



Research paper

Systemic inflammation and priming of peripheral blood leukocytes persist during clinical remission in horses with heaves^{☆,☆☆}

Anouk Lavoie-Lamoureux^a, Guy Beauchamp^a, Sylvain Quessy^b, James G. Martin^c, Jean-Pierre Lavoie^{a,*}

^a Faculté de médecine vétérinaire, Département de Sciences cliniques, U. of Montreal, 3200 rue Sicotte St-Hyacinthe, Quebec, Canada

^b Faculté de médecine vétérinaire, Département de pathologie et microbiologie, U. of Montreal, 3200 rue Sicotte St-Hyacinthe, Quebec, Canada

^c Meakins-Christie Laboratories, McGill U., 3626 St-Urbain, Montreal, Quebec, Canada

ARTICLE INFO

Article history:

Received 24 October 2011

Received in revised form

22 December 2011

Accepted 23 January 2012

Keywords:

Heaves

Innate immunity

Neutrophils

Pro-inflammatory cytokines

Systemic inflammation

ABSTRACT

Objective: To compare innate immune responses of peripheral blood leukocytes from healthy and asymptomatic heaves-affected horses.

Animals: Heaves-affected horses ($n = 5-6$) and healthy controls ($n = 4-5$) kept under low dust environments (pasture or shavings and pellets).

Methods: Blood neutrophil and neutrophil-depleted cell populations were isolated using MACS system. Cells were incubated with or without bacterial products (lipopolysaccharide (LPS), 100 ng/mL and fMLP, 5 ng/mL, 5 h). Cytokine (IL-1 β , IL-8, TNF, IL-4, INF γ and IL-10) and receptor (TLR4) mRNA expression was assessed by qPCR. TNF concentration in culture supernatants and serum samples was assessed using equine specific ELISA. Apoptotic rate of resting and stimulated neutrophils was assessed by flow cytometry using AnnexinV and 7-AAD (18 h) and correlated with early pro-inflammatory cytokine expression in the same cells (5 h).

Results: Stimulation with bacterial-derived products resulted in overexpression of pro-inflammatory cytokines in both neutrophils (IL-1 β and TNF) and neutrophil-depleted leukocytes (IL-1 β and IL-8) from heaves-affected horses. Neutrophil survival (18 h) was associated with their early TNF expression, but not IL-8. Neutrophil-depleted leukocytes from these horses also had significantly increased basal TNF mRNA levels. Serum TNF concentration was also significantly higher in heaves-affected horses compared to healthy horses kept in similar environment.

Conclusions: Altered innate immune response to bacterial products is observable *ex vivo* in peripheral blood leukocytes from asymptomatic heaves-susceptible horses and is associated with high serum TNF concentration. It remains to be determined if this phenomenon is caused by intrinsic differences in innate immune responses or to cellular priming caused by systemic inflammation.

© 2012 Elsevier B.V. All rights reserved.

Abbreviations: BAL, bronchoalveolar lavage; CD, cluster of differentiation; fMLP, Nformyl-Met-Leu-Phe; LPS, lipopolysaccharide; mRNA, messenger RNA; NF- κ B, nuclear factor-kappaB; PAMPs, pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cells; PRR, PAMPs-related receptor; TLR, Toll-like receptor.

[☆] This manuscript represents a portion of a thesis submitted by A. L.-L. to the University of Montreal, Department of Clinical Sciences, as partial fulfillment of the requirements for a PhD of Science degree.

^{☆☆} Presented in abstract form by A. L.-L. at the 2008 International Conference of the American Thoracic Society, Toronto, Canada, May 2008.

* Corresponding author at: 3200 rue Sicotte, St-Hyacinthe, Québec, J2S 2M2, Canada. Tel.: +1 450 773 8521x8292; fax: +1 450 778 8102.

E-mail address: Jean-pierre.lavoie@umontreal.ca (J.-P. Lavoie).

1. Introduction

Heaves is a chronic airway disease of horses featuring episodes of reversible bronchoconstriction upon exposure to barn-derived organic dusts (Robinson, 2001). This disease shares with human asthma several pathophysiological features including reversible airway obstruction and inflammation. The inflammatory event in heaves is characterized by a neutrophilic recruitment in the airway lumen (Robinson, 2001). The etiopathogenesis of the disease remains unknown although studies have shown the presence of adaptative immune responses to moldy hay components (molds, fungi) through the recruitment of activated lymphocytes (McGorum et al., 1993), the expression of cytokines associated with the *helper* T lymphocytes subsets (Th) (Ainsworth et al., 2003; Debrue et al., 2005; Lavoie et al., 2001), as well as specific antibody production in the airways (Halliwell et al., 1993; Schmallenbach et al., 1998). Bacterial endotoxins and β -glucans also present in high concentration in exacerbating environments are propounded as contributing factors to heaves aetiology.

