Pulmonary intravascular macrophages as proinflammatory cells in heaves, an asthma-like equine disease

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Aharonson-Raz K, Lohmann KL, Townsend HG, Marques F, Singh B. Pulmonary intravascular macrophages as proinflammatory cells in heaves, an asthma-like equine disease. Am J Physiol Lung Cell Mol Physiol 303: L189 –L198, 2012. First published June 1, 2012; doi:10.1152/ajplung.00271.2011.—Heaves, an obstructive neutrophilic airway inflammation of horses, is triggered by dust components such as endotoxin and has similarities to human asthma. Pulmonary intravascular macrophages (PIMs) increase horses’ sensitivity to endotoxin-induced lung inflammation; however, their role in an airborne pathology remains unknown. Therefore, we investigated the role of PIMs in the development of heaves in horses. Clinical and inflammatory responses were evaluated following induction of heaves by moldy hay exposure and PIM depletion with gadolinium chloride (GC). Mares (N = 9) were exposed to four treatments: alfalfa cubes (Cb), alfalfa cubes + GC (Cb-GC), moldy hay (MH), and moldy hay + GC (MH-GC). Clinical scores and neutrophil concentrations in bronchoalveolar lavage (BAL) fluid were higher when mares received MH compared with MH-GC. BAL cells from MH-GC-treated mares had significantly lower IL-8 and TLR4 mRNA expression compared with MH-treated mares. In vitro LPS challenge significantly increased IL-8 but not TLR4 mRNA expression in BAL cells recovered from horses fed with MH, but not from the MH-GC treatment. In summary, PIM depletion attenuated clinical scores, reduced the alveolar migration of neutrophils, and decreased the expression of proinflammatory molecules in BAL cells of heaves horses, suggesting a proinflammatory role of PIMs in the development of airborne pathology.

lung inflammation; endotoxin; recurrent airway obstruction; horse; gadolinium chloride

HEAVES, OR RECURRENT AIRWAY OBSTRUCTION (RAO), is a chronic respiratory tract disease of horses commonly characterized by coughing, mucopurulent discharge from both nostrils, dyspnea, and exercise intolerance. Horses older than 7 years of age are most frequently affected, and higher incidence is observed in horses in the Northern hemisphere (12). Susceptible horses, upon exposure to high concentrations of mold and endotoxin in hay, show neutrophilic airway inflammation and bronchospasm (36–38, 46). Clinical signs are reversed within few days of placing the horses on pasture or improving ventilation in the stables (12, 40). The pathogenesis of equine heaves is comparable to asthma (20). Inhaled endotoxins are an important cause of human pulmonary disease, with the severity of pulmonary inflammation and clinical symptoms experienced by subjects exposed to organic dusts being related to the endotoxin concentration of the inhaled dust (50, 55). In addition, the severity of human asthma has been related to the level of endotoxin exposure (29, 39), suggesting that inhaled endotoxin may potentiate the inflammatory response to allergens in atopic subjects.

The most consistent cytological finding in bronchoalveolar lavage (BAL) fluid from horses with heaves is neutrophilic accumulation, making 50–70% of the total cell count (40, 42). Alveolar macrophages eliminate inhaled particles, and continuous phagocytosis of dust particles activates these cells to secrete IL-8, which attracts neutrophils into the alveoli (17). Although these neutrophils assist with the elimination of the inhaled particles, they may also cause tissue damage and airway gland hypersecretion leading to obstructive airway disease (8). It was previously reported that bronchiolar neutrophilia in heaves may be the consequence of secretion of chemotactic molecules by activated pulmonary macrophages (17). A more novel study (21) suggests that alveolar macrophages can contribute to the airway inflammation resulting from stabling in horses by the release of IL-8 and macrophage inflammatory protein-2 (MIP-2), but that the release of these chemokines is unlikely to be responsible for the marked airway neutrophilia observed in heaves.

