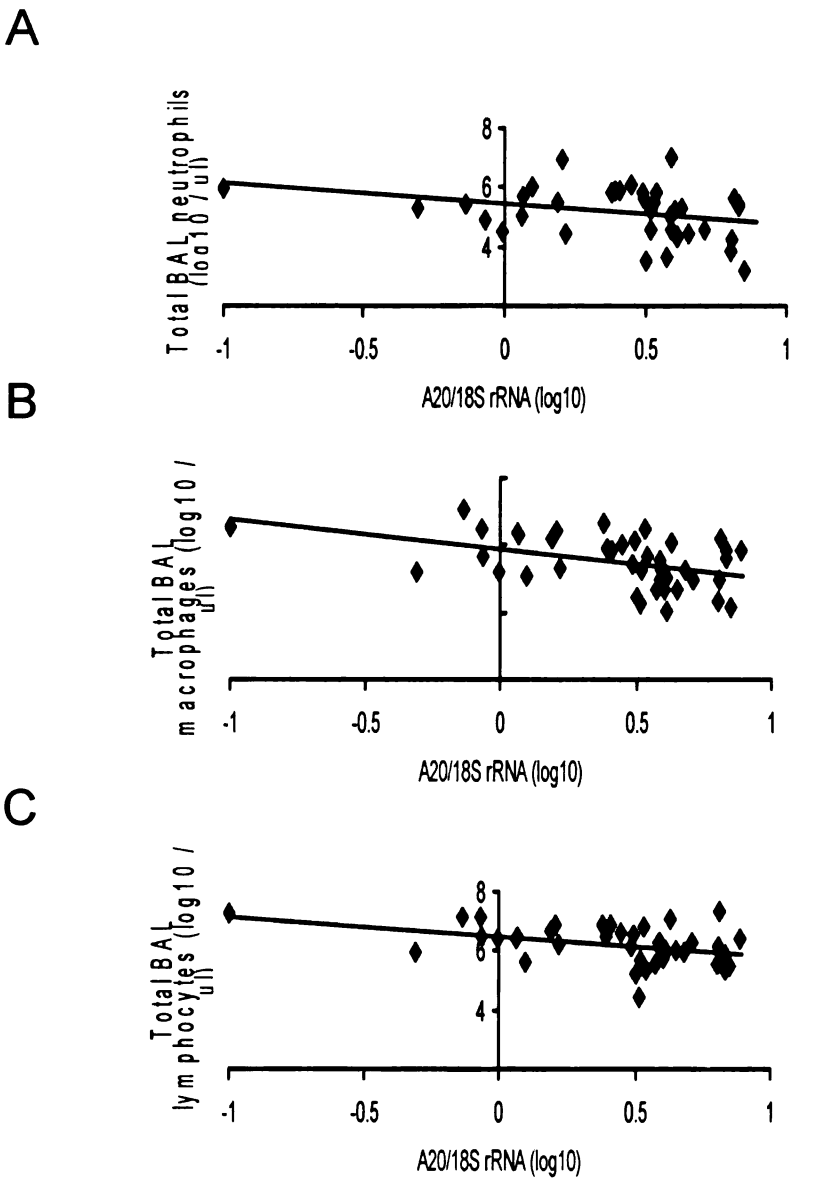


Figure 6-3: Associations between epithelial-derived A20 mRNA expression and total numbers of bronchoalveolar lavage (BAL) neutrophils (A), macrophages (B) and lymphocytes (C). A20 mRNA expressions is shown in relation to 18S rRNA (reference gene) expressions. Curves represent the linear associations between variables.



CHAPTER 7

REGRESSION AND PATHWAY ANALYSIS AND MODEL ADJUSTMENT OF TLR SIGNALING PATHWAYS IN EQUINE BRONCHIAL EPITHELIAL CELLS

Summary

In this dissertation, I propose a hypothetical model for innate immune mechanisms that are potentially involved in the pathogenesis of neutrophilic airway inflammation of RAO-affected horses. We have shown that RAO-affected and control horses did not differ in their inflammatory cell count either during stabling or on pasture. Therefore, in this chapter, I sought to adjust the given model for the general horse population by combining the data obtained in previous chapters. I analyzed TLR4, TLR2, IL-8, and A20 mRNA expressions and total bronchoalveolar lavage inflammatory cell counts using Spearman correlation and stepwise regression analyses. I concluded that airway inflammation in the general horse population is at least partially due to stimulation of TLR4 leading to IL-8 production and neutrophil accumulation. The latter may contribute to accumulations of macrophages. Finally, I propose that stimulation of TLR2 leads to A20 production, which is able to decrease BAL macrophage stimulation, which might be involved in protection against exaggerated airway inflammation in healthy control horses during stabling.

Introduction

Horses affected by recurrent airway obstruction develop neutrophilic airway inflammation, airway obstruction, and mucus accumulation. Since the latter two have been shown to be a result of inflammatory processes, with the research presented in this dissertation I was particularly interested in the pathogenesis of airway inflammation.

Although mechanisms for adaptive immune mechanisms have been reported elsewhere, we and others gained evidence that innate immune mechanisms are also involved in the pathogenesis of RAO. In chapter 1, I proposed a hypothetical model for innate mechanisms that are potentially involved in neutrophilic airway inflammation (Figure 1-1). Briefly, in RAO-affected horses, the production of IL-8, which is a potent neutrophilic chemoattractant in the equine lung, might be increased due to elevated expression of the TLR4 and TLR2, which are central in the detection of inhaled pathogens (e.g., bacteria, fungi, yeast). Stimulation of IL-8 production due to stimulation of TLR4 and 2 by their ligands is negatively regulated by the zinc finger protein A20, which itself is active during early stages of acute inflammation. Stable dust has been reported to contain a mixture of respirable pathogens. Furthermore, in chapter 2 we showed that concentrations of endotoxin, which is the primary ligand for TLR4, are higher in stables than on pasture. Finally, cell culture studies provide evidence that LPS stimulation can lead to up-regulation of TLR4 and 2 mRNA expressions.

In the studied horse population and under the published protocol we found that the exposure of horses to a stable environment causes neutrophilic airway inflammation. The stables were shown to be richer in endotoxin in comparison to the pasture (chapter

2), where these horses airway inflammation resisted. Although stabling caused an increase in neutrophils in the bronchoalveolar lavage fluid (BAL) of all horses (RAO-affected and control horses), this increase was higher in affected than control horses when considering the percentage of neutrophils (chapter 4), but did not differ when considering the total numbers of neutrophils (chapter 5). The significant difference in percentage of neutrophils between RAO-affected and control horses might have been rather the result of the accompanied decrease in the percentage of lymphocytes than an actual difference in the neutrophil numbers. Nevertheless, in the past others have also reported that stabling causes neutrophilic accumulation not only in the airways from RAO-affected horses but also in those from healthy control animals.

Similarly to our observation of an increase in total BAL neutrophils, we showed that the amount of epithelial-derived IL-8 mRNA expression was elevated in both RAO-affected and control horses due to stabling. Interleukin-8 is a neutrophilic chemoattractant in the airways of RAO-affected horses during acute exacerbations. Our results were supportive as we observed a correlation between the epithelial-derived IL-8 mRNA expression and BAL neutrophils in RAO-affected horses (chapter 4). However, this was not true for our control horse population.

In addition to the total numbers of BAL neutrophils and the IL-8 mRNA expression in airway epithelium, the expression of TLR4 mRNA was also elevated in RAO-affected and control horses during stabling than compared to pasture (chapter 4). However, the expression of TLR2 and A20 mRNA was unaffected by stabling. Toll-like receptor 4 expression strongly correlated with IL-8 mRNA expression in both RAO-affected and control animals. Although TLR2 expression did not correlate with IL-8, it

was negatively associated with the numbers of neutrophils, macrophages, and lymphocytes when all horses were pooled. Similarly, A20 was negatively associated with these BAL inflammatory cells after pooling RAO-affected and control horses.

Therefore, these studies show evidence that the hypothetical model I developed in chapter 1 is partially true, in that RAO-affected horses in acute exacerbation exhibit a pronounced expression of airway epithelial TLR4 expression, which leads to increased IL-8 production and accumulation of neutrophils in the airway lumen. In addition, we and others have reported that stabled horses without clinical signs or a history of airway disease experience influx of inflammatory cells into the airway lumen (29, 31, 57, 73, 184) On the other hand, we have shown that RAO-affected horses did not differ from control horses in their BAL inflammatory cell counts when they are kept in the same environment. Therefore, in this chapter I sought to adjust the given model for the general horse population by combining the data obtained in chapters 4, 5 and 6 in a overall statistical analysis.

Statistical analysis

The relative amount of mRNA was calculated using the relative standard curve method. Quantitative means and their standard deviations (S.D.) for TLR4, TLR2, IL-8 and A20 of each BB sample were standardized to the reference gene (18S rRNA). If standardized quantitative mRNA expression data and BAL total cell counts were not normally distributed (Kolmogorov-Smirnov $P \leq 0.05$), they were log10 transformed.

Correlations between single variables were determined using Spearman correlation analysis. Full models for regression analysis were developed according to Figure 7-1. Each variable was considered as the outcome of the combination of all possible predictors (i.e., in Figure 7-1: all variables that are at the same or a lower level than the defined outcome are considered to be predictors). For example, numbers of neutrophils is the outcome of a combination of TLR4, TLR2, IL-8, and A20 as well as numbers of macrophages and lymphocytes, or IL-8 is the outcome of a combination of TLR4, TLR2 and A20. Estimates, standard errors, t-values, and the R^2-value are reported for these full models. For each full model, I then sought to find subsets of predictors using a stepwise regression model to limit the possible models to a smaller number. For each step in the stepwise models estimates, F- and P-values are reported. For all statistical analyses, data from RAO-affected and control horses were pooled. Data were analyzed by use of SAS v. 9.1. Differences were considered significant for P-values ≤ 0.05.

Results

TLR4, TLR2, IL-8, and A20 mRNA expression: Toll-like receptor 4 and 2 mRNA expression are reported in chapter 4 and 5, respectively. Interleukin-8 mRNA expression is reported in both chapters 4 and 5. Whereas in chapter 4 only bronchial brushing samples with more than 80% of epithelial cells were used in data analysis, here we included all samples with detectable TLR4 expression. There were no differences in the results of TLR4 data between the two methods. Epithelial-derived A20 expression is discussed in chapter 6.

Bronchoalveolar lavage neutrophils, macrophages and lymphocytes: Total inflammatory cell counts are reported in chapters 4, 5 and 6.

Correlations between BAL inflammatory cell counts and bronchial brushing mRNA expressions: Correlations of pooled data between total BAL neutrophils and TLR2 or IL-8 are shown in chapter 5 and between BAL inflammatory cells and A20 as well as among inflammatory cells are reported in chapters 6. Beside the negative correlation between TLR2 and total BAL neutrophils (chapter 5), in the present analysis we also found negative correlations between TLR2 and total BAL macrophages and lymphocytes (Table 7-1). As in chapter 4, where we reported an association between epithelial-derived TLR4 and IL-8 mRNA in RAO-affected horses, we also found a very strong and highly significant correlation between those variables when data from all horses were pooled. Finally, we observed a strong and highly significant correlation between A20 and TLR2 mRNA expression (Table 7-1 and Figure 7-2).