Whether innate immune responses are altered in heaves-affected horses and contribute to the development of aberrant acquired immune responses to barn-derived antigens remains to be determined. Limited evidences of altered innate immune responses are highlighted by the finding that inhalation of lipopolysaccharides (LPSs) induces a more pronounced airway neutrophilia and obstruction in horses with heaves compared to healthy controls (Pirie et al., 2001). LPS also enhances the airway obstruction and inflammation induced by aerosolized moldy hay suspensions (Nevalainen et al., 2002; Pirie et al., 2003a) or purified allergen extracts (Pirie et al., 2003b) in susceptible horses. Furthermore, lower doses of LPS are required to induce significant release of gelatinolytic metalloproteinase (MMP)-9 in the airways of heaves-affected horses compared to normal horses (Simonen-Jokinen et al., 2005). An *ex vivo* study also showed that alveolar macrophages from these horses exhibit enhanced pro-inflammatory cytokine expression compared to macrophages isolated from healthy controls following inhalational challenge with LPS (Laan et al., 2006). Lastly, a genetic component of heaves in certain breeds of horse (Marti et al., 1991) and polymorphisms and/or mutations in the TLR4 and CD14 genes are reported (Vychodilova-Krenkova et al., 2005; Werners et al., 2006).

Equine neutrophils are rapidly recruited to the airways after allergen challenge and endotoxin inhalation (within 5 h). These cells possess receptors for LPS (TLR4) as well as other specific microbial-derived molecule receptors including receptors for formylated peptides (FPR). These agonists induce equine neutrophil activation through degranulation, increased reactive oxygen species production, and pro-inflammatory cytokine and chemokine expression (Brazil et al., 1998; Joubert et al., 2001; Weiss and Evanson, 2002). In pathological conditions, neutrophils activation and survival is unnecessarily prolonged (Haslett, 1999), phagocytosis by tissue macrophages is inhibited (Brazil et al., 2005), and excessive release of toxic mediators contributes to tissue destruction. We hypothesized that innate immune responses of horses affected with heaves

are altered and contribute to disease exacerbation. To verify this hypothesis, we compared the *ex vivo* pro-inflammatory cytokine response of peripheral blood neutrophils from control and heaves-susceptible horses to bacterial-derived stimuli, LPS and synthetic formylated peptide fMLP (N-formyl-Met-Leu-Phe), and its functional consequence on cell survival *in vitro*. We chose to study peripheral blood neutrophils as airway neutrophils would be activated by transmigration and by the local inflammatory milieu which may differ between healthy and diseased animals. Also, we used asymptomatic heaves-affected horses as antigen challenge was reported to induce peripheral neutrophil activation (Marr et al., 1997, 2002). The pro-inflammatory responses of other leukocyte populations to microbial stimuli were also compared between groups as well as their expression of prototypical cytokines of the Th1, Th2 and immunosuppressive phenotypes as these could modulate innate immune responses.

2. Material and methods

2.1. Animals

Well characterized control horses and heaves-affected horses belonging to the Respiratory Cellular and Molecular Biology Laboratory research herd were studied. Control horses had no previous history of lung diseases and did not develop airway obstruction upon challenge with moldy hay in previous studies, in contrast to heaves-affected horses. Horses were kept in the same environment and regularly vaccinated and de-wormed. Sampling was performed when heaves-affected horses were unexposed to moldy hay/straw (clinical remission). During these periods, horses were housed in a well-ventilated barn (Study 2) or in pasture/paddocks (Study 1 and 3) and were fed with pellets and sweet feed twice a day for more than 2 months. Remission of heaves was assessed by clinical scoring as described earlier (Robinson et al., 2000). Briefly, a score from 0 to 4 was attributed to abdominal movement (0: no abdominal effort; 4: severe and marked abdominal movement) and nasal flaring (0: no flaring; 4: severe, continuous flaring). Abdominal and nostril scores were added for a maximal score of 8. Scores ≥ 5 indicated the presence of airway dysfunction. All experimental procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and were approved by the Animal Care Committee of the Faculty of Veterinary Medicine of the Université de Montréal.

2.2. Study design

Different groups of horses were studied over a 3-year period and clinical remission was assured by performing clinical scores at each time point. In Study 1, neutrophils were isolated from control horses ($n=4$) and heaves-affected horses ($n=6$) in order to evaluate their gene expression after culture with bacterial products. One heaves-affected horse was recruited as a control but was diagnosed with heaves after antigen challenge, explaining the inequality of animal number in the two groups. In Study 2, neutrophil-depleted leukocytes were obtained from

controls ($n=5$) and heaves-affected horses ($n=5$) for similar cell culture and gene expression experiments. Serum samples were also obtained from these horses at this time. In Study 3, neutrophils were isolated from heaves-affected horses ($n=5$) to perform gene expression analysis and apoptosis assay. Control horses were not studied at this time point as only the relationship between neutrophil cytokine expression and survival was of interest.

2.3. Positive and negative isolation of leukocyte populations from peripheral blood using immunomagnetic selection (MACS)

Blood was drawn from the jugular vein in heparinized sterile tubes (BD Vacutainer). Neutrophils were isolated as previously described using positive immunomagnetic selection (Joubert et al., 2001). Briefly, after sedimentation of blood for 1 h at room temperature, neutrophils were retrieved from the leukocyte-rich supernatant by sequential incubation with primary monoclonal antibody (#DH24A, VMRD Inc.) and secondary rat anti-mouse IgM antibody conjugated to paramagnetic microbeads (MACS, Miltenyi Biotec) before being loaded on a ferromagnetic LS separation column (MACS, Miltenyi Biotec).