Horses, among other species, have resident population of fully differentiated pulmonary intravascular macrophages (PIMs) (2, 4, 7, 44, 51). These and other PIM-containing species, and rats upon recruitment of PIMs under physiological stress, are highly susceptible to acute lung inflammation (19). The role of macrophages including PIMs in inflammation has been studied through their in vivo depletion with chemicals such as gadolinium chloride (GC) and clodronate (18, 48, 54). We have used GC, a heavy metal lanthanide, to deplete PIMs in cattle, horses and sheep (33, 47, 48). Since PIMs are reconstituted within 72–120 h after a single treatment with GC, multiple treatments with GC are required to maintain PIM depletion in long-term studies. The vascular location of PIMs makes them prime target of GC administered in jugular vein because they phagocytose most of the GC. GC kills macrophages through induction of apoptosis and not necrosis, thus minimizing any bystander effects of cell death (30). We and others have shown that PIM depletion attenuates the development of acute lung inflammation induced by bacteria and endotoxin administered intravenously (9, 18, 33). Equine PIMs endocytose intravenously infused Escherichia coli LPS and express proinflammatory cytokines (33). Recently, we showed that PIM depletion significantly reduced the expression of Toll-like receptors 4 and 9 (TLR4, TLR9) mRNA in the equine lung, implicating the crucial role of these cells in lung innate immune system (45, 49). Furthermore, their depletion inhibited LPS-induced pulmonary arterial hypertension and accumulation of inflammatory cells in the lungs (33). Interestingly, normal human and rat lungs lack PIMs, and...
Kupffer cells are the dominant vascular phagocytic cells (25, 56). However, rats were shown to recruit PIMs under physiological stress and consequently become more susceptible to endotoxin-induced lung injury and mortality (19, 53). There are data showing increased localization of radiotracers and presence of PIMs in the lungs of patients with liver disease, alluding to the possibility of PIMs in humans (23, 53).

The role of PIMs in lung inflammation induced by vascular endotoxins and bacteria is well established. However, to our knowledge, PIMs’ role in an airborne pulmonary pathology, such as heaves in horses, is unknown. We hypothesize that PIMs promote lung inflammation and clinical signs associated with heaves in horses. The objectives of this study were to investigate the hitherto unknown role of PIMs in equine heaves.

MATERIALS AND METHODS

Animals/total clinical score. All animal protocols were approved by the Animal Research Ethics Board of the University of Saskatchewan’s Committee on Animal Care and Supply. Nine light-type mares (Quarterhorse, Morgan, Arab; 400–600 kg), 10–20 yr of age, with a history of RAO in the last two years but free of any systemic disease, were studied. Mares were kept in outdoor paddocks with sheds and were fed either 2-yr-old moldy hay for 7 days to induce heaves or with alfalfa cubes for 3 wk to achieve remission. Clinical evaluations were performed before and after treatments and prior to all BALs and included measurements of heart rate, respiratory rate, and rectal temperature; evaluation of nasal flaring and nasal discharge; lung auscultation with and without a rebreathing bag; and evaluation of abdominal effort during breathing. Total clinical score (TCS) evaluation was adapted and modified from previous studies (6, 13, 31, 42); the intensities of the following clinical signs were scored (from normal to severely abnormal): abdominal effort (1–3), nasal flaring (1–3), coughing (0, 1), auscultation (1–3), and auscultation after a rebreathing bag (1–3) (Table 1).

Table 1. Clinical scoring system: definition of individual components of the clinical signs

<table>
<thead>
<tr>
<th>Grade 0</th>
<th>Normal</th>
<th>Abdominal Effort</th>
<th>Normal</th>
<th>Auscultation</th>
<th>Auscultation Following Rebreathing Bag</th>
<th>Coughing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>Normal</td>
<td>Normal to moderate abdominal component</td>
<td>Normal</td>
<td>Increased abnormal sounds</td>
<td>Severe weezes and crackles</td>
<td>No coughing</td>
</tr>
<tr>
<td>Grade 2</td>
<td>Slight to moderate flaring</td>
<td>Severe abdominal component</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>Severe continuous flaring during each respiration</td>
<td>Marked abdominal component</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In addition, the effect of the treatments on each clinical sign was evaluated by calculating the sum of scores for individual clinical signs from all mares.

Study design. The study design was a modified crossover resulting in all horses ultimately receiving all the treatments (Fig. 1). Horses were randomly assigned to two treatment groups with five mares in one group and the remaining four in the second group. The order of the treatments differed in each group. The treatments included 1) pasture/cubes no GC treatment (Cb), remission or non-heaves-induced horses; 2) moldy hay no GC treatment (MH), heaves-induced horses; 3) pasture/cubes and intravenous administration of GC (Cb-GC); and 4) moldy hay and intravenous administration of GC (MH-GC). Following induction of heaves, horses were fed with alfalfa cubes for 3 wk to achieve remission. In addition, after BALs were performed, horses were fed with cubes for 2 wk to allow reconstitution of the alveolar cells as well as recovery from the possible irritation that occurred during the BAL procedure. The MH-GC treatment comprised of a total of three treatments of GC; the first was administered 2 days prior to the MH exposure while the horses were still fed with cubes; the additional two GC treatments were given at 96-h intervals, and thus the third administration occurred 1 day prior to the BAL procedure. Whole blood was collected into EDTA tubes prior to each BAL for complete blood cells counts and differentials.

