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The Role of Alveolar Macrophages in the Pathogenesis of Recurrent Airway Obstruction in Horses

Tamarinde T.J.M. Laan, Sarah Bull, R. Pirie, and Johanna Fink-Gremmels

When challenged with allergens and pro-inflammatory agents, such as *Aspergillus fumigatus* (AF), hay dust solution (HDS) and lipopolysaccharide (LPS), the innate immune response will not only activate the immune system but also increase the amount of pro-inflammatory cytokines in the bronchoalveolar space. The aim of this study was to assess the response of equine alveolar macrophages to different aerosolized challenges and to investigate the differences in this response between horses susceptible or nonsusceptible to recurrent airway obstruction (RAO). Seven susceptible and 5 nonsusceptible horses were challenged with saline, LPS, HDS, or AF, and bronchoalveolar lavage (BAL) cytology, total cell counts, and lung function were assessed. In addition, alveolar macrophages were isolated 6 and 24 hours after challenge, and macrophage mRNA expression of tumor necrosis factor (TNF)- α and interleukins (IL) IL-1 β , IL-6, IL-8, and IL-10 were measured by means of real-time (RT) polymerase chain reaction (PCR). There was a significant difference in lung function, neutrophil ratios, and total cell counts in the bronchoalveolar lavage fluid between RAO-susceptible and nonsusceptible horses. In addition, the expression of TNF- α , IL-1 β , and IL-8 by alveolar macrophages after challenges were higher in susceptible horses, than in nonsusceptible horses. In contrast, IL-6, considered an anti-inflammatory cytokine, showed a higher expression in nonsusceptible horses 6 hours after inhalation challenge with allergens and pro-inflammatory antigens. These data suggest that the differences between susceptible and nonsusceptible horses to RAO are not only dependent on adaptive immunity but also start with an innate immune response.

Key words: Aerosolized challenges; *Aspergillus fumigatus*; Cytokine expression; Hay dust solution; Heaves; Lipopolysaccharide.

Recurrent airway obstruction (RAO) is a well-known respiratory disease in horses that manifests with many physiopathologic similarities to chronic obstructive pulmonary disease (COPD) and asthma in humans.^{1–4} However, there are also marked differences, as eosinophils predominate in human asthma, whereas neutrophils are the major cell type in the bronchoalveolar space during exacerbations of equine RAO.^{5–7}

The airway inflammatory response in RAO contributes to clinical features, such as coughing and increased expiratory effort,^{3,8} which occur following exposure of the horse to known airborne antigens such as the mold conidia, found in respirable airborne stable dust.^{9,10} Mold-derived particles (such as conidia) are, however, not the only component in the stable dust to which horses respond with bronchiolitis. Lipopolysaccharides (LPS) of varying origins and other organic dust particles have been proven to contribute to the disease.^{10–12} In RAO equine patients, the influx of neutrophils into the bronchoalveolar space has been described for many years, however, the exact mechanism by which these neutrophils are attracted remains to be elucidated. Two

paradigms have been discussed. The 1st and most commonly supported hypothesis classifies RAO as a Th2 disorder because Th2 cell-related cytokines, such as interleukin (IL)-4 and IL-13, are increased in cellular components of bronchoalveolar lavage fluid (BALF) during exacerbations of the disease.^{13,14} Furthermore, there is evidence of increased BALF immunoglobulin (Ig)-E levels, together with a rise in BALF mast cell counts and histamine levels, in horses during disease exacerbation.^{15–17} However, recent work by Ainsworth et al¹⁸ showed an increase in IL-8 and interferon (IFN)- γ during exacerbations of RAO, without a parallel increase in IL-4 and IL-13. In addition, Giguere et al¹⁹ showed an increase of tumor necrosis factor (TNF)- α , IL-1 β , and IL-8 in RAO-susceptible horses, whereas values of IL-4 and IFN- γ varied, and IL-2, IL-5, and IL-10 were not detected. Although these findings do not directly implicate the alveolar macrophage in the etiopathogenesis of RAO, one could speculate from the obtained results that alveolar macrophages may play a significant role. Franchini et al^{20,21} suggested this hypothesis earlier, when they demonstrated that IL-8 and macrophage inhibitory protein (MIP)-2 were required in neutrophil attraction, thus implicating the involvement of the alveolar macrophage in the initial reaction. These macrophages are, together with neutrophils, the dominant effector cells of the innate immune system, responsible for the 1st defense mechanism on the surface of the lungs. The innate immune system is also responsible for initiating an inflammatory response that further regulates the onset of a well-balanced, adaptive immunity. Taken together, these findings suggest that macrophage activation is the initial response to airborne challenges, followed by a neutrophil invasion, which is partly due to the innate immune response. Hence, the aim of this study was to characterize the cytokine response of the equine alveolar macrophage to different

From the Department of Equine Science (Laan), and the Department of Veterinary Pharmacology, Pharmacy & Toxicology (VFFT) (Bull, Fink-Gremmels), Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; the Department of Health, Toxicology Unit, Imperial College, London, UK (Bull); and the R(D)SVS, Easter Bush Veterinary Centre, Roslin, Midlothian UK (Pirie); and the Veterinary Clinic "DeLingehoeve," Lienden, The Netherlands (Laan).