Regression analyses: **Full models** for regression analyses are shown in Table 7-2. In the full models for the outcomes of total numbers of neutrophils and lymphocytes in BAL

the t-values were highest for the macrophages (t-value = 3.95 and 3.88, respectively). In the model for the outcome of the total numbers of macrophages, t-values for BAL lymphocytes (t-value = 3.88) and BAL neutrophils (t-value = 3.95) were higher than from other predictors. In the models for the outcomes of epithelial-derived IL-8 and A20 mRNA, TLR4 and TLR2 mRNA, respectively, are the coefficients with the highest t-values (t-value = 10.59 and 7.2, respectively).

Stepwise models are presented in Table 7-3. Whereas the numbers of macrophages dominated in comparison to any other predictors in the full model for the outcome of total numbers of neutrophils in BAL, the stepwise analysis showed that the combination of BAL macrophage numbers in combination with the amount of epithelial–derived TLR4 mRNA resulted in a higher R^2-value than in a model with BAL macrophages alone (R^2-value = 0.64 than 0.33, respectively). The R^2-value of the former was similar to the one we observed in the full model (R^2-value = 0.66).

When using the stepwise regression procedure for the outcome of total macrophage numbers in BAL, we observed the highest R^2-value of a model including not only numbers of neutrophils and lymphocytes, but also IL-8 and A20 mRNA expressions (R^2-value = 0.8). As compared to the R^2-values in steps 1 through 3, this R^2-value was the same as for the full model. Although there was an elevation of the R^2-value when adding A20 in the model (step 4), its P-value was only significant at a level of 0.1. In addition, both IL-8 and A20 were negatively associated with the macrophage number.

For the outcomes of total numbers of BAL lymphocytes and epithelial-derived IL-8 mRNA there was no additional information than those reported for the full models. The R^2-

values in the stepwise models are similar or the same than those in full models (Lym: R^2-value = 0.62 and 0.63, respectively; IL-8: R^2-value = 0.81 and 0.81, respectively).

A combination of TLR2 and TLR4 mRNA expression significantly contributes to the regression model that explains the amount of epithelial-derived A20 mRNA. Whereas the amount of TLR2 was positively associated with the outcome, TLR4 seemed to contribute in a negative manner. In comparison to the full model for A20, by using the stepwise procedure TLR4 was added. However, its P-value is not as small and its F-value is not as high as the appropriate values reported for the predictor TLR2 (TLR4: P-value = 0.02, F-value = 5.6; TLR2: P-value = < 0.0001, F-value = 53.35). This might explain the small increase in the R^2-value from step 1 to step 2 (R^2-value = 0.57 and 0.63, respectively). Nevertheless, the R^2-value of the model in step 2 resembles the one for the full model.

Discussion

With the results of these analyses I developed a potential model for predictors of airway inflammation in the general horse population (Figure 7-3). In chapter 4 we reported a positive correlation between the percentage of neutrophils and TLR4 or IL-8 mRNA expression in RAO-affected horses. In the present study we found that the correlation between the total numbers of BAL neutrophils and TLR4 mRNA expression or IL-8 mRNA expression at least tended to be significant as well (P = 0.07 or 0.06, respectively). We showed in chapter 5 that total BAL neutrophils correlated negatively with epithelial-derived TLR2 mRNA, and in chapter 6 that total BAL neutrophils also

correlated with BAL macrophages or BAL lymphocytes. The regression analyses we performed here showed that the number of macrophages in BAL predicts the number of neutrophils even when other possible predictors for neutrophils in the airways (e.g., IL-8, TLR4, TLR2) are present as well. However, the full model's R^2-value was only achieved when macrophages and TLR4 mRNA expression were combined during the stepwise analysis. Therefore, besides TLR4 signaling in airway epithelium, macrophages in BAL may also contribute to the accumulation of neutrophils within the airways. However, since the numbers of BAL macrophages were not different during stabling than on pasture, other characteristics than the macrophage number might be important in determination of the amount of neutrophils, such as the release of chemoattractants due to activation. For example, it has been reported that MIP-2 or IL-8, which are potent neutrophilic attractants in the horse lung, are released by equine alveolar macrophages upon stimulation.

In our studies in chapter 6 we observed that total BAL macrophages correlate positively with total BAL neutrophils and lymphocytes as well as negatively with epithelial-derived A20 mRNA expression. In the present analyses we also showed that it correlated negatively with TLR2. The R^2-value of the full regression model that explains BAL macrophage numbers was reached during the stepwise regression analysis when BAL neutrophils and lymphocytes were combined with IL-8 and A20. While neutrophils and lymphocytes were positively associated with macrophage numbers, IL-8 and A20 contributed with negative associations to macrophage numbers in the selected model. Mechanisms by which neutrophils can contribute to the accumulation of macrophages in the airway lumen are, for example, the release of proteases, which may function in tissue

destruction and, therefore, would ease the trans-endothelial migration of inflammatory cells. Proteases, such as equine elastase 2-A or matrix metalloproteinase (MMPs, especially, MMP9), have been shown to be associated with airway inflammation in equine airways. Macrophages are inflammatory cells that belong to the early inflammatory response and might contribute to attraction of lymphocytes in subsequent inflammatory processes, such as the presentation of pathogenic peptides to T-lymphocytes. This might explain the inclusion of lymphocytes in the regression model for macrophages, which is supported by the regression model for total BAL lymphocyte numbers. Stimulation of TLR4 causes expression of the monocyte chemoattractant CCL2 (monocyte chemotactic protein 1 [MCP-1]) (111). Since TLR4 signals through activation of NF-κB and because A20 inhibits the NF-κB translocation it might explain the negative association between BAL macrophage numbers and A20 mRNA expression. Investigation of production of chemoattractants for monocytes due to TLR signaling in airway epithelial or other airway cells should be the subject of future studies.

In chapter 4 we reported a strong correlation between TLR4 and IL-8 mRNA expressions. Here we showed that indeed TLR4 mRNA expression explains the IL-8 production.

Finally, the regression models for A20 mRNA expression show that epithelial-derived TLR2 mRNA is the strongest predictor, since the R^2-value in the stepwise model that only includes TLR2 as predictor (R^2-value = 0.57) contributes over 90% to the full model R^2-value (R^2-value = 0.63). In addition, we observed a highly significant negative correlation between epithelial-derived TLR2 and A20 mRNA expression (Table 7-1 and Figure 7-2). To the authors' knowledge this association has not been reported previously.

However, a strong correlation between TLR2 and A20 mRNA expression is not unexpected. Indeed, A20 was originally characterized as a TNF-α-inducible gene (135), and subsequently, it was shown that it is a NF-κB target gene. As such, A20 is inducible by a wide variety of stimuli (92). While TLR2 stimulation in human airway epithelial cells leads to IL-8 production by NF-κB activation (60), A20 is a negative regulator of inflammation by inhibiting nuclear NF-κB translocation (134). Our data show that TLR2 and A20 expression are associated with each other. One may assume that A20 is involved in modulation of TLR2-induced inflammation. As a matter of fact we showed here and in the previous chapters that both A20 and TLR2 are negatively correlated with total numbers of BAL neutrophils, macrophages, and lymphocytes. Alternatively, a common stimulus for or the use of a common pathway that leads to TLR2 and A20 expression could also explain a correlation between these variables.

In summary, I propose that airway inflammation in the general horse population is at least partially due to stimulation of TLR4, which may cause IL-8 production and neutrophilic airway accumulation. The latter may contribute to accumulations of macrophages by, for example, release of mediators, such as proteases, or due to decreasing macrophage apoptosis processes. In addition, stimulation of TLR2 may cause A20 production, which is able to decrease BAL macrophage accumulation and which may protect against exaggerated airway inflammation in healthy control horses during stabling.

Table 7-1: Correlation matrix of Spearman correlations between total numbers of bronchoalveolar lavage (BAL) inflammatory cells (neutrophils, macrophages and lymphocytes) and epithelial-derived TLR4, TLR2, A20, and IL-8 mRNA after pooling data from RAO-affected and control horses.

Neu = Total BAL neutrophil count (/ul); Mac = Total BAL macrophage count (/ul); Lym = Total BAL lymphocyte count (/ul); TLR4 = Toll-like receptor 4; TLR2 = Toll-like receptor 2; A20 = zinc finger protein A20; IL-8 = interleukin 8; SCC = Spearman correlation coefficient; P = P-value; N = number of samples; n/a = not applicable

		Neu	Mac	Lym	TLR4	TLR2	A20	IL-8
Neu	SCC	1.00						
	P	n/a						
	N	44.00						
Mac	SCC	0.62	1.00					
	P	<0.0001	n/a					
	N	44.00	47.00					
Lym	SCC	0.48	0.83	1.00				
	P	0.00	<0.0001	n/a				
	N	44.00	47.00	47.00				
TLR4	SCC	0.30	-0.24	-0.25	1.00			
	P	0.07	0.14	0.12	n/a			
	N	37.00	40.00	40.00	41.00			
TLR2	SCC	-0.36	-0.56	-0.53	0.21	1.00		
	P	0.02	<0.0001	0.00	0.19	n/a		
	N	43.00	46.00	46.00	41.00	47.00		
A20	SCC	-0.39	-0.46	-0.42	-0.13	0.83	1.00	
	P	0.01	0.00	0.01	0.43	<0.0001	n/a	
	N	39.00	42.00	42.00	37.00	43.00	43.00	
IL-8	SCC	0.29	-0.14	-0.23	0.84	0.13	-0.11	1.00
	P	0.06	0.36	0.13	<0.0001	0.41	0.47	n/a
	N	42.00	45.00	45.00	40.00	46.00	42.00	46.00

Table 7-2: Full models for regression analysis. For each outcome a model combining possible predictors is shown. Predictors were chosen according to the flow diagram shown in Figure 7-1. Each variable was considered as an outcome of the combination of all possible predictors (i.e., in Figure 7-1: all variables that are at the same or a lower level than the defined outcome are considered to be predictors). The estimate, standard error, and t-value are reported for each predictor in the model. The overall R^2-value is reported for each full model.

Neu = Total BAL neutrophil count (/ul); Mac = Total BAL macrophage count (/ul); Lym = Total BAL lymphocyte count (/ul); TLR4 = Toll-like receptor 4; TLR2 = Toll-like receptor 2; A20 = zinc finger protein A20; IL-8 = interleukin 8

Outcomes	Predictors	Estimate	Standard Error	t-value	P-value	R^2-value
Neu	Intercept	1.82	1.24	1.47	0.15	0.66
	Mac	0.92	0.23	3.95	0.0006	
	Lym	-0.12	0.24	-0.51	0.61	
	TLR4	0.79	0.41	1.93	0.06	
	TLR2	-0.26	0.26	-1.00	0.33	
	A20	0.09	0.09	1.00	0.32	
	IL-8	0.12	0.32	0.39	0.80	
Mac	Intercept	0.16	0.87	0.18	0.86	0.80
	Neu	0.42	0.11	3.95	0.0006	
	Lym	0.49	0.13	3.88	0.0007	
	TLR4	-0.25	0.29	-0.85	0.40	
	TLR2	0.06	0.18	0.34	0.74	
	A20	-0.08	0.06	-1.38	0.18	
	IL-8	-0.19	0.21	-0.90	0.38	
Lym	Intercept	2.46	0.97	2.55	0.02	0.63
	Neu	-0.09	0.17	-0.51	0.61	
	Mac	0.76	0.20	3.88	0.0007	
	TLR4	0.05	0.37	0.14	0.89	
	TLR2	-0.13	0.23	-0.56	0.58	
	A20	0.04	0.07	0.48	0.63	
	IL-8	-0.03	0.27	-0.11	0.91	
IL-8	Intercept	0.87	0.16	5.40	<0.0001	0.81
	TLR4	1.15	0.11	10.59	<0.0001	
	TLR2	0.02	0.14	0.15	0.88	
	A20	-0.01	0.04	-0.14	0.89	
A20	Intercept	-0.14	0.97	-0.15	0.88	0.63
	TLR4	-0.89	0.99	-0.91	0.37	
	TLR2	2.69	0.37	7.20	<0.0001	
	logIL8	-0.10	-0.10	-0.14	0.89	

Table 7-3: Stepwise regression analysis. For each full model reported in Table 7-1 a stepwise analysis procedure was applied. The estimate, F-, P-, and R^2-values are reported for each predictor in individual steps.