GC treatment. GC (GdCl3·6H2O, Sigma-Aldrich, Oakville, ON, Canada) was administered at a dose of 10 mg/kg intravenously in 500 ml of saline over 30 min, as was previously shown to cause a significant depletion of PIMs (34). Heart rates were recorded before and after the infusion.

Collection of total airborne dust and endotoxin evaluation. Total dust samples were collected for 1 h from each environmental setting.
(cubes and moldy hay) within 20–40 cm of the nostrils of the animals as previously described (10, 22). Gravimetric analysis for total dust [milligrams of dust/cubic meter of air (mg/m³)] was performed. Airborne endotoxin and dust endotoxin concentration [endotoxin units/cubic meter of air (EU/m³) and endotoxin units per milligram of dust (EU/mg)] were determined by the Limulus amoebocyte lysate assay (E. coli O55:B5; QCL-1000 kits; Cambrex BioScience, Walkersville, MD) (10).

**BAL procedure, cells, and fluid processing.** Horses were sedated with acepromazine (10 mg intravenously) followed by detomidine (5 mg intravenously) and butorphenol (5 mg intravenously). A 3-m videoscope (Olympus SIF-100, Olympus Canada, Markham, ON, Canada) was passed intranasally and directed to the right cranial lobar bronchus to instill 500 ml of warm solution of sodium chloride 0.9%, sodium bicarbonate 0.06%, pH 6.5, at 37°C. Following instillation, the fluid was aspirated and collected with a pump (Millipore GE 5KH33DN16X mechanical pump, Mississauga, ON, Canada). The same procedure was repeated for the left lung. In each side of the lungs, prior to instillation of the lavage fluid, 20 ml of 2% Lidocaine HCl were administered to reduce irritation and minimize coughing during the procedure. Total volumes of recovered BAL fluid (BALF) were recorded.

BAL fluid was centrifuged at 700 g for 10 min, aliquoted and supernatants were stored at −80°C until further analyses (Avant J-E Beckman Coulter Centrifuge). The cells were washed with RPMI-1640 medium (Invitrogen, Burlington, ON, Canada) containing 2 mM L-glutamine, 10 mM HEPEs, and 5 µg/ml gentamicin (Invitrogen) and resuspended in the same media supplemented with 10% horse serum (Invitrogen).

Total nucleated cell counts and concentration (number of cells/ml) were determined by hemocytometer. Differential cell counts were determined by examination of 300 consecutive leukocytes on a cytospin preparation stained with Giemsa.

**In vitro LPS challenge.** BAL cells (5 × 10⁶ cells/ml) were stimulated with E. coli LPS (1 µg/ml, strain 0127:B8) (Sigma-Aldrich, Oakville, ON, Canada) or media (control) for 6 h. Following incubation the cells were spun down at 2,000 g for 30 s. The supernatants and cells were collected separately and stored at −80°C until further analyses.

**RNA isolation and cDNA synthesis.** Total RNA was extracted from BAL cells by using the RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s instructions. Integrity and concentration of RNA were evaluated by use of a Thermo Scientific A260/280 ratio spectrophotometer. Integrity and concentration of RNA were determined by examination of 300 consecutive leukocytes on a cytospin preparation stained with Giemsa.