Neutrophil-depleted leukocytes eluted in the negative fraction of cell isolation were pelleted and the remaining RBCs were removed by two treatments with 0.83% ammonium chloride (Sigma–Aldrich). The cells were washed twice in PBS (w/o $\text{Ca}^{2+}/\text{Mg}^{2+}$, pH 7.4, GIBCO) using low speed centrifugation (900 rpm, 15 min, GS-6R Centrifuge, Beckman) to remove platelets.

Cell counts and viability assessment were performed using a hemacytometer and Trypan blue dye (Invitrogen). Cytospins were prepared for differential counting (Cytospin2, Shadon) and stained with Protocol Hema 3 (Fisher Scientific). A minimum of 400 cells were counted.

2.4. Cell culture conditions for gene expression analysis

Neutrophils and neutrophil-depleted leukocytes were suspended at 5×10^6 cells/mL in culture medium RPMI 1640 supplemented with 10% heat inactivated low-endotoxin FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (all products from GIBCO) and cultured in 6- or 12-well plates (non-treated plastic, Ultident, Canada) in the presence or absence of Lipopolysaccharide from *Escherichia coli* 0:111B4 (100 ng/mL) and Nformyl-Met-Leu-Phe (fMLP, 5 ng/mL,

both from Sigma–Aldrich) for 5 h in a CO_2 incubator at 37°C . These concentrations were shown to induce pro-inflammatory gene expression by equine neutrophils (Joubert et al., 2001). Cell viability was assessed before homogenization in TRIzol[®] Reagent (10^7 cells/test, Invitrogen). Samples were kept at -80°C until further use.

2.5. RNA extraction and reverse transcription (RT)

RNA extraction was performed according to the manufacturer instructions using 3 steps nucleic acid precipitation with 0.2 volume chloroform, 1 volume isopropanol and ethanol. RNA pellets were air-dried, suspended in nuclease-free water and total RNA concentration and purity was evaluated using spectrophotometry (GeneQuantpro, Biochrom). Five hundred nanograms of total RNA was used for reverse transcription as described earlier (Lavoie-Lamoureux et al., 2010b) using Oligo(dT)_{12,18} primers (Invitrogen) and AMV reverse transcriptase (Roche Diagnostic). cDNA samples were stored at -20°C . For gene analysis using primers that did not span exon–intron boundaries (IL-4, INF γ and IL-10), RNA samples from neutrophil-depleted leukocytes were further treated with amplification Grade DNase1 (Invitrogen) following manufacturer's instructions before reverse transcription.

2.6. qPCR

Real-time PCR was performed using Quantitect SYBR Green PCR kit (QIAGEN) as described earlier (Lavoie-Lamoureux et al., 2010b) on the RotorGene 3000 thermal cycler (Corbett Research, Montreal-Biotec). PCR runs included a first 10 min denaturation step at 95°C followed by a maximum of 50 amplification cycles. Quantification was performed using optimized gene-specific standard curves made of serial dilutions ($10\times$) of PCR products (QIAGEN's Gel Extraction Kit) with reproducible efficiency coefficient over 90% and a calibrator sample in the target run. Absolute values were corrected relatively to ubiquitin expression used as a reference gene. Primers are listed in Table 1.

2.7. Apoptosis assays

The association between gene expression and cell survival was studied in neutrophils from heaves-affected horses during clinical remission ($n=5$). Briefly, isolated

Table 1
Primers used for gene expression quantification using qPCR.

	Sens (5' → 3')	Anti-sens (5' → 3')	References
IL-1 β	GAC TGA CAA GAT ACC TGT GGC CT	AGA CAA CAG TGA AGT GCA GCC T	Giguere and Prescott (1999)
IL-4	TCG TGC ATG GAG CTG ACT GTA	GCC CTG CAG ATT TCC TTT CC	Ainsworth et al. (2003)
IL-8	CTT TCT GCA GCT CTG TGT GAA G	GCA GAC CTC AGC TCC GTT GAC	Lavoie-Lamoureux et al. (2010b)
IL-10	TGC TAT GTT ACC TGG TCT TCC TGG	TAG TAG AGT CAC CGT CCT GGA TGC	Giguere and Prescott (1999)
INF γ	TCT TTA ACA GCA GCA CCA GCA A	GCG CTG GAC CTT CAG ATC AT	Ainsworth et al. (2003)
TNF α	CTT GTG CCT CAG CCT CTT CTC CTT C	CTTGTGCCTCAGCCTCTTCCTCTC	
TLR2	GAA TCA CTG GAC AAT GCC ACA TGC	AGA CAC CAG TGG GAT CAC AAG ACA	
TLR4	TGG GAC TCT GAT CCC AGC CAT	AGG TCC AGT TCC TTG GTT GAT GTG	Lavoie-Lamoureux et al. (2010b)
Ubiquitin	TAG CAG TTT CTT CGT GTC CGT	TGT AAT CGG AAA GAG TGC GG	Huang et al. (2007)