Neu = Total BAL neutrophil count (/ul); Mac = Total BAL macrophage count (/ul); Lym = Total BAL lymphocyte count (/ul); TLR4 = Toll-like receptor 4; TLR2 = Toll-like receptor 2; A20 = zinc finger protein A20; IL-8 = interleukin 8

Outcome	Predictors		1st step	2nd step	3rd step	4th step
Neu	Intercept	Estimate	1.61	1.69		
		F-value	2.82	5.55		
		P-value	0.10	0.03		
	Mac	Estimate	0.65	0.82		
		F-value	14.82	39.33		
		P-value	0.0006	<0.0001		
	TLR4	Estimate		0.87		
		F-value		24.73		
		P-value		<0.0001		
		R^2-value	0.33	0.64		
Mac	Intercept	Estimate	0.24	-0.40	-0.17	0.51
		F-value	0.10	0.31	0.06	0.46
		P-value	0.75	0.58	0.80	0.50
	Lym	Estimate	0.86	0.73	0.55	0.50
		F-value	49.17	37.41	19.90	16.66
		P-value	<0.0001	<0.0001	0.0001	0.0004
	Neu	Estimate		0.28	0.42	0.39
		F-value		8.60	17.97	15.93
		P-value		0.01	0.0002	0.0005
	IL-8	Estimate			-0.32	-0.34
		F-value			8.12	9.74
		P-value			0.01	0.0043
	A20	Estimate				-0.06
		F-value				2.98
		P-value				0.10
		R^2-value	0.62	0.71	0.77	0.80
Lym	Intercept	Estimate	2.20			
		F-value	14.13			
		P-value	0.0007			
	Mac	Estimate	0.72			
		F-value	49.17			
		P-value	<0.0001			
		R^2-value	0.62			
IL-8	Intercept	Estimate	0.88			
		F-value	46.32			
		P-value	<0.0001			
	TLR4	Estimate	1.15			
		F-value	141.77			
		P-value	<0.0001			
		R^2-value	0.81			
A20	Intercept	Estimate	1.08	-0.23		
		F-value	6.09	0.12		
		P-value	0.02	0.74		
	TLR2	Estimate	2.62	2.69		
		F-value	44.77	53.35		
		P-value	<0.0001	<0.0001		
	TLR4	Estimate		-1.01		
		F-value		5.60		
		P-value		0.02		
		R^2-value	0.57	0.63		

Figure 7-1: Flow diagram for developing full models for regression analyses. All variables that are at the same or at a lower level than the defined outcome are considered to be predictors. All variable were considered to be outcomes.

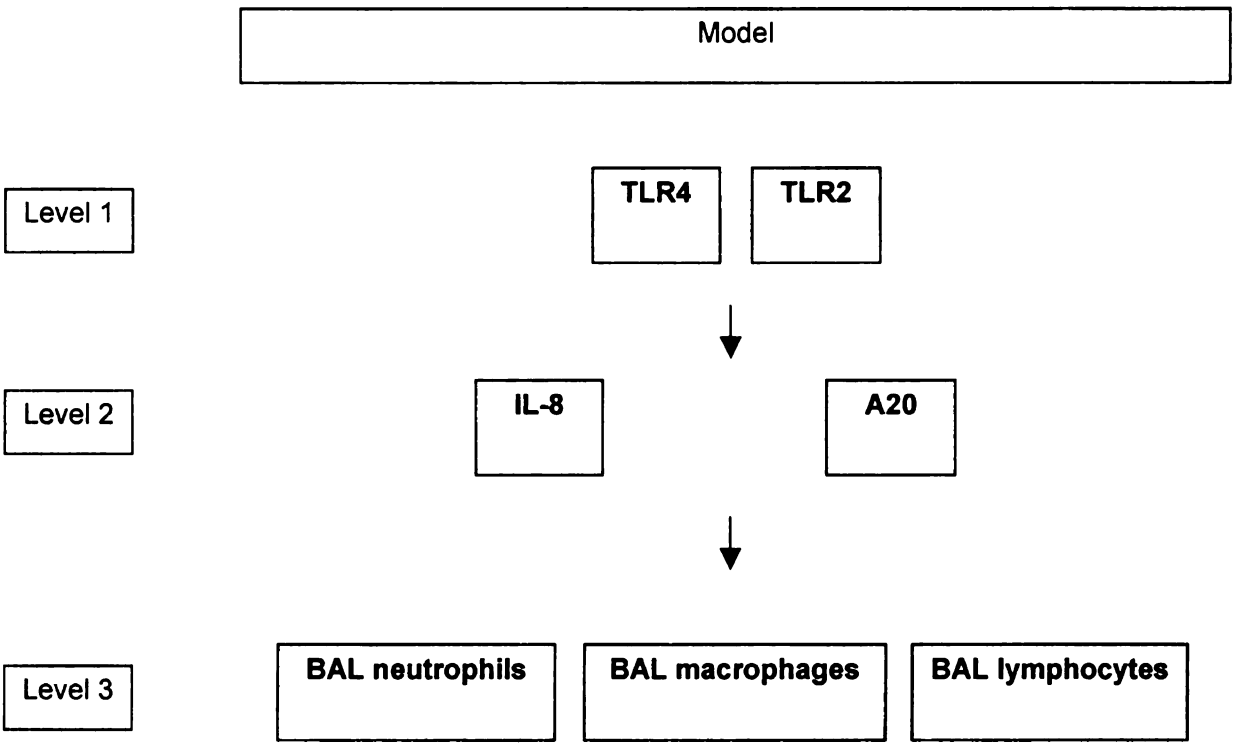


Figure 7-2: Association between TLR2 and A20 mRNA expressions in bronchial brushing samples (BBs) from all horses. TLR2 and A20 mRNA expressions are shown in relation to 18S rRNA (reference gene) expressions.

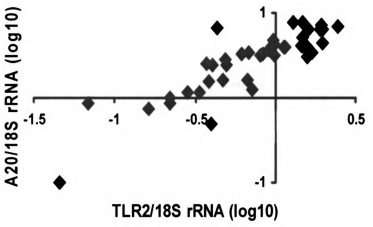
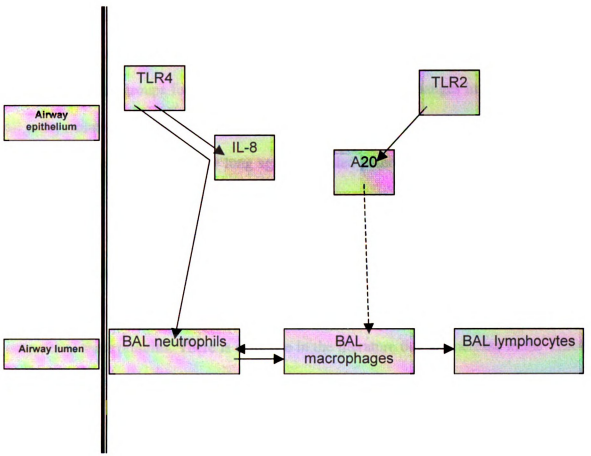


Figure 7-3: A potential model for predictors of airway inflammation in the general horse population.



CHAPTER 8

INTERPRETATION AND OUTLOOK

The research of this dissertation contributed to the understanding of the complex nature of immune mechanisms that are involved in the pathogenesis of RAO. Whereas in the past, research groups focused on cytokine and chemokine changes in BAL cells (e.g., all cells, isolated macrophages, or lymphocytes), I demonstrated that epithelial cells may also play a role in protecting the lung against inhaled pathogens. In addition, the current studies showed that key players in the innate immune response (e.g., TLR-4 and IL-8) are associated with airway neutrophilia. Finally, I observed an association between the regulatory protein A20 with inflammatory cells in BAL. Despite the fact of increased TLR4 and IL-8 mRNA expression, we did not have evidence of a parallel increase in the amounts of their proteins. There is evidence in the literature that an increase in the TLR4 mRNA does not necessarily lead to an elevation of the TLR4 protein (125). However, an increase in TLR4 and IL-8 mRNAs that were accompanied by an elevation of their proteins due to LPS injection or natural challenge, respectively, has been shown for equine lung tissue (4, 170). Therefore, it can be predicted that the changes in mRNA expression that are reported in my dissertation are associated with quantitative differences in the receptor protein.

Although the pathogenesis of RAO still remains ill defined, it becomes more and more clear that a genetic disease predisposition may underlie aberrant immune mechanisms that contribute to the RAO phenotype (i.e., airway neutrophilia, mucus

accumulation, and hyper-responsiveness). Therefore, the challenge in the future will be to find genes or traits that eventually are associated with those phenotypic outcomes and to understand the complexity of their interconnecting pathways. For these purposes whole equine genome sequence information, advanced analytical (e.g., genome or targeted expression chips), statistical and bioinformatic tools, and searchable databases need to become the center of our focus. The amount of information that is gained from whole-genome sequencing projects and expression analyses can only be understood in the context of detailed verification experiments on the molecular and cellular levels using technologies, such as qRT-PCR, ELISAs, and immunohistochemistry.

Areas for future research

Our understanding that RAO can be inherited originated with the report by Marti et al. (112) and this area will be able to be investigated in more detail with the public online access of the complete equine genome information in the near future. Since the beginning of sequencing efforts for the whole *Equus caballus* genome in March 2006, equine sequences are currently available at the Trace Archive at NCBI under the web address:

(http://0-www.ncbi.nlm.nih.gov.library.vu.edu.au/Traces/trace.cgi). With about 31 x 10^6 traces of about 700 base pairs per trace there are about 2.17 x 10^{10} base pairs reported for the equine genome today. Since mammalian genomes are about 3 x 10^9 base pairs in

length, the current online equine genome sequence information provides approximately 7.2-fold coverage.

Because of the similarity to human asthma it can be assumed that the phenotypes of RAO are quantitative rather than discrete traits. For the former it is characteristic to be under the influence of many genes, gene-gene, and gene-environment interactions. In addition, quantitative traits vary over a wide range within a population, which can be the basis for identifying disease-dominating genotypic variations (phenotype-driven strategies for gene identification). In contrast to mouse strains that were inbred to generate homozygosity of their genomes, the equine population represents a pool of phenotypic and, therefore, genotypic variation that would make it an appropriate animal model for quantitative trait analysis (QTL analysis). Disadvantages, such as high maintenance costs, the large size of these animals, and their long gestation and generation time, in comparison to other laboratory animals (e.g., mice, rats) are limiting factors. The still existing gaps in the database information for the equine genome (e.g., number of SNPs) further limit appropriate experimental design today. However, the latter may be overcome in the next few years, considering the fast growth of information since the completion of the human and mouse genome projects in 2000-2003 and 2002, respectively. Therefore, it is worthwhile to mention some theoretical considerations when using QTLs as a technique to identify genes that are potentially involved in causing the RAO phenotypes, such as airway neutrophilia, mucus accumulation, or airway hyperreactivity.