**Quantitative RT-PCR.** The cDNA from the BAL cells was used for quantitative RT-PCR analysis for the expression of IL-8 (GenBank accession no. AF062377), and TLR4 (GenBank accession no. AF062377), and TLR4 (GenBank accession no. AY005808) genes using the QuantiFast SYBR Green PCR kit (Qiagen). The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH; GenBank accession no. AF157626) was used as the reference housekeeping gene. The reactions were performed using the primer pairs; 5’-ATGATCTGAACGCTCTTCT-3’ and 5’-GGGACACCACGACAATAACTT-3’ for GAPDH; 5’-TCACATCTCTCAGGAG-3’ and 5’-TCTTGTTGGGGCAG-3’ for IL-8; 5’-GCCTGACA TCTCAGAGACTTCCATT-3’ and 5’-GGGACACCACGACAATAACTT-3’ for TLR4; and 5’-TCACATCTCTCAGGAG-3’ and 5’-TCTTGTTGGGGCAG-3’ for GAPDH. Real-time PCR analysis was performed by using the MX3005P LightCycler (Stratagene, La Jolla, CA). Results were standardized to control (Cb)-treated animals and given as relative fluorescence over control mRNA levels (fold difference) after correction for expression of GAPDH.

**Statistical analysis.** The investigator performing the clinical examinations was not blinded to the treatments but the investigators performing rest of the experiments (cells counts, mRNA levels, secondary challenges with LPS) were blinded to the treatments. All results were analyzed by use of computerized statistical software (Student Statistix version 8). The effect of drug, feed, and drug by feed interaction was analyzed by using the analysis of variance test with a mare block. Differences between groups were analyzed using all-pairwise comparison test. Differences were considered significant at P < 0.05.

## RESULTS

**Airborne dust and endotoxin concentrations.** The moldy hay environment, instigator of heaves clinical signs, contained a higher concentration of dust (3-fold), endotoxin (20-fold), and endotoxin per milligram of dust (7-fold) than the cubes environment (Table 2).

**Effect of PIM depletion on the development of clinical signs of heaves.** Total blood cell counts evaluated prior to each BAL were not different between the various treatments (data not shown). Total clinical scores before each BAL were significantly higher in the mares fed moldy hay compared with those given cubes or fed moldy hay and treated with GC. GC treatment alone had no effect on the clinical scores in mares fed with cubes. Whereas coughing, abdominal effort, auscultation, and auscultation after a rebreathing bag were affected by the treatments, nasal flaring was not affected and hence does not have an effect on the total clinical scores. Although Table 3 shows the effect of different treatments on each clinical sign and contribution of each clinical sign to the scoring system, Fig. 2 depicts the comparisons of total clinical scores of the different mares in the different treatments. The symbols in Fig. 2 are used such that each mare can be compared with itself.

**Effect of PIM depletion on inflammatory cell recruitment in heaves in horses.** Total cell concentration and neutrophil concentration in the BAL fluid were significantly higher in mares fed moldy hay compared with those given alfalfa cubes (Figs. 3 and 4, respectively). Although GC treatment did not affect the

<table>
<thead>
<tr>
<th>Clinical Sign</th>
<th>Possible Range (sum of mares)</th>
<th>Actual Sum of Mares</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abd. Eff. (1–3)</td>
<td>9–27</td>
<td>11</td>
</tr>
<tr>
<td>Nas. Flar. (1–3)</td>
<td>9–27</td>
<td>10</td>
</tr>
<tr>
<td>Coughing (0,1)</td>
<td>0–9</td>
<td>0</td>
</tr>
<tr>
<td>Ausc. (1–3)</td>
<td>9–27</td>
<td>10</td>
</tr>
<tr>
<td>Rebreath. (1–3)</td>
<td>9–27</td>
<td>10</td>
</tr>
</tbody>
</table>

**Treatments:** Cubes (Cb); Cubes + Gadolinium chloride (Cb-GC); Moldy hay (MH), Moldy hay + Gadolinium chloride (MH-GC). Clinical signs (from normal to severe): abdominal effort (Abd. Eff. 1–3), nasal flaring (Nas. Flar. 1–3), coughing (0, 1), auscultation (Ausc 1–3), and auscultation after a rebreathing bag (Rebreath. 1–3).

**Table 2. Airborne dust and endotoxin concentrations and endotoxin content in dust**

<table>
<thead>
<tr>
<th>Dust Type</th>
<th>Airborne Dust Concentration (mg/m³)</th>
<th>Airborne Endotoxin Concentration (ng/m³)</th>
<th>Endotoxin Content in Dust (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cubes</td>
<td>0.31</td>
<td>0.87</td>
<td>2.8</td>
</tr>
<tr>
<td>Moldy hay</td>
<td>0.94</td>
<td>18.25</td>
<td>19.4</td>
</tr>
</tbody>
</table>

**Table 3. Effects of treatments on the respiratory effort determined by calculating the sum of all mares for each clinical sign**
**DISCUSSION**

We provide the first evidence of an important role of PIMs in heaves in horses. This information is of interest because so far PIMs have been studied only in the context of lung inflammation associated with blood-borne bacteria and endotoxins whereas alveolar macrophages have been credited with airway pathophysiology such as heaves in horses.