Reprint requests: Tamarinde Laan, Hogeweg 32, Dreumel BN 6621 The Netherlands; e-mail: t.laan@delinge.hoeve.nl.

Submitted November 10, 2004; Revised March 22, 2005; Accepted July 22, 2005.

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0891-6640/06/2001-0021/\$3.00/0

aerosolized challenges and to describe the differences in this response between RAO-susceptible horses and healthy nonsusceptible horses.

Materials and Methods

Statement of Animal Care

The committee on ethical considerations in animal experiments of the Faculty of Veterinary Science of the University of Utrecht, The Netherlands, approved all experiments under project number 0301.0602.

Animals

Twelve adult Dutch warmblood horses, ten mares and two geldings (mean body weight 610 kg, range 570–700 kg, age 7–11 years), were used in this study. Seven horses were classified as being RAO-susceptible and five horses were considered free of respiratory disease. Classification of the horses was done on the basis of anamnesis and complete clinical examination including, endoscopy, pulmonary function testing and bronchoalveolar lavage (BAL), historical treatment results, and a natural challenge experiment, during which the horses were stabled for 24 hours in a dusty environment and offered moldy hay, followed by a BAL and cytologic examination of the BAL fluid. Horses were classified as RAO-susceptible when, following natural challenge, lung function was significantly impaired, tracheal secretions increased, and the neutrophil ratio in the BAL fluid increased from less than 5%, before the challenge, to more than 20% after challenge. These criteria used for the classification of horses are comparable to the criteria defined in the consensus report for experimental studies in heaves by Robinson.²² Before the main experiments, horses were kept on shavings and fed silage and pelleted feed for a minimum of 2 months, were vaccinated, received routine anthelmintic treatment, and were accustomed to the wearing of the facemask used in the forthcoming challenge procedures. Throughout the study, the horses were individually stabled but shared a common airspace. Horses were evaluated clinically and, when necessary, endoscopically for upper airway disease before each challenge. The minimum interval between 2 individual challenges was 2 weeks because earlier pilot experiments (data not shown) had indicated that during that period all parameters returned to baseline values.

Challenge Solutions

Saline (0.9%) 2.5 mL served as a control. Two thousand micrograms of *Escherichia coli* (*E. coli*)-derived lipopolysaccharide (LPS),^a diluted from a stock (1 mg/mL) with 2.5 mL sterile saline, was given immediately before nebulization. The hay dust suspension (HDS) used in this study was a combination of 2 suspensions that had previously been successfully used to induce RAO in susceptible horses and to differentiate RAO-susceptible horses from healthy controls following inhalation challenge.^{23,24} Details of HDS preparation and analysis are described elsewhere.²⁵ Briefly, fine organic dust particles were collected from batches of visibly moldy hay, suspended in sterile isotonic saline, and analyzed by microscopic examination for particulate, endotoxin, and glucan concentrations. Particulate concentration of 0–40 μm^3 was measured at $741 \times 10^9/\text{mL}$ and at 40–300 μm^3 at $184 \times 10^6/\text{mL}$. Protein concentration consisted of 0.47 mg/mL, and endotoxin and β -glucan concentration were measured at 18.4 and 390 $\mu\text{g}/\text{mL}$, respectively.

The HDS particulates used primarily ranged between 0 and 40 μm . Assuming that most particulates are spherical, this correlates to a particulate diameter of 0–4 μm . For each challenge, 1 mL of HDS was used, diluted to a total volume of 2.5 mL with

saline. *Aspergillus fumigatus* (AF)-antigen was supplied by Greer Laboratories.^b Each challenge contained 200 μg of the antigen-protein dissolved in 2.5 mL sterile saline.

Challenges

Horses received an inhalation challenge with saline, LPS, HDS, or AF antigen in a randomized crossover design. The aerosol was generated using a compressor,^c with a calibrated output of 9 L/min, connected to a nebulizer cup. According to the manufacturer's information, 80% of the aerosol generated in this manner is in the respirable range ($<5 \mu\text{m}$). The aerosol was delivered to a T-piece that was connected to an equine Aeromask.^d Sedation of the horses was not considered necessary because they had been trained to wear the facemask using saline nebulization. The total volume of each challenge consisted of 2.5 mL.