A QTL study would need appropriate parents (one of the parents should be RAO affected), back- or F2 intercrosses, pheno- and genotyping of the offsprings for

polymorphic markers, and regression analysis (165). The goal is to find associations between allelic and phenotypic variations. Single nucleotide polymorphisms (SNPs), which are natural sequence variations, are the markers of choice in many gene mapping experiments these days. For use in a QTL analysis SNP markers should be chosen so that they are evenly spaced throughout the genome.

Levels of gene expression can also be considered as a quantitative trait (155). Expression QTLs (eQTLs) can be obtained by the use of microarray data. Besides identification of genes that underlie a QTL, eQTLs and microarrays are especially important in pathway analysis. The combination of data from genetic and gene-expression analyses may lead to identification of key regulatory genes that primarily determine the phenotypic outcome (37). An alternative to using microarray data for eQTL analysis is the Microsoft Excel-based software algorythm termed Global Pattern Recognition (GPR), which is available at the following web address: http://www.jax.org/staff/roopenian/labsite/gene_expression.html.

The amount of information we might gather from whole genome sequencing or expression analyses cannot be interpreted without detailed studies on the molecular and cellular level. The following recommendations for future research questions that result from the present thesis may lead us to another level of understanding of immunological processes in the lung of horses.

Questions resulting from chapter 2:

- How do different types of lipopolysaccharides (endotoxin) or fungi interact with airway epithelial cells?
- What is the effect of LPS inhalation on TLR expression?

Questions resulting from chapter 4:

- Are TLRs other than TLR4 and TLR2 expressed in the equine lung, and is their expression profile associated with the pathogenesis of RAO?

Questions resulting from chapter 5:

- What is the expression profile and function of the TLR2 co-receptors TLR1 and TLR6?
- Where are the TLRs localized within a cell?
- Which transcription factor binding sites can be determined in the promoter region of TLRs?
- Does the TLR expression and stimulation differ in different airway cells, such as macrophages, neutrophils, epithelial cells, dendritic cells, and airway smooth muscle cells?
- Which effector molecules in the TLR signaling pathway (e.g., MyD88, TRAF-6 etc.) are important in the pathogenesis of RAO?

Questions resulting from chapter 6:

- What are the stimulatory and regulatory mechanisms for A20?
- What is the dynamic of A20 expression at early time points in RAO-affected and control horses?
- What regulatory mechanisms are involved in modulating the early influx of neutrophils into the airways in control horses?
- What are the mechanisms that prevent an exaggerated airway neutrophilia in control horses?

Examples of immunological studies in the field of RAO by different research groups are given in Table 8-1. It is apparent that comparisons and interpretations between studies are currently difficult due to the use of different protocols and methods. Especially for the above-discussed future areas of research, study designs (animals and protocols) and methodology standardization need to be considered. Although a standardization of protocols and methodologies may seem to limit the scientific variety, a broader range of information might be gained over a shorter period of time. Other factors, such as financial, time, and labor efforts, may also limit globally accepted agreements for common protocols. Nevertheless, efforts for the acceptance of a common definition of the RAO phenotype (151) could pave the way for further interactive research efforts.

Some areas in which a consensus would enable us to compare results between studies are mentioned in the following discussion:

- Develop a common definition of baseline. After stabling, how long do RAO-affected horses need to be maintained on pasture to be non-differentiable from control horses (remission)? Although it has been reported that the numbers of BAL inflammatory cells and lung function do not differ between RAO-affected and control horses after returning horses to pasture for two weeks (13), there is evidence for continued subclinical inflammatory processes on pasture (102, 24). For example, Sandersen et al. reported that the transcription factor NF-κB activity in RAO-affected horses does not return to baseline activity after 3 weeks on pasture (161). Furthermore, Dewachi et al. showed that the receptors for IL-5 and IL-9 on equine peripheral neutrophils were elevated in RAO-affected horses compared to control horses after more than 3 months on pasture (34). Finally, it

will be of interest to determine if anti-inflammatory drugs are able to speed up or are necessary to initiate the processes toward complete remission.

- Determine biologically important time points for sample collection. Since we are still not able to study pathological changes in whole biological systems during disease development in real time, we have to rely on data obtained at different time points, during which important changes in the disease pathogenesis may occur. Using animal models of allergic lung diseases, a response to inhaled pathogens occurs within minutes of inhalation by susceptible animals (early response) (75). Although the amount of IgE is elevated in lungs from RAO-affected horses (40, 63, 162), the early allergic response seems to be absent in the RAO pathogenesis. Subsequently, the late-phase inflammatory reaction can be observed after six to nine hours of inhalation challenge, which is characterized by influx of inflammatory cells into the airway lumen, production and release of cytokines, and development of sustained airway obstruction (17, 106). Indeed Fairbain et al. showed an influx of neutrophils into equine airways beginning at 6 hours of exposure (42). Furthermore, time-course studies of cytokine expressions may reveal additional time points during or after stabling, during which it might be worthwhile to obtain data.
- Using the best match of diseased and control animals. Recurrent airway obstruction is a pulmonary disorder of primarily mature horses older than eight years (19). Since we currently do not understand the processes that are involved in the development of the disease, the use of age- and breed-matched horse pairs is of advantage in case control studies. Although cost and time intensive,

establishing herds of known pedigrees could help in determination of age-related difference, familial associations, breed associations, etc. Although laboratory animal models, such as the mouse, are valuable for high-throughput research, because of its small size, cost effective maintenance, and short gestation time (~ 19 to 20 days), horses affected by RAO provide the advantage of their naturally close proximity to the phenotypes of acute and occupational asthma. Gathering information from longitudinal studies by using horses with well-defined pedigree and disease history may yield informative data about the complexity in the pathogenesis that we might not be able to obtain from laboratory animal models or cell culture experiments.

In conclusion, with this dissertation I contributed to our understanding of innate immune mechanisms in equine airways. First, I showed that in our stables, animals are exposed to higher endotoxin concentrations than on pasture and that the endotoxin receptor (TLR4) is more abundant in airway epithelium when horses are kept in this environment as compared to an outdoor environment. Similarly, when exposed to stable dust, IL-8 expression was also elevated in bronchial epithelial cells, most likely due to TLR4 signaling. Interestingly, all horses showed neutrophilic influx into the airway lumen; however, only the RAO-susceptible animals developed airway obstruction. Although, RAO-affected horses had in general a higher IL-8 production, other mechanisms, such as negative inflammatory regulators, like A20, or activation of other airway located inflammatory cells, such as alveolar macrophages, may be important players in the pathogenesis. Therefore, it is evident that the complex nature of these processes cannot

be discussed and understood in detail by studying a "tiny" subset of potentially important genes in such a complex biological system. Future studies that will analyze expression profiles and pathways on a basis of whole genome sequence availability will become available in the near future and will possibly incorporate new challenges, such as the use of bioinformatical tools.

Table 8-1: Examples of immunological studies in the field of RAO by different research groups.

TNF-α = Tumor necrosis factor alpha; IL-1β = Interleukin 1 beta; IL-6 = Interleukin 6; IL-8 = Interleukin 8; IL-10 = Interleukin 10; NF-κB = Nuclear factor kappa B; I- κB = Inhibitor of NF-κB; BBs = bronchial brushing samples; BAL = bronchoalaveolar lavage; ICAM-1 = Intracellular adhesion molecule 1; GM-CSF = Ganulocyte macrophage-colony-stimulating factor; STAT-5 = Signal transducer and activator of transcription 5; MIP-2 = Macrophage inflammatory protein-2; IL-4 = Interleukin 4; IFN-γ = Interferon gamma; IL-17 = Interleukin 17; IL-5R = Interleukin 5 receptor; IL-9R = Interleukin 9 receptor; TLR4 = Toll-like receptor 4; TLR2 = Toll-like receptor 2; A20 = zinc finger protein A20; CXCL1 = CXC chemokine ligand 1; G-CSF = Granulocyte-colony stimulating factor; LPS = lipopolysaccharide; AF = Aspergillus fumigatus; HDS = Hay dust suspension; CB = clenbuterol; PBS = Phosphate buffered saline; n.m. = not measured; ΔPplmax = Difference between inspiratory and expiratory pleural pressure; EMSA = Electromobility shift ssay; ISH = *In-situ* hybridization

Molecules of interest	Challenge method	Sample collection baseline	length of challenge	after challenge	Method	Ref.
TNF-α, IL-1β	LPS, AF, HDS (cell culture)	PBS	24 hrs	n.m.	Bioassays	(97)
TNF-α, IL-1β, IL-6, IL-8, IL-10	LPS, AF, HDS (inhalation)	> 2 Mon: shavings, sillage	n.m.	6, 24 hrs	q-PCR	(95)
TNF-α, IL-1β, IL-6, IL-8, IL-10	LPS, AF, HDS (inhalation) after 3 days pre-tx with CB	> 2 Mon: shavings, sillage	n.m.	6, 24 hrs	q-PCR	(96)
NF-κB, I- κB (in BBs)	hay (stable)	n.m.	24 hrs after onset of crisis	3 weeks	EMSA, Immunoplots	(22)
NF- κB, ICAM-1 (in BBs)	hay (stable)	n.m.	24 hrs after onset of crisis	3 weeks	EMSA, Immunoplots	(21)
NF- κB (in BAL)	hay (stable)	n.m.	24 hrs after onset of crisis	3 weeks	EMSA	(161)
GM-CSF, STAT-5	hay (stable)	n.m.	during crisis	n.m.	Apoptosis assay, EMSA	(185)
IL-8, MIP-2	LPS (AM)	before adding LPS		1.5, 3, 5, 7, 24 h	semi q-PCR	(51)
IL-8	hay, straw		check-up horses		chemotaxis assay	(50)
TNF-α, IL-1β, IL-8, IL-6, IL-4, IFN-γ	hay	ΔPplmax < 10 cm H_2O	ΔPplmax > 20 cm H_2O, after 3 weeks		dot-blot assay q-PCR, bioassay dot-blot assay	(59)
IL-4, IL-5, IFN-γ	hay, straw	n.m.	> 3 Mon		ISH	(99)
IL-4, IL-5, IFN-γ	hay, straw	> 3 Mon pasture	1 and 9 days		ISH	(29)
IL-17	hay, straw	> 4 Mon pasture	35 days		q-PCR	(31)
IL-5R, IL-9R	hay, straw	> 3 Mon pasture	21 days		IHC	(34)
IL-13, IFN-γ, IL-8	hay, shavings	> 3 Mon pasture	1 day, 3 weeks		q-PCR	(3)
IL-17, TLR4	hay, shavings	> 3 Mon pasture	1, 14, 35, 49 days		q-PCR	(4)
IL-8, CXCL1, GM-CSF, G-CSF					IHC, biopsy	
TLR4, TLR2, IL-8, A20	hay, straw	n.m.	ΔPplmax > 15 cm H_2O	1, 2, 4 weeks	q-PCR, brushings	(13)

BIBLIOGRAPHY

1. **Abrams L, Seixas N, Robins T, Burge H, Muilenberg M, and Franzblau A.** Characterization of metalworking fluid exposure indices for a study of acute respiratory effects. *Appl Occup Environ Hyg* 15: 492-502, 2000.

2. **Aggarwal N, and Holmes MA.** Characterisation of equine T helper cells: demonstration of Th1- and Th2-like cells in long-term equine T-cell cultures. *Res Vet Sci* 66: 277-279, 1999.

3. **Ainsworth DM, Grunig G, Matychak MB, Young J, Wagner B, Erb HN, and Antczak DF.** Recurrent airway obstruction (RAO) in horses is characterized by IFN-gamma and IL-8 production in bronchoalveolar lavage cells. *Vet Immunol Immunopathol* 96: 83-91, 2003.