neutrophils were cultured with or without microbial products for 18 h in supplemented RPMI, as described above. Aliquots of the cells (10^7) cultured in separate plates were homogenized in TRIzol[®] Reagent after 5 h for mRNA analysis. Apoptosis was assessed after 18 h using APC-conjugated AnnexinV and 7-AAD (BD Biosciences) as markers of cell apoptosis and death, respectively, following the manufacturer's instructions. Briefly, neutrophils were washed twice in cold PBS (w/o) $\text{Ca}^{2+}\text{Mg}^{2+}$ and 100 μL aliquots were incubated in the dark with AnnexinV-APC and/or 7-AAD for 15 min at room temperature in $1 \times$ binding buffer (BD Biosciences). The cells were placed on ice after adding 400 μL of binding buffer and analysed immediately on a FACSCalibur flow cytometer (BD Biosciences). Data were acquired on 10 000 events and analysed using BD CellQuest[™] Pro Software (BD Biosciences). APC-AnnexinV fluorescence was detected in FL-4 channel (690 nm) and 7-AAD in FL-3 channel (650 nm). Fluorescence threshold levels were set using single-labelled neutrophils.

2.8. Determination of TNF concentrations in serum and culture supernatants by ELISA

TNF was used as a marker of systemic inflammation in serum samples (Higashimoto et al., 2008). Blood was collected by venipuncture in dry sterile Vacutainers tubes (BD Vacutainer). Blood samples were allowed to clot at room temperature and were centrifuged for 10 min at 1500 rpm (GS-6R Centrifuge, Beckman). Serum was collected and frozen at -80°C within 2 h of collection. TNF dosage in serum samples and culture supernatants were performed using equine TNF- α /TNFSF1A DuoSet[®] ELISA (RnD Systems) with minor modification to the manufacturer's protocol, as described elsewhere (Lavoie-Lamoureux et al., 2010a). Serum samples diluted in Bio-Plex Pro Isotyping Diluent (Bio-Rad) 1:4 to 1:256 and culture supernatants (1:1) in reagent diluent (bovine serum albumin 1% in PBS). The assay detection limit was 3.9 pg/ml.

2.9. Statistical analysis

Data obtain from PCR experiments were analysed using repeated-measures linear models using SAS v.9.1. (Cary, NC). Stimulation (resting versus LPS + fMLP) was treated as within-subject factors and the group (controls versus heaves) as a between-subject factor. Graphpad Prism 5 software (GraphPad Software Inc.) was used to perform all other analysis. Pearson's correlation analysis was used to assess the association between cytokine expression by neutrophils and their % of viability on \log_{10} -transformed data. One-way paired *t*-test was performed on \log_{10} -transformed neutrophils viability parameters as increased survival was expected with stimulation. A repeated measures linear model with stimulation and group treated as within- and between-subject factors, respectively, was used to analyse \log_{10} -transformed TNF concentrations in neutrophil and mononuclear cell culture supernatants. Serum TNF data were \log_{10} transformed and mean concentrations between controls and heaves-affected horses were compared using a two-tailed unpaired *t*-test. For statistical analysis purposes, a value of 3.9 pg/ml corresponding

to the assay detection threshold was assigned to samples with undetectable TNF in ELISAs. Graphs show raw data, expressed as mean \pm SEM. *p* Values less than 0.05 were considered statistically significant.

3. Results

3.1. Animals

Horses did not show signs of pulmonary dysfunction at times of sampling as assessed by clinical scoring: total scores were ≤ 4 for all horses in all experiments.

3.2. Enhanced pro-inflammatory gene expression by peripheral blood neutrophils and neutrophil-depleted leukocytes from horses with heaves

We first compared the innate immune response of neutrophils from healthy and heaves-affected horses by assessing pro-inflammatory cytokine expression. The purity of isolated neutrophils was $97.80 \pm 0.38\%$ and $98.00 \pm 0.17\%$ for controls and heaves respectively, and viability was $95.35 \pm 1.01\%$ and $97.08 \pm 1.27\%$, respectively. Neutrophils expressed significantly more IL-8 and TLR4 mRNA after stimulation with bacterial products for 5 h in both group of horses ($p < 0.05$, Fig. 1). However, only neutrophils from heaves-affected horses showed significant up-regulation of TNF mRNA and IL-1 β expression.

In order to evaluate if the differences observed in gene expression patterns were restricted to neutrophils, gene expression by neutrophil-depleted leukocytes from the two horse groups was also studied. This population consisted primarily of mononuclear cells (>85%) (Table 2). Cell viability was $99.02 \pm 0.08\%$ and $99.14 \pm 0.06\%$ for controls and heaves, respectively. As for neutrophils, the other leukocyte populations showed a different gene expression profile whether they were isolated from diseased or healthy horses. Indeed, stimulation with microbial products significantly enhanced the relative IL-1 β and IL-8 mRNA expression in neutrophil-depleted leukocytes from heaves-affected horses only ($p < 0.01$, Fig. 2A). Surprisingly, TNF expression was not increased by stimulation in neutrophil-depleted leukocytes from either group. This phenomenon was reproduced using different horses and stimulation with LPS only (100 ng/ml, $n = 4$, not shown) suggesting that mononuclear cells have different kinetics for TNF gene expression compared to neutrophils in horses. However, in these cells, baseline TNF expression was significantly higher in heaves-affected horses compared to

Table 2
Differential cell counts in neutrophil-depleted leukocytes.