Validation of the study design. We used a randomized crossover design to investigate the role of PIMs in heaves. This is a powerful design since each horse receives all treatments and acts as its own control. To achieve this, we triggered heaves in horses, as indicated by higher clinical scores, by feeding them moldy hay, and induced remission by feeding alfalfa cubes. In this study, we did not confirm remission of heaves with BAL or other relevant tests nor did we confirm reconstitution of PIMs in PIM-depleted lung. In fact, the percentage of neutrophils found in BALs from alfalfa cubes-fed mares (see Appendix Table A1) suggests presence of airway neutrophilia even after the 3-wk washout period. That does leave a possibility that mares did not achieve complete remission or reconstitution of PIMs that might blur the differences between groups resulting in a failure to identify anything but the most pronounced changes in end points, which actually would strengthen the significant differences that were found in this study. The reconstitution of PIMs can be determined through either examination of lung tissues collected after euthanasia or infusion of tracers. While considering the design of the study, we could not euthanize the horses after each treatment, and the infusion of tracers such as endotoxins or particulate material would activate PIMs and affect the obser-
vations. Nevertheless, we have previously shown reconstitution of PIMs in horses, cattle, and sheep within 72–120 h of a single treatment with GC (34).

The moldy hay environment in the present experiment contained slightly lower levels of airborne dust and airborne endotoxin compared with what has been reported for conventional stables (0.94 vs. 2.74 mg/m³, 18.2 vs. 19.76 ng/m³, respectively) but had a higher endotoxin concentration in the dust (19.4 vs. 7.57 ng/mg) (28). The alfalfa cubes environment in our study contained less endotoxin in dust than was reported for a pasture environment (2.8 vs. 4.85 ng/mg) (28), which is considered, to date, the ideal environment to achieve remission from heaves. Our observations are in agreement with previous findings that heaves is triggered by a dusty endotoxin-rich environment (36–38).

Depletion of PIMs attenuates the development of heaves. The role of PIMs in lung inflammation has previously been studied by depleting or inactivating these cells with agents such as clodronate (18) and tyloxapol (52). We used a previously validated protocol in which we found that a single treatment with GC induces apoptosis and causes significant depletion of horse PIMs within 72 h of the treatment (34). Because phagocytosis of GC is a prerequisite for its efficacy, vascular macrophages such as PIMs and Kupffer cells become prime targets following intravenous administration of GC (48). Preferential phagocytosis of blood-borne material by PIMs following injection into the jugular vein due to the “first pass” in the lung makes PIMs the primary target of action of GC with minimal damage to Kupffer cells (15, 47, 51). There is a possibility of leakage of GC from the vasculature into the alveolar space and interstitium, which will ultimately have an effect on the alveolar or interstitial macrophages. Although a single intravenous administration of GC did not affect alveolar or interstitial macrophages in rats (5), there was some GC-like material in the endosomes of alveolar macrophages and some signs of damage to macrophages following 36 weekly injections in lambs (47). A continuous decline of PIMs was previously observed at 72 h after GC administration (34). Therefore, to avoid a possibility of insufficient clearance of the GC by the PIMs and consequently its leakage and effect on Kupffer cells.

Fig. 4. A: Giemsa-stained cytospin preparation (×40) of BALF from 1 representative mare that received the 4 treatments. B: neutrophil concentration (mean) in the BALF collected from mares exposed to different treatments. Error bars represent SE. Different superscripts represent significant differences between treatments (P < 0.05). AM, alveolar macrophages; Eo, eosinophils; Neu, neutrophils; SMC, small mononuclear cells.
and alveolar macrophages, GC was administered every 96 h. The four injections of GC in our experiments altered neither the total numbers of alveolar macrophages nor the numbers of apoptotic cells in BAL fluid (data not shown). Therefore, it is unlikely that GC had an effect on the alveolar macrophages. The effect of GC on other cells in the lungs possessing phagocytic capabilities, such as neutrophils, dendritic cells, and endothelial cells was not assessed in this study. The consequences of inhibiting or depleting such cells would be similar to those seen in our study (i.e., attenuation of the inflammatory processes), because of a decrease in the chemotactic gradient and decrease in adhesion complexes expressed on the endothelium. Since we did not rule out the effect of GC on other cells in the lung that could contribute to the inflammatory process, we must take caution in ascribing the outcomes of this study solely to the depletion of the PIMs. However, given the phagocytic abilities of PIMs to scavenge most materials during their first pulmonary pass following administration into jugular vein, there may be very little effect on other blood-borne or tissue phagocytic cells.