Pulmonary Function

At 6 and 24 hours postchallenge, measurements of respiratory mechanics were performed as described previously.²⁶ Briefly, airflow rate was determined using a heated pneumotachograph (Fleisch No. 4) fitted to a fiberglass facemask placed over the horse's nose. The maximum change in esophageal pressure (ΔP_L) was measured using a balloon sealed over the end of a polyethylene catheter attached to a pressure transducer. The balloon catheter was placed in the esophagus through a nasogastric tube and positioned midthorax to estimate pleural pressure. The signals were amplified and passed through a converter to a computer using data acquisition software. Values of pulmonary lung function (transpulmonary pressure, ΔP_L ; pulmonary resistance, R_L ; and dynamic compliance, C_{dyn}) were obtained from 5 consecutive breaths, chosen on the basis of loop closure and uniformity, from which a mean was calculated.

Bronchoalveolar Lavage

Immediately following each lung function test, BALF was collected under standing chemical restraint (10 $\mu\text{g}/\text{kg}$ detomidine) via a 2.2-m-long by 9-mm-wide endoscope, passed via the trachea and wedged in a secondary or tertiary bronchus. During the endoscopic-guided BAL procedure, the trachea was instilled with a 1:5 dilution of 2% lidocaine (4 mg/mL) to minimize the cough reflex. Following positioning of the tip of the scope in an appropriate bronchus, 5 successive 60-mL aliquots of warmed saline containing 200 $\mu\text{g}/\text{mL}$ gentamicin and 2.5 $\mu\text{g}/\text{mL}$ amphotericin B were instilled and withdrawn immediately by manual suction using 60-mL syringes. The lavage fluid was pooled, decanted into sterile bottles, and placed on ice. A 10-mL aliquot was used for BALF cytology. BALF was collected from the right lung at 6 hours postchallenge and from the left lung at 24 hours postchallenge.

Preparation of Microscopic Slides

Cytospin slides were used for microscopic evaluation of the cellular components of the BALF. Briefly, samples were filtered through a double layer of sterile gauze and washed twice by centrifugation in cold phosphate-buffered saline (PBS) at $1,100 \times g$ for 5 minutes at 4°C. Two milliliters of the washed sample were placed in cytochambers and centrifuged at $50 \times g$ for 5 minutes. Slides were air-dried before staining with May-Grünwald Giemsa stain and a cover slip was placed over the cells using DePeX mounting medium. Differential cell counts (DCC) were based on observation of 400 cells. Cells were identified as macrophages, lymphocytes, neutrophils, mast cells, or eosinophils.

Table 1. Nucleotide sequences of equine specific primers. F is forward, R is reverse.

Gene	Primer	Sequence (5'-3')	T (ann) (°C)	Accession number
GAPDH	F	TGGCATGGCCTTCCGTGTCC	63.0	AF157626
	R	GCCCTCCGATGCCTGCTTCAC		
β -actin	F	CAAGGCCAACC GCGAGAAGATGAC	63.5	AF035774
	R	GCCAGAGGCGTACAGGGACAGCA		
TNF- α	F	GCCTTTCCCGCCCTCCTCTCG	61.5	M64087
	R	CTCTCCTGGCTGGTCCCCTGTTGC		
IL-1 β	F	TGGCAGAGGGGAATAGAAGGGTTTG	59.0	U92480
	R	ATAGGGAAGGCAGCTGGGCATTGA		
IL-6	F	ATGAGTGGCTGAAGAACAACAAC	61.0	AF005227
	R	AGGAATGCCCATGAATAACAACAAT		
IL-8	F	TGGCCTCTTCCTGCTTTCTG	64.0	AF062377
	R	GGTTTGGAGTGCCTCTTGATGC		
IL-10	F	GAGAACCACGGCCAGACATCAAG	64.5	U38200
	R	GACAGCGCCGAGCCTCACT		

Total Cell Counts

Total cell count (TCC) measurement in filtered BALF was performed using a dual threshold Coulter counter. Urea concentration was measured in both heparinized venous blood samples collected immediately before BAL and in BALF supernatant using commercial kits.^c Measurement of urea in BALF was adapted from a method described earlier by McGorum et al²⁷ and validated for horses before the initiation of the experiments. The proportion of pulmonary epithelial lining fluid in each BALF sample was calculated from the formula: (BALF urea concentration $\times$ 100) $\div$ (plasma urea concentration).²⁷