	Controls		Heaves	
	Mean (%)	SEM	Mean (%)	SEM
Mononuclear cells	86.91	3.68	88.45	3.98
Lymphocytes	76.66	3.90	79.65	4.01
Monocytes	10.25	0.56	8.80	0.83
Granulocytes	13.09	3.68	11.55	3.98
Eosinophils	9.60	2.67	4.60	1.97
Neutrophils	3.49	1.90	6.94	2.43

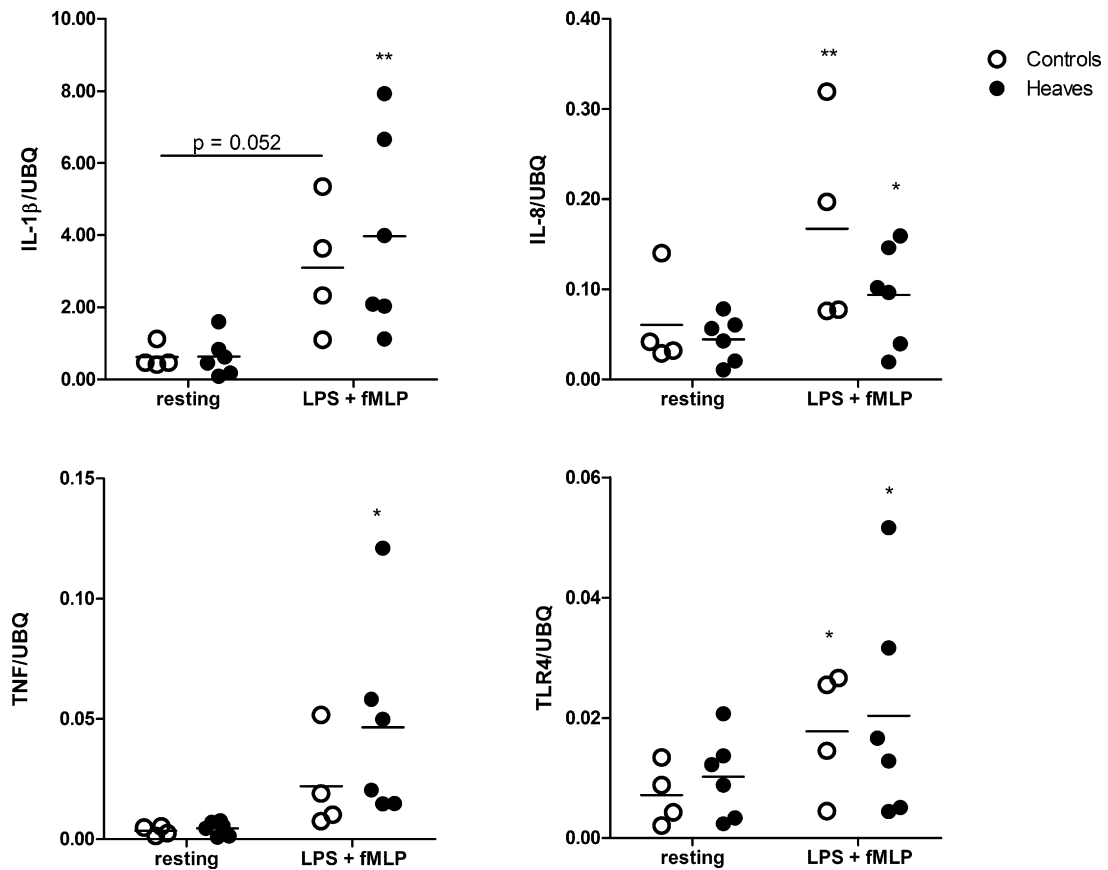


Fig. 1. Gene expression in neutrophils from control and heaves-affected horses after stimulation with microbial products. Neutrophils were isolated from the peripheral blood of healthy controls ($n=4$) and heaves-affected horses ($n=6$) during clinical remission. Cells were stimulated with microbial products (LPS 100 ng/ml and fMLP 5 ng/ml) for 5 h. Quantitative PCR for pro-inflammatory cytokines (IL-1 β , IL-8, TNF) and TLR4 was performed to compare the innate immune responses of neutrophils from both groups. * $p < 0.05$, ** $p < 0.01$.

control horses ($p < 0.05$, Fig. 2A). Another contrasting observation was that LPS + fMLP did not significantly induce TLR4 up-regulation in neutrophil-depleted leukocytes after culture for 5 h.

Prototypical cytokines of the Th1 (INF- γ), Th2 (IL-4) and immunosuppressive (IL-10) phenotypes were also studied (Fig. 2B). Differences in peripheral blood levels of these cytokines have been reported during both disease remission and exacerbation (Horohov et al., 2005). Here, we found no difference between groups in the expression of INF- γ and IL-10 in unstimulated cells. Stimulation with microbial products for 5 h did not affect INF- γ expression whereas a slight but significant decrease in IL-4 expression was observed in neutrophil-depleted leukocytes from heaves-affected horses only. LPS + fMLP significantly enhanced the expression of IL-10 mRNA in both groups.