4. **Ainsworth DM, Wagner B, Franchini M, Grunig G, Erb HN, and Tan JY.** Time-dependent alterations in gene expression of interleukin-8 in the bronchial epithelium of horses with recurrent airway obstruction. *Am J Vet Res* 67: 669-677, 2006.

5. **Akhtar M, Watson JL, Nazli A, and McKay DM.** Bacterial DNA evokes epithelial IL-8 production by a MAPK-dependent, NF-kappaB-independent pathway. *Faseb J* 17: 1319-1321, 2003.

6. **Akira S, and Takeda K.** Toll-like receptor signalling. *Nat Rev Immunol* 4: 499-511, 2004.

7. **Alexopoulou L, Holt AC, Medzhitov R, and Flavell RA.** Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413: 732-738, 2001.

8. **Altieri DC, and Edgington TS.** A monoclonal antibody reacting with distinct adhesion molecules defines a transition in the functional state of the receptor CD11b/CD18 (Mac-1). *J Immunol* 141: 2656-2660, 1988.

9. **Andersen-Nissen E, Smith KD, Bonneau R, Strong RK, and Aderem A.** A conserved surface on Toll-like receptor 5 recognizes bacterial flagellin. *J Exp Med* 204: 393-403, 2007.

10. **Andonegui G, Bonder CS, Green F, Mullaly SC, Zbytnuik L, Raharjo E, and Kubes P.** Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. *J Clin Invest* 111: 1011-1020, 2003.

11. **Bals R, and Hiemstra PS**. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *Eur Respir J* 23: 327-333, 2004.

12. **Basu S, and Fenton MJ**. Toll-like receptors: function and roles in lung disease. *Am J Physiol Lung Cell Mol Physiol* 286: L887-892, 2004.

13. **Berndt A, Derksen FJ, Venta P, Ewart S, Yuzbasiyan-Gurkan V, and Robinson NE**. ELEVATED AMOUNT OF TOLL-LIKE RECEPTOR 4 mRNA IN BRONCHIAL EPITHELIAL CELLS IS ASSOCIATED WITH AIRWAY INFLAMMATION IN HORSES WITH RECURRENT AIRWAY OBSTRUCTION. *Am J Physiol Lung Cell Mol Physiol* 2006.

14. **Beyaert R, Heyninck K, and Van Huffel S**. A20 and A20-binding proteins as cellular inhibitors of nuclear factor-kappa B-dependent gene expression and apoptosis. *Biochem Pharmacol* 60: 1143-1151, 2000.

15. **Bhatia M, and Moochhala S**. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. *J Pathol* 202: 145-156, 2004.

16. **Boone DL, Turer EE, Lee EG, Ahmad RC, Wheeler MT, Tsui C, Hurley P, Chien M, Chai S, Hitotsumatsu O, McNally E, Pickart C, and Ma A**. The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. *Nat Immunol* 5: 1052-1060, 2004.

17. **Bousquet J, Jeffery PK, Busse WW, Johnson M, and Vignola AM**. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 161: 1720-1745, 2000.

18. **Bowles KS, Beadle RE, Mouch S, Pourciau SS, Littlefield-Chabaud MA, Le Blanc C, Mistric L, Fermaglich D, and Horohov DW**. A novel model for equine recurrent airway obstruction. *Vet Immunol Immunopathol* 87: 385-389, 2002.

19. **Bracher V, von Fellenberg R, Winder CN, Gruenig G, Hermann M, and Kraehenmann A**. An investigation of the incidence of chronic obstructive pulmonary disease (COPD) in random populations of Swiss horses. *Equine Vet J* 23: 136-141, 1991.

20. **Brazil TJ, and McGorum BC**. Molecules and mediators of inflammation in equine heaves: mechanisms and markers of disease. *Equine Vet J* 33: 113-115, 2001.

21. **Bureau F, Bonizzi G, Kirschvink N, Delhalle S, Desmecht D, Merville MP, Bours V, and Lekeux P**. Correlation between nuclear factor-kappaB activity in bronchial brushing samples and lung dysfunction in an animal model of asthma. *Am J Respir Crit Care Med* 161: 1314-1321, 2000.

22. **Bureau F, Delhalle S, Bonizzi G, Fievez L, Dogne S, Kirschvink N, Vanderplasschen A, Merville MP, Bours V, and Lekeux P**. Mechanisms of persistent

NF-kappa B activity in the bronchi of an animal model of asthma. *J Immunol* 165: 5822-5830, 2000.

23. **Carter JD, Ghio AJ, Samet JM, and Devlin RB**. Cytokine production by human airway epithelial cells after exposure to an air pollution particle is metal-dependent. *Toxicol Appl Pharmacol* 146: 180-188, 1997.

24. **Carty CL, Gehring U, Cyrys J, Bischof W, and Heinrich J**. Seasonal variability of endotoxin in ambient fine particulate matter. *J Environ Monit* 5: 953-958, 2003.

25. **Castellan RM, Olenchock SA, Hankinson JL, Millner PD, Cocke JB, Bragg CK, Perkins HH, Jr., and Jacobs RR**. Acute bronchoconstriction induced by cotton dust: dose-related responses to endotoxin and other dust factors. *Ann Intern Med* 101: 157-163, 1984.

26. **Castellan RM, Olenchock SA, Kinsley KB, and Hankinson JL**. Inhaled endotoxin and decreased spirometric values. An exposure-response relation for cotton dust. *N Engl J Med* 317: 605-610, 1987.

27. **Christiani DC, Ye TT, Zhang S, Wegman DH, Eisen EA, Ryan LA, Olenchock SA, Pothier L, and Dai HL**. Cotton dust and endotoxin exposure and long-term decline in lung function: results of a longitudinal study. *Am J Ind Med* 35: 321-331, 1999.

28. **Chun DT, Chew V, Bartlett K, Gordon T, Jacobs RR, Larsson BM, Larsson L, Lewis DM, Liesivuori J, Michel O, Milton DK, Rylander R, Thorne PS, White EM, and Brown ME**. Preliminary report on the results of the second phase of a round-robin endotoxin assay study using cotton dust. *Appl Occup Environ Hyg* 15: 152-157, 2000.

29. **Cordeau ME, Joubert P, Dewachi O, Hamid Q, and Lavoie JP**. IL-4, IL-5 and IFN-gamma mRNA expression in pulmonary lymphocytes in equine heaves. *Vet Immunol Immunopathol* 97: 87-96, 2004.

30. **Davenpeck KL, Steeber DA, Tedder TF, and Bochner BS**. P- and L-selectin mediate distinct but overlapping functions in endotoxin-induced leukocyte-endothelial interactions in the rat mesenteric microcirculation. *J Immunol* 159: 1977-1986, 1997.

31. **Debrue M, Hamilton E, Joubert P, Lajoie-Kadoch S, and Lavoie JP**. Chronic exacerbation of equine heaves is associated with an increased expression of interleukin-17 mRNA in bronchoalveolar lavage cells. *Vet Immunol Immunopathol* 105: 25-31, 2005.

32. **Delclaux C, Delacourt C, D'Ortho MP, Boyer V, Lafuma C, and Harf A**. Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane. *Am J Respir Cell Mol Biol* 14: 288-295, 1996.

33. **Derksen FJ, Scott JS, Slocombe RF, and Robinson NE.** Micropolyspora faeni causes airway inflammation but not hyperresponsiveness in sensitized ponies. *J Appl Physiol* 62: 1398-1404, 1987.

34. **Dewachi O, Joubert P, Hamid Q, and Lavoie JP.** Expression of interleukin (IL)-5 and IL-9 receptors on neutrophils of horses with heaves. *Vet Immunol Immunopathol* 109: 31-36, 2006.

35. **Donham KJ, Cumro D, Reynolds SJ, and Merchant JA.** Dose-response relationships between occupational aerosol exposures and cross-shift declines of lung function in poultry workers: recommendations for exposure limits. *J Occup Environ Med* 42: 260-269, 2000.

36. **Donham KJ, Reynolds SJ, Whitten P, Merchant JA, Burmeister L, and Popendorf WJ.** Respiratory dysfunction in swine production facility workers: dose-response relationships of environmental exposures and pulmonary function. *Am J Ind Med* 27: 405-418, 1995.

37. **Drake TA, Schadt EE, and Lusis AJ.** Integrating genetic and gene expression data: application to cardiovascular and metabolic traits in mice. *Mamm Genome* 17: 466-479, 2006.

38. **Driscoll KE, Simpson L, Carter J, Hassenbein D, and Leikauf GD.** Ozone inhalation stimulates expression of a neutrophil chemotactic protein, macrophage inflammatory protein 2. *Toxicol Appl Pharmacol* 119: 306-309, 1993.

39. **Duits LA, Nibbering PH, van Strijen E, Vos JB, Mannesse-Lazeroms SP, van Sterkenburg MA, and Hiemstra PS.** Rhinovirus increases human beta-defensin-2 and -3 mRNA expression in cultured bronchial epithelial cells. *FEMS Immunol Med Microbiol* 38: 59-64, 2003.

40. **Eder C, Crameri R, Mayer C, Eicher R, Straub R, Gerber H, Lazary S, and Marti E.** Allergen-specific IgE levels against crude mould and storage mite extracts and recombinant mould allergens in sera from horses affected with chronic bronchitis. *Vet Immunol Immunopathol* 73: 241-253, 2000.

41. **Evans AG, Paradis MR, and O'Callaghan M.** Intradermal testing of horses with chronic obstructive pulmonary disease and recurrent urticaria. *Am J Vet Res* 53: 203-208, 1992.

42. **Fairbairn SM, Marr KA, Lees P, Cunningham FM, and Page CP.** Effects of platelet activating factor on the distribution of radiolabelled leucocytes and platelets in normal horses and asymptomatic horses with chronic obstructive pulmonary disease. *Res Vet Sci* 61: 107-113, 1996.

43. **Fan J, Frey RS, and Malik AB.** TLR4 signaling induces TLR2 expression in endothelial cells via neutrophil NADPH oxidase. *J Clin Invest* 112: 1234-1243, 2003.

44. **Fan J, and Malik AB.** Toll-like receptor-4 (TLR4) signaling augments chemokine-induced neutrophil migration by modulating cell surface expression of chemokine receptors. *Nat Med* 9: 315-321, 2003.

45. **Fernandez N, Renedo M, Garcia-Rodriguez C, and Sanchez Crespo M.** Activation of monocytic cells through Fc gamma receptors induces the expression of macrophage-inflammatory protein (MIP)-1 alpha, MIP-1 beta, and RANTES. *J Immunol* 169: 3321-3328, 2002.

46. **Fernandez S, Jose P, Avdiushko MG, Kaplan AM, and Cohen DA.** Inhibition of IL-10 receptor function in alveolar macrophages by Toll-like receptor agonists. *J Immunol* 172: 2613-2620, 2004.

47. **Foster AP, Cunningham FM, and Lees P.** Inflammatory effects of platelet activating factor (PAF) in equine skin. *Equine Vet J* 24: 208-214, 1992.

48. **Foster AP, Lees P, Andrews MJ, and Cunningham FM.** Effects of WEB 2086, an antagonist to the receptor for platelet-activating factor (PAF), on PAF-induced responses in the horse. *Equine Vet J* 24: 203-207, 1992.

49. **Foster AP, Lees P, and Cunningham FM.** Platelet activating factor is a mediator of equine neutrophil and eosinophil migration in vitro. *Res Vet Sci* 53: 223-229, 1992.