The main objective of this study was to determine the effect of PIMs on the development of heaves in horses and to determine whether their depletion would attenuate the development of heaves. Although exposure to moldy hay resulted in higher clinical scores, GC-induced PIM depletion decreased the median clinical scores in heaves horses. Nasal flaring is often used in diagnosis and in clinical scoring systems to determine the severity of heaves (11, 13, 43), yet in the present study nasal flaring was hardly noticeable following different treatments and therefore did not affect the total clinical scores. However, we found that coughing was affected by the different treatments, which is in contrast to previous findings that this clinical sign cannot be assessed accurately by counting during brief periods because of its sporadic nature (41). The investigator performing the clinical examinations was not blinded to the treatments, which might raise the concern of biased results; nevertheless, the rest of the experiments (cell counts and mRNA levels) were carried out by an investigator blinded to the treatments. Therefore, although the issue of bias may have played a role on the clinical assessment of horses, it may not be of significance since the cellular and molecular results were parallel to the clinical scores. Similar to previous studies, we did not find any alterations in the hormones of mares following induction of heaves (14) and following GC treatments (34).

Mares exposed to moldy hay showed an increase in the total cell and neutrophil concentrations in the BAL fluid; however, interestingly, GC treatment kept the neutrophil concentration similar to the Cb (non-heaves-induced) levels, despite the exposure to moldy hay. This finding correlates with the above finding on the total clinical scores since clinical signs of heaves are linked to bronchoconstriction and accumulation of mucus and neutrophils in the airways (41). It is well documented that activation of PIMs by intravascular LPS enhances the migration of neutrophils into the lung (33, 48, 56). However, this is the first study reporting the effect of inhaled endotoxin and other irritating molecules on the equine PIMs that are embedded in lung vascular endothelium, indicating a potential important role of the PIMs in the development of an airborne pathology. One of the explanations for such involvement of PIMs could be the transport of an airborne pathogen or infectious agent or the transport of inflammatory mediators, produced by activated airway epithelium and macrophages, across the blood-air barrier, leading to their interaction with PIMs. Intratracheally instilled Mannheimia hemolytica leukotoxin was detected in PIMs of cattle, which suggests leukotoxin transfer across the blood-air barrier (57). The TLR4 expressed in equine PIMs will enable them to respond to endotoxin transported across the blood-air barrier through production of inflammatory mediators and recruitment of other inflammatory cells (49). Previously, transport of intra-alveolar TNF-α across the air-blood barrier has been shown in rats (24). Although the mechanism remains unclear, the data presented here show reduced lung inflammation in GC-treated moldy hay-fed horses, indicating activation of PIMs and their proinflammatory role in heaves.

TLR4 is believed to play an essential role in heaves pathogenesis due to the presence of endotoxin in the stable environment or the moldy hay in the present study. We found a significant increase in TLR4 mRNA in BAL cells of moldy hay-fed mares. Increase in TLR4 expression, which is a crucial receptor of LPS (1), would suggest an inflammatory response potentially triggered by endotoxin in moldy hay and also increased susceptibility to subsequent exposure to endotoxins. Recently, Berndt and colleagues (6) showed an increase in TLR4 mRNA expression in bronchial epithelial cells from heaves horses exposed to stable dust, which correlated with IL-8 mRNA expression as well as with neutrophilic airway inflammation. In addition, Ainsworth and colleagues (3) found a 3- and 10-fold increase in gene expression of IL-8 in epithelial and BALF cells in RAO-susceptible horses after 14 days of challenge exposure. Our study, showing reduced TLR4 and IL-8 mRNA expression in BAL cells obtained from GC-treated moldy hay-fed horses and reduced clinical scores in these horses, indicates a potential beneficial impact of depletion of PIMs in the pathogenesis of heaves.