3.3. TNF release by neutrophils and neutrophil-depleted leukocytes in culture supernatants

ELISA was used to evaluate if increased release of TNF in cell cultures from heaves-affected horses could attest of or contribute to paracrine cellular priming. As shown

in Fig. 3, TNF remained undetectable in most neutrophil supernatants (mean \pm SEM: 3.90 ± 0.00 pg/ml for all unstimulated neutrophil supernatants; 5.50 ± 1.60 pg/ml versus 12.38 ± 5.06 pg/ml in stimulated neutrophil supernatants from controls and heaves, respectively). Neither stimulation nor group effect reached statistical significance. All but one mononuclear cell culture supernatants had detectable levels of TNF. The concentration of TNF was 95.64 ± 32.64 pg/ml in controls versus 88.14 ± 26.98 pg/ml in heaves-affected horses unstimulated neutrophil-depleted leukocytes and 233.74 ± 57.84 pg/ml versus 415.20 ± 128.82 pg/ml in supernatants from LPS + fMLP-stimulated neutrophil-depleted leukocytes from controls and heaves-affected horses, respectively. In contrast to mRNA data, microbial stimulation resulted in significant release of TNF by neutrophil-depleted leukocytes from both groups and no difference in baseline TNF release was observed.

3.4. LPS + fMLP-induced TNF expression by neutrophils, but not IL-8, correlates with their survival

Microbial stimulation delays spontaneous apoptosis in neutrophils (Colotta et al., 1992) partly through