50. **Franchini M, Gill U, von Fellenberg R, and Bracher VD.** Interleukin-8 concentration and neutrophil chemotactic activity in bronchoalveolar lavage fluid of horses with chronic obstructive pulmonary disease following exposure to hay. *Am J Vet Res* 61: 1369-1374, 2000.

51. **Franchini M, Gilli U, Akens MK, Fellenberg RV, and Bracher V.** The role of neutrophil chemotactic cytokines in the pathogenesis of equine chronic obstructive pulmonary disease (COPD). *Vet Immunol Immunopathol* 66: 53-65, 1998.

52. **Frevert CW, Farone A, Danaee H, Paulauskis JD, and Kobzik L.** Functional characterization of rat chemokine macrophage inflammatory protein-2. *Inflammation* 19: 133-142, 1995.

53. **Frevert CW, Huang S, Danaee H, Paulauskis JD, and Kobzik L.** Functional characterization of the rat chemokine KC and its importance in neutrophil recruitment in a rat model of pulmonary inflammation. *J Immunol* 154: 335-344, 1995.

54. **Gao B, and Tsan MF.** Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor alpha release by murine macrophages. *J Biol Chem* 278: 174-179, 2003.

55. **Gao B, and Tsan MF.** Recombinant human heat shock protein 60 does not induce the release of tumor necrosis factor alpha from murine macrophages. *J Biol Chem* 278: 22523-22529, 2003.

56. **Gerber V.** Nature and causes of mucus accumulation in equine lower airway disease. *Dissertation Thesis* 2003.

57. **Gerber V, Lindberg A, Berney C, and Robinson NE.** Airway mucus in recurrent airway obstruction--short-term response to environmental challenge. *J Vet Intern Med* 18: 92-97, 2004.

58. **Gern JE, French DA, Grindle KA, Brockman-Schneider RA, Konno S, and Busse WW.** Double-stranded RNA induces the synthesis of specific chemokines by bronchial epithelial cells. *Am J Respir Cell Mol Biol* 28: 731-737, 2003.

59. **Giguere S, Viel L, Lee E, MacKay RJ, Hernandez J, and Franchini M.** Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. *Vet Immunol Immunopathol* 85: 147-158, 2002.

60. **Gon Y, Asai Y, Hashimoto S, Mizumura K, Jibiki I, Machino T, Ra C, and Horie T.** A20 inhibits toll-like receptor 2- and 4-mediated interleukin-8 synthesis in airway epithelial cells. *Am J Respir Cell Mol Biol* 31: 330-336, 2004.

61. **Guillot L, Medjane S, Le-Barillec K, Balloy V, Danel C, Chignard M, and Si-Tahar M.** Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. *J Biol Chem* 279: 2712-2718, 2004.

62. **Guo RF, and Ward PA.** Mediators and regulation of neutrophil accumulation in inflammatory responses in lung: insights from the IgG immune complex model. *Free Radic Biol Med* 33: 303-310, 2002.

63. **Halliwell RE, McGorum BC, Irving P, and Dixon PM.** Local and systemic antibody production in horses affected with chronic obstructive pulmonary disease. *Vet Immunol Immunopathol* 38: 201-215, 1993.

64. **Hasday JD, Bascom R, Costa JJ, Fitzgerald T, and Dubin W.** Bacterial endotoxin is an active component of cigarette smoke. *Chest* 115: 829-835, 1999.

65. **Hauber HP, Tulic MK, Tsicopoulos A, Wallaert B, Olivenstein R, Daigneault P, and Hamid Q.** Toll-like receptors 4 and 2 expression in the bronchial mucosa of patients with cystic fibrosis. *Can Respir J* 12: 13-18, 2005.

66. **Hawn TR, Verbon A, Janer M, Zhao LP, Beutler B, and Aderem A.** Toll-like receptor 4 polymorphisms are associated with resistance to Legionnaires' disease. *Proc Natl Acad Sci U S A* 102: 2487-2489, 2005.

67. **Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, and Aderem A.** The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410: 1099-1103, 2001.

68. **Hertz CJ, Wu Q, Porter EM, Zhang YJ, Weismuller KH, Godowski PJ, Ganz T, Randell SH, and Modlin RL.** Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. *J Immunol* 171: 6820-6826, 2003.

69. **Heyninck K, and Beyaert R.** The cytokine-inducible zinc finger protein A20 inhibits IL-1-induced NF-kappaB activation at the level of TRAF6. *FEBS Lett* 442: 147-150, 1999.

70. **Heyninck K, De Valck D, Vanden Berghe W, Van Criekinge W, Contreras R, Fiers W, Haegeman G, and Beyaert R.** The zinc finger protein A20 inhibits TNF-induced NF-kappaB-dependent gene expression by interfering with an RIP- or TRAF2-mediated transactivation signal and directly binds to a novel NF-kappaB-inhibiting protein ABIN. *J Cell Biol* 145: 1471-1482, 1999.

71. **Hirschfeld M, Ma Y, Weis JH, Vogel SN, and Weis JJ.** Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J Immunol* 165: 618-622, 2000.

72. **Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO, Goode J, Lin P, Mann N, Mudd S, Crozat K, Sovath S, Han J, and Beutler B.** Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 424: 743-748, 2003.

73. **Holcombe SJ, Jackson C, Gerber V, Jefcoat A, Berney C, Eberhardt S, and Robinson NE.** Stabling is associated with airway inflammation in young Arabian horses. *Equine Vet J* 33: 244-249, 2001.

74. **Holcombe SJ, Robinson NE, Derksen FJ, Bertold B, Genovese R, Miller R, de Feiter Rupp H, Carr EA, Eberhart SW, Boruta D, and Kaneene JB.** Effect of tracheal mucus and tracheal cytology on racing performance in Thoroughbred racehorses. *Equine Vet J* 38: 300-304, 2006.

75. **Holgate ST.** The role of mast cells and basophils in inflammation. *Clin Exp Allergy* 30 Suppl 1: 28-32, 2000.

76. **Hollander A, Heederik D, and Kauffman H.** Acute respiratory effects in the potato processing industry due to a bioaerosol exposure. *Occup Environ Med* 51: 73-78, 1994.

77. **Horohov DW, Beadle RE, Mouch S, and Pourciau SS.** Temporal regulation of cytokine mRNA expression in equine recurrent airway obstruction. *Vet Immunol Immunopathol* 108: 237-245, 2005.

78. **Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, and Akira S.** Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 162: 3749-3752, 1999.

79. **Housley DJ, Zalewski ZA, Beckett SE, and Venta PJ.** Design factors that influence PCR amplification success of cross-species primers among 1147 mammalian primer pairs. *BMC Genomics* 7: 253, 2006.

80. **Iigo Y, Suematsu M, Higashida T, Oheda J, Matsumoto K, Wakabayashi Y, Ishimura Y, Miyasaka M, and Takashi T.** Constitutive expression of ICAM-1 in rat microvascular systems analyzed by laser confocal microscopy. *Am J Physiol* 273: H138-147, 1997.

81. **Imasato A, Desbois-Mouthon C, Han J, Kai H, Cato AC, Akira S, and Li JD.** Inhibition of p38 MAPK by glucocorticoids via induction of MAPK phosphatase-1 enhances nontypeable Haemophilus influenzae-induced expression of toll-like receptor 2. *J Biol Chem* 277: 47444-47450, 2002.

82. **Jaattela M, Mouritzen H, Elling F, and Bastholm L.** A20 zinc finger protein inhibits TNF and IL-1 signaling. *J Immunol* 156: 1166-1173, 1996.

83. **John M, Au BT, Jose PJ, Lim S, Saunders M, Barnes PJ, Mitchell JA, Belvisi MG, and Chung KF.** Expression and release of interleukin-8 by human airway smooth muscle cells: inhibition by Th-2 cytokines and corticosteroids. *Am J Respir Cell Mol Biol* 18: 84-90, 1998.

84. **Kaisho T, and Akira S.** Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice. *Trends Immunol* 22: 78-83, 2001.

85. **Karakurum M, Shreeniwas R, Chen J, Pinsky D, Yan SD, Anderson M, Sunouchi K, Major J, Hamilton T, Kuwabara K, and et al.** Hypoxic induction of interleukin-8 gene expression in human endothelial cells. *J Clin Invest* 93: 1564-1570, 1994.

86. **Kindler S, Wang H, Richter D, and Tiedge H.** RNA transport and local control of translation. *Annu Rev Cell Dev Biol* 21: 223-245, 2005.

87. **Kinjyo I, Hanada T, Inagaki-Ohara K, Mori H, Aki D, Ohishi M, Yoshida H, Kubo M, and Yoshimura A.** SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity* 17: 583-591, 2002.

88. **Klein CL, Bittinger F, Kohler H, Wagner M, Otto M, Hermanns I, and Kirkpatrick CJ.** Comparative studies on vascular endothelium in vitro. 3. Effects of cytokines on the expression of E-selectin, ICAM-1 and VCAM-1 by cultured human endothelial cells obtained from different passages. *Pathobiology* 63: 83-92, 1995.

89. **Klugewitz K, Ley K, Schuppan D, Nuck R, Gaehtgens P, and Walzog B.** Activation of the beta2 integrin Mac-1 (CD11b/CD18) by an endogenous lipid mediator of human neutrophils and HL-60 cells. *J Cell Sci* 110 (Pt 8): 985-990, 1997.

90. **Knapp S, Wieland CW, van 't Veer C, Takeuchi O, Akira S, Florquin S, and van der Poll T.** Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense. *J Immunol* 172: 3132-3138, 2004.

91. **Kobayashi K, Hernandez LD, Galan JE, Janeway CA, Jr., Medzhitov R, and Flavell RA.** IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell* 110: 191-202, 2002.

92. **Krikos A, Laherty CD, and Dixit VM.** Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappa B elements. *J Biol Chem* 267: 17971-17976, 1992.

93. **Kruse-Elliott KT, Albert DH, Summers JB, Carter GW, Zimmerman JJ, and Grossman JE.** Attenuation of endotoxin-induced pathophysiology by a new potent PAF receptor antagonist. *Shock* 5: 265-273, 1996.

94. **Kullman GJ, Thorne PS, Waldron PF, Marx JJ, Ault B, Lewis DM, Siegel PD, Olenchock SA, and Merchant JA.** Organic dust exposures from work in dairy barns. *Am Ind Hyg Assoc J* 59: 403-413, 1998.

95. **Laan TT, Bull S, Pirie R, and Fink-Gremmels J.** The role of alveolar macrophages in the pathogenesis of recurrent airway obstruction in horses. *J Vet Intern Med* 20: 167-174, 2006.

96. **Laan TT, Bull S, Pirie RS, and Fink-Gremmels J.** The anti-inflammatory effects of IV administered clenbuterol in horses with recurrent airway obstruction. *Vet J* 171: 429-437, 2006.

97. **Laan TT, Bull S, Pirie RS, and Fink-Gremmels J.** Evaluation of cytokine production by equine alveolar macrophages exposed to lipopolysaccharide, Aspergillus fumigatus, and a suspension of hay dust. *Am J Vet Res* 66: 1584-1589, 2005.

98. **Larson VL, and Busch RH.** Equine tracheobronchial lavage: comparison of lavage cytologic and pulmonary histopathologic findings. *Am J Vet Res* 46: 144-146, 1985.

99. **Lavoie JP, Maghni K, Desnoyers M, Taha R, Martin JG, and Hamid QA.** Neutrophilic airway inflammation in horses with heaves is characterized by a Th2-type cytokine profile. *Am J Respir Crit Care Med* 164: 1410-1413, 2001.