Isolation of Alveolar Macrophages

Bronchoalveolar lavage fluid was processed within 30 minutes after sample collection. The lavage fluid was filtered through a double layer of sterile gauze and centrifuged in a swing-out rotor centrifuge at $150 \times g$ for 30 minutes at 4°C. The fluid was discarded, and the cell pellet washed twice by centrifugation in cold PBS at $150 \times g$ for 15 minutes at 4°C. After washing, the cells were resuspended in 4 mL of warm Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% fetal bovine serum (FBS), 100 U/mL penicillin, 200 μ g/mL gentamicin, and 2.5 μ g/mL amphotericin B. This cell suspension was layered over Ficoll-Hypaque using a ratio of 6:4 and centrifuged at $3,000 \times g$ at 22°C for 20 minutes. The cells were recovered from the RPMI-Ficoll interface and washed by centrifugation in warm medium at $150 \times g$ for 15 minutes at 22°C. The alveolar macrophages were finally resuspended in 1 mL of medium, counted, and their viability assessed by trypan-blue staining. Cells were seeded into 6-well plates, using a cell density of 1×10^6 cells/well and incubated at 37°C and 5% carbon dioxide (CO₂) for 1.5 hours to allow cells to adhere; after which, the medium and the nonadherent cells were removed. The monolayer was washed and harvested in cold PBS. The cell suspension was placed in RNase-free eppendorf vials and centrifuged at $15,000 \times g$ for 5 minutes. Cell pellets were snap-frozen in liquid nitrogen and stored at -70°C until further analysis. The cell cultures contained more than 95% macrophages, as determined by differential staining with May-Grünwald Giemsa.

RNA Isolation and cDNA Synthesis

Total cellular RNA was isolated from the above-mentioned samples using the RNeasy mini kit^f according to the manufacturer's instructions. The RNA concentration was measured by optical density at 260 nm. All RNA samples were treated with

amplification-grade DNase according to the manufacturer's instructions to remove any traces of DNA. The cDNA was synthesized with the reverse transcription (RT) system from Promega^g following the manufacturers protocol. Briefly, 1 μ g of total RNA was mixed with 0.5 μ g of Oligo(dT)₁₅ primer and 15 units of AMV reverse transcriptase. Reverse transcription was carried out at 42°C for 45 minutes, followed by heating to 99°C for 5 minutes and incubation at 0–5°C for 5 minutes; after which, the sample was diluted and stored at 4°C until PCR amplification.

Quantitative PCR Measurements

Quantitative real-time PCR was performed on equine glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -actin, TNF- α , IL-1 β , IL-6, IL-8, and IL-10. The real-time PCR method was based on the high-affinity, double-stranded DNA-binding dye SYBR green I^h and was performed in triplicate in a spectrofluorometric thermal cycler. For each PCR reaction, 2 μ L of cDNA was used in a reaction volume of 50 μ L containing $1 \times$ manufacturer's buffer, 2 mM MgCl₂, 0.5 $\times$ SYBR green I, 200 μ M dNTP, 20 pmol of both primers, and 1.25 units of AmpliTaq Goldⁱ on 96-well iCycler iQ plates. PCR primer pairs specific for equine GAPDH, β -actin, TNF- α , IL-1 β , IL-6, IL-8, and IL-10 were designed by PrimerSelect software based on their GenBank accession numbers (Table 1). Melting curves were used to examine each sample for purity, and negative controls were included in the amplification reactions to check for contamination. Standard sequencing procedures were used to verify the analytical specificity of the PCR products. All genes were examined in each experimental sample. GAPDH and β -actin were determined as housekeeping genes.

Statistical Analysis

The distribution of data was assessed using the Kolmogorov-Smirnov test and were found to be normally distributed. When, during either the clinical or laboratory procedure, circumstances indicated that the results might not be representative, the Grubb's or Dixon's test was applied to exclude statistical outliers.

To test whether the LPS, HDS, or AF challenge influenced pulmonary lung function, absolute cell numbers, neutrophil ratios, or cytokine production, data were subjected to repeated measurements using analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test comparing the data against saline challenge. To evaluate the difference between RAO-susceptible and nonsusceptible horses, data were analyzed using a two-tailed, nonpaired Student's *t*-test. Data was considered statistically significant when $P < .05$. Statistical analyses of data were

Table 2. Lung function variables from 7 recurrent airway obstruction (RAO) susceptible horses 6 and 24 hours after challenge with saline, *Escherichia coli*-derived lipopolysaccharide (LPS), hay dust suspension (HDS) and *Aspergillus fumigatus* (AF). Data are presented as mean (range).