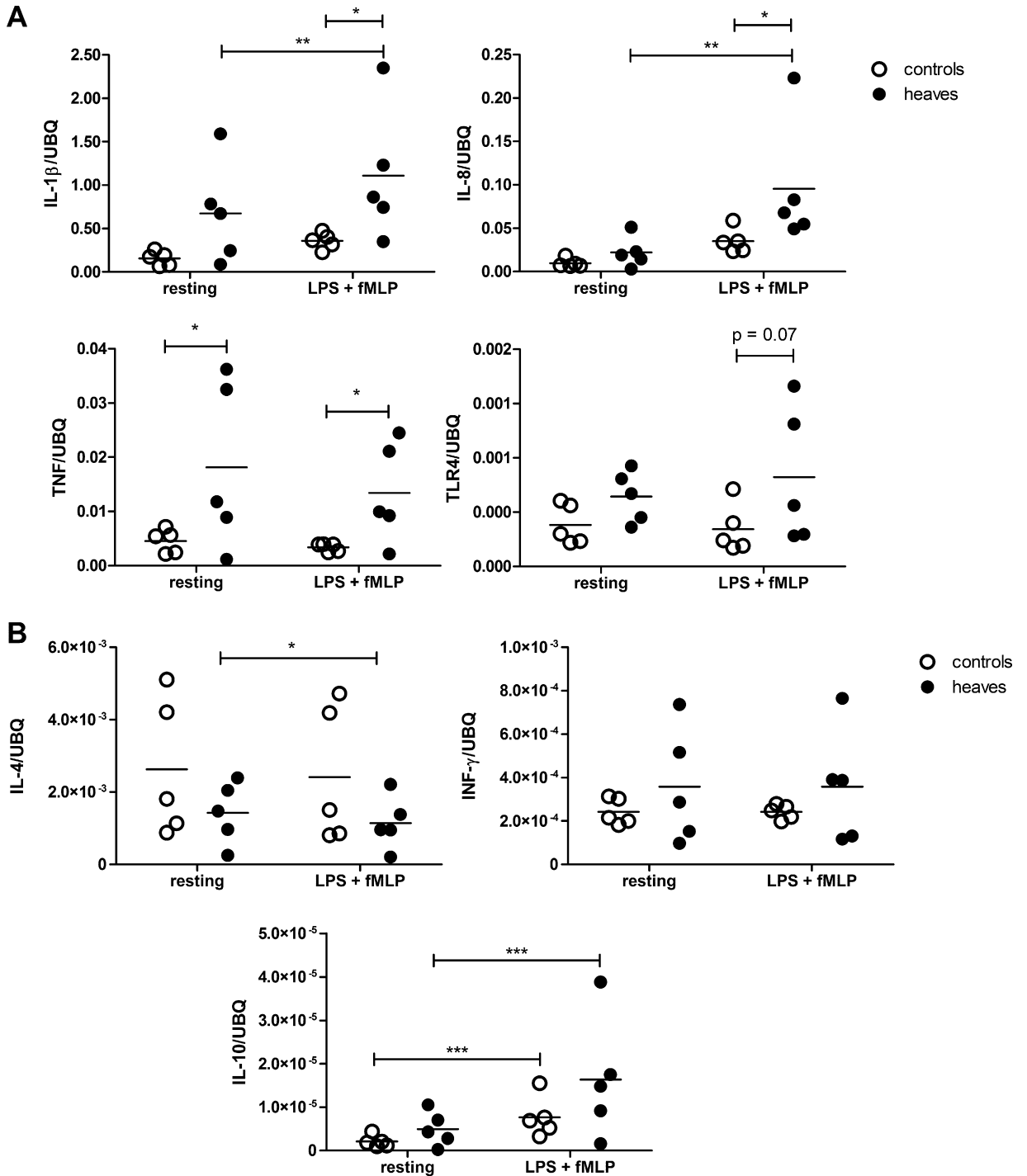


Fig. 2. Gene expression in neutrophil-depleted leukocytes from control and heaves-affected horses after stimulation with microbial products. Neutrophil-depleted leukocytes were retrieved from the negative fraction of neutrophil isolation using MACS in healthy controls ($n=5$) and heaves-affected horses ($n=5$) during clinical remission. Cells were stimulated with microbial products (LPS 100 ng/ml and fMLP 5 ng/mL) for 5 h. (A) Quantitative PCR for pro-inflammatory cytokines (IL-1 β , IL-8, TNF) and TLR4 was performed to compare the innate immune responses of neutrophil-depleted leukocytes from both groups. (B) INF γ , IL-4 and IL-10 were quantified as prototypical cytokines of the Th1, Th2 and immunosuppressive adaptative phenotypes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

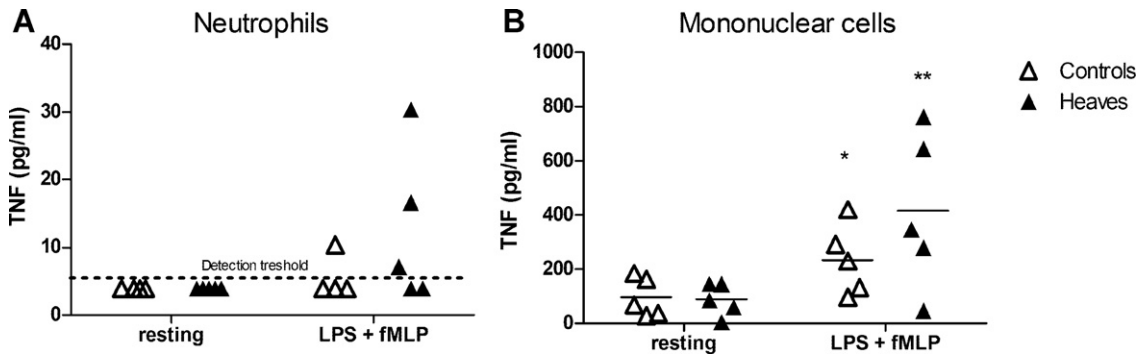


Fig. 3. TNF concentration in culture supernatants measured by ELISA. (A) TNF detection in neutrophil culture supernatants. Controls ($n = 4$), heaves-affected horses ($n = 5$). (B) TNF detection in mononuclear cell culture supernatants. Controls ($n = 5$), heaves-affected horses ($n = 5$). * $p < 0.05$, ** $p < 0.01$.

paracrine/autocrine release of pro-inflammatory cytokines (Bureau et al., 2000; Colotta et al., 1992; Cowburn et al., 2004). As expected, we found an effect of LPS + fMLP on neutrophil's survival after culture for 18 h (Fig. 4). Increased % of live cells was observed in stimulated neutrophils from 4 of the 5 horses compared to non-stimulated neutrophils ($21.80 \pm 6.84\%$ versus $30.01 \pm 7.63\%$, $p = 0.06$, Fig. 4B). A significantly decreased % of dead cells ($4.14 \pm 0.55\%$ versus $2.69 \pm 0.53\%$, $p < 0.01$) was observed in stimulated compared to non-stimulated neutrophils whereas the decreased % of apoptotic cells did not reach significance ($73.97 \pm 7.23\%$ versus $67.13 \pm 7.61\%$). Fig. 4C

shows that the % of viable cells after 18 h was significantly correlated with TNF expression after 5 h, but not with IL-8, in stimulated neutrophils. This association was not found in unstimulated neutrophils (not shown).

3.5. Increased serum TNF concentration in asymptomatic heaves-affected horses compared to controls

The presence of systemic inflammation in asymptomatic heaves-affected horses could also contribute to peripheral blood leukocyte priming. We thus compared serum TNF concentration as a marker of systemic inflam-