100. **Lee EG, Boone DL, Chai S, Libby SL, Chien M, Lodolce JP, and Ma A.** Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. *Science* 289: 2350-2354, 2000.

101. **Li X, Abdi K, Rawn J, Mackay CR, and Mentzer SJ.** LFA-1 and L-selectin regulation of recirculating lymphocyte tethering and rolling on lung microvascular endothelium. *Am J Respir Cell Mol Biol* 14: 398-406, 1996.

102. **Li XC, Miyasaka M, and Issekutz TB.** Blood monocyte migration to acute lung inflammation involves both CD11/CD18 and very late activation antigen-4-dependent and independent pathways. *J Immunol* 161: 6258-6264, 1998.

103. **Lindberg A, Robinson NE, Nasman-Glaser B, Jensen-Waern M, and Lindgren JA.** Assessment of leukotriene B4 production in leukocytes from horses with recurrent airway obstruction. *Am J Vet Res* 65: 289-295, 2004.

104. **Lindsay GK, Roslansky PF, and Novitsky TJ.** Single-step, chromogenic Limulus amebocyte lysate assay for endotoxin. *J Clin Microbiol* 27: 947-951, 1989.

105. **Liu YC, Penninger J, and Karin M.** Immunity by ubiquitylation: a reversible process of modification. *Nat Rev Immunol* 5: 941-952, 2005.

106. **MacKay RJ, King RR, Dankert JR, Reis KJ, and Skelley LA.** Cytotoxic tumor necrosis factor activity produced by equine alveolar macrophages: preliminary characterization. *Vet Immunol Immunopathol* 29: 15-30, 1991.

107. **Malik AB, and Lo SK.** Vascular endothelial adhesion molecules and tissue inflammation. *Pharmacol Rev* 48: 213-229, 1996.

108. **Mambula SS, Sau K, Henneke P, Golenbock DT, and Levitz SM.** Toll-like receptor (TLR) signaling in response to Aspergillus fumigatus. *J Biol Chem* 277: 39320-39326, 2002.

109. **Manabe H, Akuta K, Okamura K, and Ohmori K.** KF19514, a phosphodieterase 4 and 1 inhibitor, inhibits PAF-induced lung inflammatory responses by inhaled administration in guinea pigs. *Int Arch Allergy Immunol* 114: 389-399, 1997.

110. **Marr KA, Fairbairn SM, Page CP, Lees P, and Cunningham FM.** A study of the effect of a platelet activating factor (PAF) receptor antagonist on antigen challenge of horses with chronic obstructive pulmonary disease. *J Vet Pharmacol Ther* 19: 233-237, 1996.

111. **Marr KA, Lees P, and Cunningham FM.** Agonist-induced adherence of equine neutrophils to fibronectin- and serum-coated plastic is CD18 dependent. *Vet Immunol Immunopathol* 71: 77-88, 1999.

112. **Marti E, Gerber H, Essich G, Oulehla J, and Lazary S.** The genetic basis of equine allergic diseases. 1. Chronic hypersensitivity bronchitis. *Equine Vet J* 23: 457-460, 1991.

113. **Mattoli S, Marini M, and Fasoli A.** Expression of the potent inflammatory cytokines, GM-CSF, IL6, and IL8, in bronchial epithelial cells of asthmatic patients. *Chest* 101: 27S-29S, 1992.

114. **McEver RP, and Cummings RD.** Role of PSGL-1 binding to selectins in leukocyte recruitment. *J Clin Invest* 100: S97-103, 1997.

115. **McGorum BC, Dixon PM, and Halliwell RE.** Evaluation of intradermal mould antigen testing in the diagnosis of equine chronic obstructive pulmonary disease. *Equine Vet J* 25: 273-275, 1993.

116. **McGorum BC, Dixon PM, and Halliwell RE.** Responses of horses affected with chronic obstructive pulmonary disease to inhalation challenges with mould antigens. *Equine Vet J* 25: 261-267, 1993.

117. **McGorum BC, Ellison J, and Cullen RT.** Total and respirable airborne dust endotoxin concentrations in three equine management systems. *Equine Vet J* 30: 430-434, 1998.

118. **McPherson EA, Lawson GH, Murphy JR, Nicholson JM, Fraser JA, Breeze RG, and Pirie HM.** Chronic obstructive pulmonary disease (COPD): identification of affected horses. *Equine Vet J* 10: 47-53, 1978.

119. **Menzies-Gow A, and Robinson DS.** Eosinophils, eosinophilic cytokines (interleukin-5), and antieosinophilic therapy in asthma. *Curr Opin Pulm Med* 8: 33-38, 2002.

120. **Milton DK, Amsel J, Reed CE, Enright PL, Brown LR, Aughenbaugh GL, and Morey PR.** Cross-sectional follow-up of a flu-like respiratory illness among fiberglass manufacturing employees: endotoxin exposure associated with two distinct sequelae. *Am J Ind Med* 28: 469-488, 1995.

121. **Milton DK, Walters MD, Hammond K, and Evans JS.** Worker exposure to endotoxin, phenolic compounds, and formaldehyde in a fiberglass insulation manufacturing plant. *Am Ind Hyg Assoc J* 57: 889-896, 1996.

122. **Milton DK, Wypij D, Kriebel D, Walters MD, Hammond SK, and Evans JS.** Endotoxin exposure-response in a fiberglass manufacturing facility. *Am J Ind Med* 29: 3-13, 1996.

123. **Monick MM, Yarovinsky TO, Powers LS, Butler NS, Carter AB, Gudmundsson G, and Hunninghake GW.** Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. *J Biol Chem* 278: 53035-53044, 2003.

124. **Morath S, Stadelmaier A, Geyer A, Schmidt RR, and Hartung T.** Synthetic lipoteichoic acid from Staphylococcus aureus is a potent stimulus of cytokine release. *J Exp Med* 195: 1635-1640, 2002.

125. **Morris GE, Whyte MK, Martin GF, Jose PJ, Dower SK, and Sabroe I.** Agonists of toll-like receptors 2 and 4 activate airway smooth muscle via mononuclear leukocytes. *Am J Respir Crit Care Med* 171: 814-822, 2005.

126. **Murthy KG, Deb A, Goonesekera S, Szabo C, and Salzman AL.** Identification of conserved domains in Salmonella muenchen flagellin that are essential for its ability to activate TLR5 and to induce an inflammatory response in vitro. *J Biol Chem* 279: 5667-5675, 2004.

127. **Muzio M, Bosisio D, Polentarutti N, D'Amico G, Stoppacciaro A, Mancinelli R, van't Veer C, Penton-Rol G, Ruco LP, Allavena P, and Mantovani A.** Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol* 164: 5998-6004, 2000.

128. **Nardoni S, Mancianti F, Sgorbini M, Taccini F, and Corazza M.** Identification and seasonal distribution of airborne fungi in three horse stables in Italy. *Mycopathologia* 160: 29-34, 2005.

129. **Nevalainen M, Raulo SM, Brazil TJ, Pirie RS, Sorsa T, McGorum BC, and Maisi P.** Inhalation of organic dusts and lipopolysaccharide increases gelatinolytic matrix metalloproteinases (MMPs) in the lungs of heaves horses. *Equine Vet J* 34: 150-155, 2002.

130. **Nocker RE, Schoonbrood DF, van de Graaf EA, Hack CE, Lutter R, Jansen HM, and Out TA.** Interleukin-8 in airway inflammation in patients with asthma and chronic obstructive pulmonary disease. *Int Arch Allergy Immunol* 109: 183-191, 1996.

131. **Nohgawa M, Sasada M, Maeda A, Asagoe K, Harakawa N, Takano K, Yamamoto K, and Okuma M.** Leukotriene B4-activated human endothelial cells promote transendothelial neutrophil migration. *J Leukoc Biol* 62: 203-209, 1997.

132. **O'Neill LA, Fitzgerald KA, and Bowie AG.** The Toll-IL-1 receptor adaptor family grows to five members. *Trends Immunol* 24: 286-290, 2003.

133. **O'Reilly SM, and Moynagh PN.** Regulation of Toll-like receptor 4 signalling by A20 zinc finger protein. *Biochem Biophys Res Commun* 303: 586-593, 2003.

134. **Onose A, Hashimoto S, Hayashi S, Maruoka S, Kumasawa F, Mizumura K, Jibiki I, Matsumoto K, Gon Y, Kobayashi T, Takahashi N, Shibata Y, Abiko Y, Shibata T, Shimizu K, and Horie T.** An inhibitory effect of A20 on NF-kappaB activation in airway epithelium upon influenza virus infection. *Eur J Pharmacol* 541: 198-204, 2006.

135. **Opipari AW, Jr., Boguski MS, and Dixit VM.** The A20 cDNA induced by tumor necrosis factor alpha encodes a novel type of zinc finger protein. *J Biol Chem* 265: 14705-14708, 1990.

136. **Pang L, and Knox AJ.** Bradykinin stimulates IL-8 production in cultured human airway smooth muscle cells: role of cyclooxygenase products. *J Immunol* 161: 2509-2515, 1998.

137. **Pang L, and Knox AJ.** Synergistic inhibition by beta(2)-agonists and corticosteroids on tumor necrosis factor-alpha-induced interleukin-8 release from cultured human airway smooth-muscle cells. *Am J Respir Cell Mol Biol* 23: 79-85, 2000.

138. **Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, and Abraham E.** Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol Chem* 279: 7370-7377, 2004.

139. **Pease JE, and Sabroe I.** The role of interleukin-8 and its receptors in inflammatory lung disease: implications for therapy. *Am J Respir Med* 1: 19-25, 2002.

140. **Pirie RS, Collie DD, Dixon PM, and McGorum BC.** Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 2: Effects of inhaled HDS on control and heaves horses. *Equine Vet J* 34: 337-342, 2002.

141. **Pirie RS, Collie DD, Dixon PM, and McGorum BC.** Inhaled endotoxin and organic dust particulates have synergistic proinflammatory effects in equine heaves (organic dust-induced asthma). *Clin Exp Allergy* 33: 676-683, 2003.

142. **Pirie RS, Dixon PM, Collie DD, and McGorum BC.** Pulmonary and systemic effects of inhaled endotoxin in control and heaves horses. *Equine Vet J* 33: 311-318, 2001.

143. **Pirie RS, Dixon PM, and McGorum BC**. Endotoxin contamination contributes to the pulmonary inflammatory and functional response to Aspergillus fumigatus extract inhalation in heaves horses. *Clin Exp Allergy* 33: 1289-1296, 2003.

144. **Pirie RS, Dixon PM, and McGorum BC**. Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 3: Effect of fractionation of HDS. *Equine Vet J* 34: 343-347, 2002.

145. **Pirie RS, McLachlan G, and McGorum BC**. Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 1: Preparation and composition of HDS. *Equine Vet J* 34: 332-336, 2002.

146. **Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, and Beutler B**. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282: 2085-2088, 1998.

147. **Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P, and Malo D**. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J Exp Med* 189: 615-625, 1999.

148. **Rhee SH, Im E, Riegler M, Kokkotou E, O'Brien M, and Pothoulakis C**. Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. *Proc Natl Acad Sci U S A* 102: 13610-13615, 2005.

149. **Rickards KJ, Page CP, Lees P, and Cunningham FM**. Differential inhibition of equine neutrophil function by phosphodiesterase inhibitors. *J Vet Pharmacol Ther* 24: 275-281, 2001.

150. **Robins T, Seixas N, Franzblau A, Abrams L, Minick S, Burge H, and Schork MA**. Acute respiratory effects on workers exposed to metalworking fluid aerosols in an automotive transmission plant. *Am J Ind Med* 31: 510-524, 1997.