Depletion of PIMs protects against a second endotoxin challenge. Horses with severe airway obstruction may develop secondary bacterial infection due to lower clearance capability (14, 26). In addition, horses suffering from heaves are repeat-

![Fig. 5. AM concentration (mean) in the BALF collected from the different treatment groups. Error bars represent SE. There were no significant differences between treatments (P < 0.05); columns sharing a similar letter are not significantly different.](image-url)
edly exposed to the same endotoxin-rich environment that
exaggerates lung inflammation. Therefore, we studied the im-
pact of GC treatment on the ability of BAL cells to respond to
an in vitro LPS challenge representing a secondary endotoxin
exposure.

Previous studies show strong chemotactic activity in the
BAL fluid partially attributed to the secretion of IL-8 and
MIP-2 by activated macrophages (17). In the present study, the
significant increase in IL-8 mRNA expression in BAL cells
isolated from mares after moldy hay exposure was even more

Fig. 6. IL-8 (A) and TLR4 (B) mRNA expression (mean of fold difference), and IL-8 (C) and TLR4 (D) mRNA expression (mean of fold difference) after 6 h
exposure to media/LPS, as determined by real-time PCR, in BAL cells recovered from mares exposed to different treatments. Error bars represent SE. Different
superscripts represent significant differences between treatments (P < 0.05); columns sharing a similar letter are not significantly different.

Table A1. Summary of BAL fluid volumes and cell counts (concentration, percentage)

<table>
<thead>
<tr>
<th>Bal volume, ml</th>
<th>Cb</th>
<th>Cb-GC</th>
<th>MH</th>
<th>MH-GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Cell concentration</td>
<td>0.31 ± 0.11</td>
<td>0.39 ± 0.11</td>
<td>1.06 ± 0.43</td>
<td>0.50 ± 0.12</td>
</tr>
<tr>
<td>Mean Neu concentration</td>
<td>10.33 ± 5.57</td>
<td>8.67 ± 1.98</td>
<td>38.78 ± 8.74</td>
<td>21.50 ± 7.49</td>
</tr>
<tr>
<td>Mean Neu concentration</td>
<td>75.17 ± 61.87</td>
<td>35.94 ± 17.58</td>
<td>600.03 ± 313.09</td>
<td>96.22 ± 39.51</td>
</tr>
<tr>
<td>Mean Neu concentration</td>
<td>61.67 ± 7.17</td>
<td>50.00 ± 2.43</td>
<td>36.67 ± 8.97</td>
<td>48.33 ± 6.43</td>
</tr>
<tr>
<td>Mean AM concentration</td>
<td>149.57 ± 30.40</td>
<td>201.60 ± 57.50</td>
<td>190.50 ± 37.00</td>
<td>278.58 ± 97.90</td>
</tr>
<tr>
<td>Mean Small Mono concentration</td>
<td>38.11 ± 10.42</td>
<td>61.67 ± 9.03</td>
<td>42.33 ± 11.54</td>
<td>50.56 ± 13.17</td>
</tr>
<tr>
<td>Small Mono concentration average</td>
<td>105.56 ± 36.71</td>
<td>223.61 ± 49.1</td>
<td>399.52 ± 159.36</td>
<td>234.27 ± 75.62</td>
</tr>
<tr>
<td>Mean Mast concentration</td>
<td>1.00 ± 0.50</td>
<td>4.89 ± 1.91</td>
<td>3.22 ± 1.07</td>
<td>3.67 ± 0.91</td>
</tr>
<tr>
<td>Mean Mast concentration</td>
<td>3.79 ± 1.91</td>
<td>19.94 ± 8.39</td>
<td>14.89 ± 3.96</td>
<td>13.98 ± 4.85</td>
</tr>
<tr>
<td>Mean %Eo</td>
<td>2.44 ± 1.67</td>
<td>1.89 ± 0.99</td>
<td>0.56 ± 0.29</td>
<td>1.44 ± 0.67</td>
</tr>
<tr>
<td>Mean Eo concentration</td>
<td>22.37 ± 19.11</td>
<td>13.78 ± 10.98</td>
<td>2.59 ± 1.37</td>
<td>8.75 ± 4.62</td>
</tr>
<tr>
<td>Mean %Apop</td>
<td>0.11 ± 0.11</td>
<td>0.11 ± 0.11</td>
<td>0.89 ± 0.42</td>
<td>0.22 ± 0.15</td>
</tr>
<tr>
<td>Mean Apop concentration</td>
<td>0.13 ± 0.13</td>
<td>0.12 ± 0.12</td>
<td>15.45 ± 12.57</td>
<td>0.89 ± 0.76</td>
</tr>
</tbody>
</table>