Susceptible Horses	Saline		LPS		HDS		AF	
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
C _{dyn}	1.68 (1.3–2.0)	1.71 (1.3–2.0)	0.83* (0.6–0.12)	0.83* (0.6–1.3)	1.13* (0.8–0.16)	1.06* (0.8–1.4)	1.61 (1.3–1.9)	1.50 (0.8–1.7)
ΔP _L	5.28 (4.0–6.0)	5.29 (4.0–6.0)	11.71* (10.0–13.0)	7.86* (6.0–11.0)	9.43* (0.8–16.0)	8.57* (7.0–11.0)	6.57 (5.0–11.0)	6.47 (5.0–10.0)
R _L	0.69 (0.5–0.8)	0.60 (0.5–0.7)	1.21* (0.70–1.5)	1.06* (0.7–1.3)	1.06* (0.8–1.3)	0.93* (0.6–1.1)	0.80 (0.6–1.2)	0.76 (0.6–1.0)

ΔP_L, maximal esophageal pressure excursions (cm H₂O); R_L, pulmonary resistance (cm H₂O/L/s); C_{dyn}, dynamic compliance (L/cm H₂O).

* Significant difference when compared with saline challenge, $P < .05$.

performed with statistical software[†]. Unless otherwise indicated, data are presented as mean ± standard deviation.

Results

Lung Function and BALF Cytology

LPS, HDS, and AF challenges had no significant effect on lung function of healthy nonsusceptible horses (C_{dyn} range, 1.1–2.0; ΔP_L range, 3.5–6.5; R_L range, 0.4–0.8). Within the RAO-susceptible group, there were significant differences in C_{dyn}, ΔP_L, and R_L at 6 and 24 hours following inhalation challenge with both LPS and HDS when compared with the saline challenge. Inhalation challenge with the AF antigen did not significantly alter any of the lung function variables (Table 2).

Compared to the saline inhalation challenge, neutrophil ratios significantly increased in RAO-susceptible horses following inhalation challenge with both LPS and HDS at both 6 and 24 hours but not following inhalation challenge with AF. In contrast, none of the inhalation challenges resulted in an increase in neutrophil ratios in nonsusceptible horses (Fig 1a). When comparing the neutrophil ratios of RAO-susceptible horses and nonsusceptible controls, significant differences were observed following inhalation challenge with both LPS and HDS at 6 and 24 hours, but not following inhalation challenge with AF (Fig 1a).

TCC were significantly increased at 24 hours following both HDS and AF inhalation challenge in RAO-susceptible horses. Furthermore, there was a significant difference in TCC between RAO-susceptible horses and nonsusceptible controls at 24 hours following LPS inhalation challenge (Fig 1b).

Absolute numbers of macrophages also significantly increased in RAO-susceptible horses at 24 hours following HDS inhalation challenge (Fig 1c). None of the inhalation challenges resulted in a significant alteration in TCC (Fig 1b) or absolute macrophage numbers (data not shown) at 24 hours in nonsusceptible horses.

Cytokine Expression in Alveolar Macrophages

Alveolar macrophages were isolated from horses at 6 and 24 hours following inhalation challenge with saline,

LPS, HDS, and AF, and TNF-α, IL-1, IL-6, IL-8, and IL-10 expression were measured by means of RT-PCR. To account for variation in mRNA expression, all results were corrected to mean values of GAPDH, serving as a housekeeping gene.

Cytokine Expression in Alveolar Macrophages Harvested from RAO-Susceptible Horses

Compared with the saline inhalation challenge, mRNA expression of TNF-α in alveolar macrophages was significantly increased following inhalation challenge with HDS at 6 hours but had returned to baseline