151. **Robinson NE**. International Workshop on Equine Chronic Airway Disease. Michigan State University 16-18 June 2000. *Equine Vet J* 33: 5-19, 2001.

152. **Robinson NE, Karmaus W, Holcombe SJ, Carr EA, and Derksen FJ**. Airway inflammation in Michigan pleasure horses: prevalence and risk factors. *Equine Vet J* 38: 293-299, 2006.

153. **Robinson NE, Olszewski MA, Boehler D, Berney C, Hakala J, Matson C, and Derksen FJ**. Relationship between clinical signs and lung function in horses with recurrent airway obstruction (heaves) during a bronchodilator trial. *Equine Vet J* 32: 393-400, 2000.

154. **Rodgers HC, Pang L, Holland E, Corbett L, Range S, and Knox AJ.** Bradykinin increases IL-8 generation in airway epithelial cells via COX-2-derived prostanoids. *Am J Physiol Lung Cell Mol Physiol* 283: L612-618, 2002.

155. **Rosa GJ, de Leon N, and Rosa AJ.** Review of microarray experimental design strategies for genetical genomics studies. *Physiol Genomics* 28: 15-23, 2006.

156. **Ross J.** mRNA stability in mammalian cells. *Microbiol Rev* 59: 423-450, 1995.

157. **Rush BR, Raub ES, Rhoads WS, Flaminio MJ, Matson CJ, Hakala JE, and Gillespie JR.** Pulmonary function in horses with recurrent airway obstruction after aerosol and parenteral administration of beclomethasone dipropionate and dexamethasone, respectively. *Am J Vet Res* 59: 1039-1043, 1998.

158. **Rylander R, Thorn J, and Attefors R.** Airways inflammation among workers in a paper industry. *Eur Respir J* 13: 1151-1157, 1999.

159. **Sabroe I, Parker LC, Wilson AG, Whyte MK, and Dower SK.** Toll-like receptors: their role in allergy and non-allergic inflammatory disease. *Clin Exp Allergy* 32: 984-989, 2002.

160. **Sampson AP.** The role of eosinophils and neutrophils in inflammation. *Clin Exp Allergy* 30 Suppl 1: 22-27, 2000.

161. **Sandersen C, Bureau F, Turlej R, Fievez L, Dogne S, Kirschvink N, and Lekeux P.** p65 Homodimer activity in distal airway cells determines lung dysfunction in equine heaves. *Vet Immunol Immunopathol* 80: 315-326, 2001.

162. **Schmallenbach KH, Rahman I, Sasse HH, Dixon PM, Halliwell RE, McGorum BC, Crameri R, and Miller HR.** Studies on pulmonary and systemic Aspergillus fumigatus-specific IgE and IgG antibodies in horses affected with chronic obstructive pulmonary disease (COPD). *Vet Immunol Immunopathol* 66: 245-256, 1998.

163. **Scholz D, Devaux B, Hirche A, Potzsch B, Kropp B, Schaper W, and Schaper J.** Expression of adhesion molecules is specific and time-dependent in cytokine-stimulated endothelial cells in culture. *Cell Tissue Res* 284: 415-423, 1996.

164. **Schwartz DA, Thorne PS, Yagla SJ, Burmeister LF, Olenchock SA, Watt JL, and Quinn TJ.** The role of endotoxin in grain dust-induced lung disease. *Am J Respir Crit Care Med* 152: 603-608, 1995.

165. **Sen S, and Churchill GA.** A statistical framework for quantitative trait mapping. *Genetics* 159: 371-387, 2001.

166. **Shibata F.** [The role of rat cytokine-induced neutrophil chemoattractants (CINCs) in inflammation]. *Yakugaku Zasshi* 122: 263-268, 2002.

167. **Shuto T, Imasato A, Jono H, Sakai A, Xu H, Watanabe T, Rixter DD, Kai H, Andalibi A, Linthicum F, Guan YL, Han J, Cato AC, Lim DJ, Akira S, and Li JD.** Glucocorticoids synergistically enhance nontypeable Haemophilus influenzae-induced Toll-like receptor 2 expression via a negative cross-talk with p38 MAP kinase. *J Biol Chem* 277: 17263-17270, 2002.

168. **Silverstein AM.** History of immunology. Cellular versus humoral immunity: determinants and consequences of an epic 19th century battle. *Cell Immunol* 48: 208-221, 1979.

169. **Simonen-Jokinen T, Pirie RS, McGorum B, and Maisi P.** Dose responses to inhalation of endotoxin, hay dust suspension and Aspergillus fumigatus extract in horses as measured by levels and activation of matrix metalloproteinase-9. *Equine Vet J* 37: 155-160, 2005.

170. **Singh Suri S, Janardhan KS, Parbhakar O, Caldwell S, Appleyard G, and Singh B.** Expression of Toll-like receptor 4 and 2 in horse lungs. *Vet Res* 37: 541-551, 2006.

171. **Smid T, Heederik D, Houba R, and Quanjer PH.** Dust- and endotoxin-related acute lung function changes and work-related symptoms in workers in the animal feed industry. *Am J Ind Med* 25: 877-888, 1994.

172. **Smid T, Heederik D, Houba R, and Quanjer PH.** Dust- and endotoxin-related respiratory effects in the animal feed industry. *Am Rev Respir Dis* 146: 1474-1479, 1992.

173. **Smiley ST, King JA, and Hancock WW.** Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *J Immunol* 167: 2887-2894, 2001.

174. **Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SL, Cookson BT, and Aderem A.** Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nat Immunol* 4: 1247-1253, 2003.

175. **Soong G, Reddy B, Sokol S, Adamo R, and Prince A.** TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells. *J Clin Invest* 113: 1482-1489, 2004.

176. **Spertini O, Luscinskas FW, Kansas GS, Munro JM, Griffin JD, Gimbrone MA, Jr., and Tedder TF.** Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. *J Immunol* 147: 2565-2573, 1991.

177. **Stijkel A, van Eijndhoven JC, and Bal R.** Drafting guidelines for occupational exposure to chemicals: the Dutch experience with the assessment of reproductive risks. *Am J Ind Med* 30: 705-717, 1996.

178. **Strunk RC, Eidlen DM, and Mason RJ.** Pulmonary alveolar type II epithelial cells synthesize and secrete proteins of the classical and alternative complement pathways. *J Clin Invest* 81: 1419-1426, 1988.

179. **Takizawa H.** Bronchial epithelial cells in allergic reactions. *Curr Drug Targets Inflamm Allergy* 4: 305-311, 2005.

180. **Tamura H, Arimoto Y, Tanaka S, Yoshida M, Obayashi T, and Kawai T.** Automated kinetic assay for endotoxin and (1-->3)-beta-D-glucan in human blood. *Clin Chim Acta* 226: 109-112, 1994.

181. **Taylor KR, Trowbridge JM, Rudisill JA, Termeer CC, Simon JC, and Gallo RL.** Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. *J Biol Chem* 279: 17079-17084, 2004.

182. **Teeuw KB, Vandenbroucke-Grauls CM, and Verhoef J.** Airborne gram-negative bacteria and endotoxin in sick building syndrome. A study in Dutch governmental office buildings. *Arch Intern Med* 154: 2339-2345, 1994.

183. **Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, Miyake K, Freudenberg M, Galanos C, and Simon JC.** Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med* 195: 99-111, 2002.

184. **Tremblay GM, Ferland C, Lapointe JM, Vrins A, Lavoie JP, and Cormier Y.** Effect of stabling on bronchoalveolar cells obtained from normal and COPD horses. *Equine Vet J* 25: 194-197, 1993.

185. **Turlej RK, Fievez L, Sandersen CF, Dogne S, Kirschvink N, Lekeux P, and Bureau F.** Enhanced survival of lung granulocytes in an animal model of asthma: evidence for a role of GM-CSF activated STAT5 signalling pathway. *Thorax* 56: 696-702, 2001.

186. **van Duin D, Mohanty S, Thomas V, Ginter S, Montgomery RR, Fikrig E, Allore HG, Medzhitov R, and Shaw AC.** Age-associated defect in human TLR-1/2 function. *J Immunol* 178: 970-975, 2007.

187. **Van Huffel S, Delaei F, Heyninck K, De Valck D, and Beyaert R.** Identification of a novel A20-binding inhibitor of nuclear factor-kappa B activation termed ABIN-2. *J Biol Chem* 276: 30216-30223, 2001.

188. **Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, and Segal DM.** Regulation of Toll-like receptors in human monocytes and dendritic cells. *J Immunol* 166: 249-255, 2001.

189. **Wagner JG, and Roth RA.** Neutrophil migration during endotoxemia. *J Leukoc Biol* 66: 10-24, 1999.

190. **Wagner JG, and Roth RA.** Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. *Pharmacol Rev* 52: 349-374, 2000.

191. **Walters M, Milton D, Larsson L, and Ford T.** Airborne environmental endotoxin: a cross-validation of sampling and analysis techniques. *Appl Environ Microbiol* 60: 996-1005, 1994.

192. **Wassef A, Janardhan K, Pearce JW, and Singh B.** Toll-like receptor 4 in normal and inflamed lungs and other organs of pig, dog and cattle. *Histol Histopathol* 19: 1201-1208, 2004.

193. **Werners AH, Bull S, Vendrig JC, Smyth T, Bosch RR, Fink-Gremmels J, and Bryant CE.** Genotyping of Toll-like receptor 4, myeloid differentiation factor 2 and CD-14 in the horse: an investigation into the influence of genetic polymorphisms on the LPS induced TNF-alpha response in equine whole blood. *Vet Immunol Immunopathol* 111: 165-173, 2006.

194. **Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, Wu P, Wiesmann C, Baker R, Boone DL, Ma A, Koonin EV, and Dixit VM.** De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. *Nature* 430: 694-699, 2004.

195. **Wolfs TG, Buurman WA, van Schadewijk A, de Vries B, Daemen MA, Hiemstra PS, and van 't Veer C.** In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN-gamma and TNF-alpha mediated up-regulation during inflammation. *J Immunol* 168: 1286-1293, 2002.

196. **Woods PS, Robinson NE, Swanson MC, Reed CE, Broadstone RV, and Derksen FJ.** Airborne dust and aeroallergen concentration in a horse stable under two different management systems. *Equine Vet J* 25: 208-213, 1993.

197. **Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, and Akira S.** Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301: 640-643, 2003.

198. **Yauch LE, Mansour MK, Shoham S, Rottman JB, and Levitz SM.** Involvement of CD14, toll-like receptors 2 and 4, and MyD88 in the host response to the fungal pathogen Cryptococcus neoformans in vivo. *Infect Immun* 72: 5373-5382, 2004.

199. **Zaas AK, and Schwartz DA.** Innate immunity and the lung: defense at the interface between host and environment. *Trends Cardiovasc Med* 15: 195-202, 2005.

200. **Zhang CH, Davis WC, Grunig G, and Antczak DF.** The equine homologue of LFA-1 (CD11a/CD18): cellular distribution and differential determinants. *Vet Immunol Immunopathol* 62: 167-183, 1998.

201. **Zhang G, and Ghosh S.** Negative regulation of toll-like receptor-mediated signaling by Tollip. *J Biol Chem* 277: 7059-7065, 2002.

202. **Zimmerman GA, Elstad MR, Lorant DE, McLntyre TM, Prescott SM, Topham MK, Weyrich AS, and Whatley RE.** Platelet-activating factor (PAF): signalling and adhesion in cell-cell interactions. *Adv Exp Med Biol* 416: 297-304, 1996.