Results are presented as means ± SE. Values within a row with different superscripts (a and b) are significantly different (P < 0.05). BAL, bronchoalveolar
lavage; Neu, neutrophils; AM, alveolar macrophages; small mono, small mononuclear cells; Eo, eosinophils; apop, apoptotic cells.
pronounced after an in vitro exposure to LPS. Interestingly, the BAL cells from GC-treated moldy hay-fed mares did not respond to the in vitro LPS challenge, and reacted similar to cells recovered from horses in the control environment. The data suggest that GC-mediated effects on PIMs either inhibit the expression of chemotactic factors produced by bronchoalveolar inflammatory cells or inhibit the migration of cells responsible for such chemotactic activity from the vasculature, thus leaving only the inactivated resident cells in the bronchoalveolar space. Nevertheless, the results presented here show an effect of GC-induced PIM depletion on the ability of BAL cells to respond to secondary endotoxin challenge.

As opposed to IL-8, TLR4 mRNA expression did not seem to be affected by the in vitro LPS challenge. It is known that both the dose of LPS as well as the length of exposure determine the level of TLR4 expression in macrophage cells lines. It was previously shown that exposure of mouse peritoneal macrophages to a high dose of LPS (100 ng/ml) for 2.5 h severely reduced the mRNA expression of TLR4; however, the expression returned to the original level after a 20 h stimulation. In addition, TLR4 mRNA expression level after 24 h of LPS treatment was almost at the same level as that of nontreated cells (32). The LPS dose used in the present study was 1 μg/ml, which is higher than the “high dose” of the above cited report. Hence, it is possible that an overstimulation of the cells in this study initially caused a shutdown or downregulation in the TLR4 expression and after 6-h exposure to LPS the TLR4 expression was restored back to its original levels (i.e., without the LPS). Although GAPDH was always considered to be the preferred housekeeping gene, recent reports indicate that in some circumstances (including stimulation with LPS), it is more accurate to use a combination of GAPDH with another housekeeping gene than the use of this gene alone (16, 35). Nevertheless, we used solely this gene in the interpretations of the RT-PCR results.

We are cognizant of the limitations of our study conducted on clinical cases of heaves in horses. As discussed elsewhere in the manuscript, the crossover study design prevented us from gaining direct evidence of PIM depletion/reconstitution. We also were constrained by the number of BAL that could be performed on the animals. Although other studies have established that GC infused into jugular vein preferentially affects PIMs, we cannot rule out the possibility of some effect on other vascular cells. Lastly, similar to the complex interplay of inflammatory cells in human asthma, we need to perform additional studies on the biology of specific cells isolated from BALF. Even with these limitations, the study does provide the first evidence of a critical proinflammatory role for PIMs in equine heaves and may open new therapeutic avenues.

Taken together, the data contained in this study confirm our hypothesis and identify PIMs as important proinflammatory cells in the development of an airborne pathology such as heaves (Fig. 7). The data indicate the beneficial effects of depletion of PIMs in reduction of clinical and inflammatory signs of heaves. Interestingly, PIM depletion also appeared to reduce sensitivity of BAL cells to LPS stimulation through reduced expression of TLR4. Because of the similarity of heaves to human asthma, this study adds data highlighting the need to study the biology of PIMs in human pulmonary diseases.

Fig. 7. Suggested involvement of pulmonary intravascular macrophages (PIMs) in the development of an airborne pathology. Inhaled dust and LPS activate the AM and the epithelial lining of the alveolar space. These activated cells produce proinflammatory mediators (i.e., TNF-α, IL-1β, IL-8), which activate the PIMs and the endothelial cells to express adhesion molecules. LPS may also penetrate the vasculature through an injured blood-air barrier, bind to the CD14-TLR4 complex, and further activate the PIMs. Activated PIMs produce inflammatory mediators that activate both circulating neutrophils as well as the endothelium. Activated neutrophils express high levels of TLR4, adhere to the endothelium, and start migrating toward a chemotactic gradient into the alveolar space. At this stage there are high numbers of activated inflammatory cells in the alveolar space; because of the increase in the TLR4 expression these cells are more responsive to the inhaled LPS, which reactivates them, and they further contribute to the ongoing inflammatory process.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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