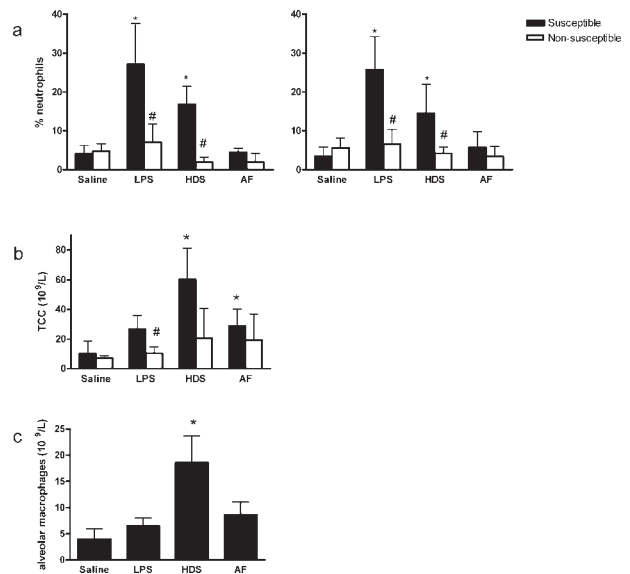


Fig 1. (a) Percentage of neutrophils in bronchoalveolar lavage fluid (BALF), 6 hours (left) and 24 hours (right) after challenge with saline, *Escherichia coli*-derived lipopolysaccharide (LPS), hay dust suspension (HDS), or *Aspergillus fumigatus* (AF). (b) Total cell counts in the BALF 24 hours after challenge with saline, LPS, HDS, or AF. (c) Number of alveolar macrophages in BALF of recurrent airway obstruction (RAO) susceptible horses 24 hours after challenge with saline, LPS, HDS, or AF. Data are expressed as mean ± standard deviation; * indicates significant differences within a group compared with the saline challenge; # indicates significant differences between RAO-susceptible ($n = 7$) and nonsusceptible horses ($n = 5$) within a challenge.

values after 24 hours. Six hours following the HDS-inhalation challenge, alveolar macrophages had a significantly greater expression of TNF- α compared with alveolar macrophages from nonsusceptible controls (Fig 2a).

IL-1 β was significantly increased 6 and 24 hours following inhalation challenge with LPS, HDS, and AF. At 6 hours following LPS inhalation challenge and at 24 hours following HDS inhalation challenge, a significantly increased IL-1 β expression was observed in the RAO-susceptible horses compared with alveolar macrophages harvested from nonsusceptible controls (Fig 2b).

mRNA expression of IL-6 was significantly increased 6 hours following inhalation challenge with HDS. At 24 hours, IL-6 expression had returned to baseline values (Fig 2c). Expression of IL-8 was significantly increased 6 hours following inhalation challenge with both LPS and HDS, although at 24 hours, expression of IL-8 had decreased to baseline values. Compared with alveolar macrophages harvested from RAO-nonsusceptible horses, IL-8 expression was significantly increased 6 hours following LPS inhalation challenge (Fig 2d). None of the challenges significantly altered mRNA expression of IL-10 either at 6 or 24 hours postchallenge (Fig 2e).

Cytokine Expression in Alveolar Macrophages Harvested from RAO-Nonsusceptible Horses

No LPS, HDS, nor AF inhalation challenge significantly altered TNF- α , IL-1 β , IL-6, IL-8, or IL-10 expression (Figs 2a–e). Interestingly, alveolar macrophages harvested from healthy, nonsusceptible horses had a significantly higher level of IL-6 expression 6 hours following inhalation challenge with saline, LPS, and AF, compared with alveolar macrophages harvested from RAO-susceptible horses (Fig 2c).

Discussion

We investigated, in this study, the expression of various pro- and anti-inflammatory cytokines in alveolar macrophages from RAO-susceptible and nonsusceptible horses in response to inhalation challenge with selected allergens and pro-inflammatory agents present in organic dust. Our results demonstrate a significant difference between RAO-susceptible and nonsusceptible horses with respect to BALF cytology and the expression of cytokines and chemokines following inhalation challenges with LPS, HDS, and AF.

In alveolar macrophages, the expression of both pro-inflammatory (TNF- α , IL-1 β , and IL-8) and anti-inflammatory cytokines (IL-6 and IL-10) is most likely initiated by a CD14 and Toll-like receptor (TLR)-dependent signaling pathway that activates the transcription factor NF κ B.^{28,29} Although NF κ B activation in the surrounding tissues may contribute to inflammatory response in the bronchoalveolar space in vivo,³⁰ the differences found in cytokine expression following different challenges in this study, as well as differences between susceptible and nonsusceptible subjects, suggest

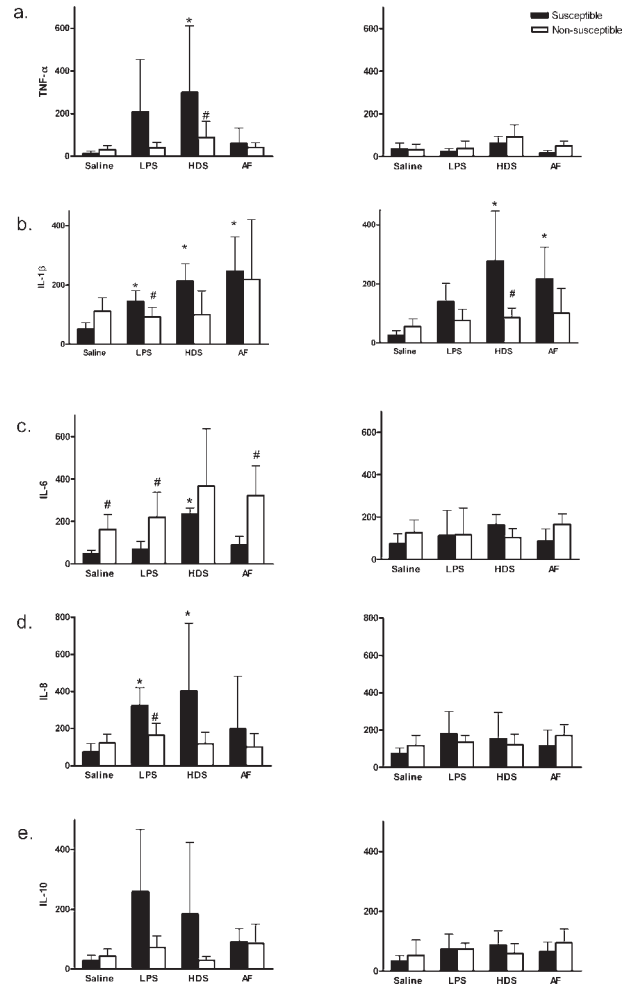


Fig 2. Expression of (a) tumor necrosis factor (TNF)- α , (b) interleukin (IL)-1 β , (c) IL-6, (d) IL-8, and (e) IL-10 mRNA (as a ratio against glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) 6 hours (left) and 24 hours (right) after challenge with saline, *Escherichia coli*-derived lipopolysaccharide (LPS), hay dust suspension (HDS), or *Aspergillus fumigatus* (AF). Data are presented as mean $\pm$ standard deviation; * indicates significant differences within a group compared with the saline challenge; # indicates significant differences between recurrent airway obstruction (RAO)-susceptible (n = 7) and nonsusceptible horses (n = 5) within a challenge.

that the alveolar macrophages play a pivotal role in the initiation of the inflammatory reaction.

Inhalation challenge with HDS induced the greatest cytokine expression. In contrast, the neutrophil infiltration of the bronchoalveolar space was most pronounced following inhalation challenge with LPS. Enhanced sensitivity to inhaled endotoxins has been proven to exist in human subjects, and a link to the activation of CD14 is proposed.³¹ Thus, possible explanations for the results in our experiment are that either the amounts of different pathogen-associated molecular patterns present in HDS stimulate different TLRs, leading to a higher expression of cytokines after challenge with HDS, or that CD14 involvement is more important in the LPS-TLR4 signaling pathway, result-

ing in an up-regulated Th2 cytokine profile and higher neutrophil percentages following a LPS challenge.

In comparable experiments, Pirie et al^{23,24} previously found a greater increase in BALF neutrophil ratios, ranging from 51 to 85% in RAO-susceptible horses, following inhalation challenge with HDS. As we used equal volumes of the same hay dust suspensions and a comparable experimental design, the differences in neutrophil ratios could be explained by interindividual variability in the inherent response status of the test horses.²³ HDS challenge did influence both neutrophil influx and mRNA expression in alveolar macrophages in our experiments. Pirie et al²⁵ showed that 23–26% of the 0–40 μm^3 particulates (ie, 0–4 μm in diameter) were successfully nebulized, and microscopic evaluation revealed the particulates to have the physical appearance of fungal spores. Thus, they are likely to be the allergic components in HDS.²⁵ The lack of neutrophil influx following AF-antigen challenge in the presented experiment is in agreement with findings of Pirie et al,¹² who also failed to show neutrophil influx after challenge with AF-antigen alone. In contrast, AF solution not depleted from LPS did result in significant differences in neutrophil influx. This was also shown in the experiments conducted with fractionated HDS, showing that the response to HDS-related AF is most likely accelerated in the presence of LPS. Other particles in HDS, present in nonfractionated HDS, resulted in a greater neutrophil influx.²⁴

Neutrophil ratios in nonsusceptible horses remained unchanged after challenge with HDS in our experiments. The lack of neutrophil infiltration in these nonsusceptible horses are in agreement with the minimal changes in mRNA expression levels, most likely reflecting the nonexacerbated, physiologic innate immune response.

AF antigen used in the *in vivo* challenge protocol, although significantly altering IL-1 β mRNA expression at 6 and 24 hours, failed to induce significant increases in expression of the other pro-inflammatory cytokine, TNF- α , or the chemokine IL-8. These results were unexpected because earlier experiments^k with an AF solution, containing both fungal conidia and hyphae, had been shown to induce the highest production of TNF- α when compared with the LPS and HDS challenge. The obvious discrepancy between the AF antigen used in the *in vivo* experiments and AF solution used in the previous *ex vivo* experiments could possibly be attributed to MyD88-dependent mechanisms if the solution was contaminated with LPS or by MyD88-independent mechanisms being activated following exposure to conidia and hyphae present in AF solutions,³² leading to a higher cytokine expression when compared with that observed following TLR activation by AF antigen alone.

Interleukin-6 has long been regarded as a pro-inflammatory cytokine, but recent results in IL-6 knock-out mice and the IL-6-related immunologic effects in these animals suggest that it also exhibits anti-inflammatory properties.^{33,34} Data from the present study demonstrate that in RAO-susceptible horses, the in-

crease in IL-6 expression after the different challenges was comparable to the increases observed in the expressions of TNF- α , IL-1 β , and IL-8. However, a remarkable difference was observed in nonsusceptible control horses when compared with RAO-susceptible horses because significantly higher levels of expression of IL-6 mRNA were found after challenge with HDS in nonsusceptible control horses, whereas TNF- α and IL-1 β remained low. IL-6 has been shown to down-regulate TNF- α and IL-1 β .^{35,36} Thus, the high IL-6 expression in nonsusceptible horses is supportive of the anti-inflammatory properties of IL-6, ultimately resulting in a lower leukocyte influx into the airways of nonsusceptible horses. The phenomenon of high IL-6 production in nonsusceptible horses has, to our knowledge, not been reported before and warrants further investigation.

Within the human pulmonary, innate immune response, IL-10 is the most important anti-inflammatory cytokine,³⁴ and its anti-inflammatory properties have been similarly demonstrated in animal models.^{37–39} Following inhalation challenge with LPS in RAO-susceptible horses, a slight but significant increase in IL-10 expression was observed, however, the values remained low. Stimulation of alveolar macrophages through TLRs has been shown to inhibit IL-10 receptor function,⁴⁰ and this mechanism is believed to be advantageous in the lung because it permits alveolar macrophages to overcome the inhibitory effects of IL-10. Likewise, we demonstrated a reduced IL-10 expression that may permit a more effective response to pulmonary infection.

Despite evidence supporting a Th1-dependent pathway, the hypothesis that the pathogenesis of RAO is mainly Th2-dependent is still the best-accepted concept explaining the influx of inflammatory cells in exacerbations of equine RAO.¹ The Th2 cytokine profile may reflect characteristics of lymphocytes already committed to this response,¹³ or this may be acquired when lymphocytes enter the bronchoalveolar space. The shift could be a result of their antigen specificity and the influences of the micro environment.¹⁴ Alexis et al³¹ have earlier suggested that airway macrophages and granulocytes, being part of this microenvironment, play such a modulatory role. They showed a CD14-dependent neutrophil response to inhaled LPS and concluded from these results that atopy plays a role in the level of expressed CD14, therefore, predicting the magnitude of neutrophilic infiltration in asthmatic subjects.³¹ With respect to these results and the results of Virchow et al,⁴¹ who showed a positive correlation between IL-4, IL-5, IL-13, and CD14 expression, the question arises whether CD14 up-regulation, a macrophage-related response, drives the allergic phenotype that is responsible for the outcome of clinical disease. Taken together, these findings suggest that macrophages play an important role in the pathogenesis of human asthma and, possibly, equine RAO.

In conclusion, these experiments revealed that equine alveolar macrophages exhibit an increased expression of cytokine mRNA following different aerosolized challenges *in vivo*. The function of these cytokines is to

enhance an inflammatory reaction, to initiate a well-balanced adaptive immunity, and possibly to drive the allergic phenotype. Hence, the presented data suggest that the differences between susceptible and nonsusceptible horses to RAO depend not only on the adaptive immunity but also on the early innate immune response.

Footnotes

- ^a O111:B4, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands
^b *Aspergillus fumigatus* antigen, Lot My3-79; Greer Laboratories Inc, Lenoir, NC
^c Porta-Neb, Medic-Aid Ltd, Bognor Regis, West Sussex, UK
^d Aeromask Trudell Medical International, Ontario, Canada
^e BALF supernatant: Infinity urea, Clindia Benelux BV, Leusden, The Netherlands
^f Qiagen Inc, Leusden, The Netherlands
^g Promega Benelux, Leiden, The Netherlands
^h SYBR green I, BMA, Rockland, ME
ⁱ Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands
^j Graphpad Prism 4.0 GraphPad Software Inc, San Diego, CA; Statgraphics plus 5.0 Manugistics Inc, Rockville, MD
^k Laan TTJM, Bull S, Fink-Gremmels J. Cytokine modulation by β_2 agonists. Proc. ACVIM Forum 2004 (abstract)
^l Horohov DW. Cytokine response in equine airways. Proc ACVIM Forum 2003 (abstract)

Acknowledgments

We would like to thank Suzanne Brons for her help with the cell cultures, Geert de Vrieze for carrying out the RT-PCR experiments, and Bart Spee for his expertise in developing the PCR protocol. The work was conducted at the Faculty of Veterinary Medicine, Department of Equine Science, Utrecht University.

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