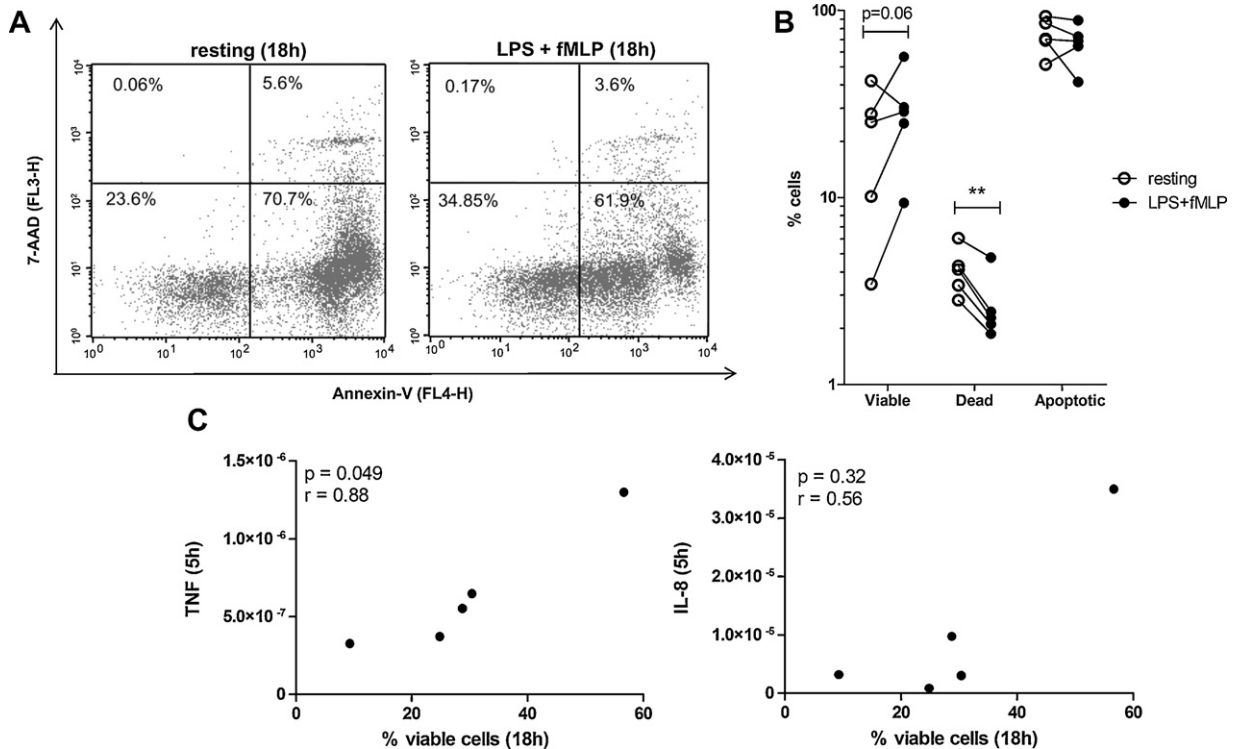


Fig. 4. The enhanced survival of peripheral blood neutrophils from asymptomatic heaves-affected horses cultured with microbial products is strongly associated with their expression of TNF. (A) Representative scatter plots showing flow cytometry analysis of the rate of spontaneous apoptosis in neutrophils with or without stimulation. Upper left: necrosis, upper right: death, lower left: viable, lower right: apoptosis. (B) Effects of stimulation with microbial products (LPS 100 ng/ml and fMLP 5 ng/ml) on neutrophils viability parameters after 18 h in heaves-affected horses ($n = 5$). (C) Correlation between absolute TNF or IL-8 mRNA expression (ng/ μ l) by stimulated neutrophils (5 h) and the percentage (%) of viable cells after 18 h ($n = 5$). ** $p < 0.01$.

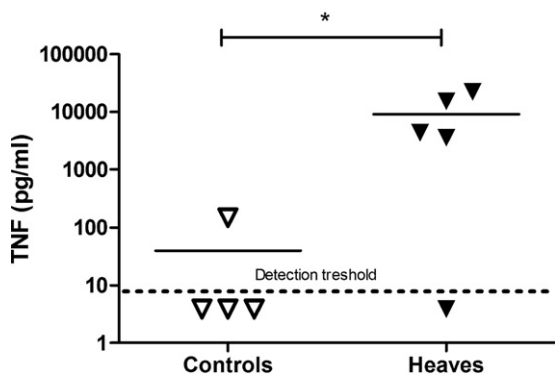


Fig. 5. Serum TNF concentration in controls ($n=4$) and heaves-affected horses ($n=5$) during clinical remission. A minimum value a 3.9 pg/ml was assigned to samples with undetectable TNF concentrations. * $p < 0.05$.

mation in normal and heaves-affected horses during clinical remission (Fig. 5). TNF was detected in 4 over 5 horses affected with heaves and 1 over 4 controls. The mean TNF concentration was significantly higher in heaves-affected horses compared to controls ($9\,125.34 \pm 4\,181.13$ versus 40.18 ± 36.28 pg/ml, respectively).

4. Discussion

In the present study, we investigated the innate immunoregulatory functions of leukocytes from horses with heaves by comparing the response of peripheral blood neutrophils and non-neutrophil leukocyte populations from diseased and healthy horses to common bacterial-derived products. We found that in horses with heaves, both cell populations were primed for enhanced pro-inflammatory responses to LPS and formylated peptides *ex vivo*. Stimulated neutrophils from asymptomatic heaves-affected horses had up-regulated IL-1 β and TNF expression; the latter was strongly associated with their survival rates. Neutrophil-depleted leukocytes from these horses also had up-regulated pro-inflammatory cytokine (IL-1 β and IL-8) expression following stimulation and showed high basal TNF mRNA levels compared to controls, suggestive of cellular priming or activation. This led us to investigate whether systemic inflammation was involved in this phenomenon. Indeed, we found significantly higher TNF concentrations in serum samples from asymptomatic heaves-affected horses compared to normal horses kept in the same environment. Taken together, these results suggest that systemic inflammation persist in heaves-affected horses despite antigenic avoidance and that this may contribute to prime peripheral blood neutrophils and mononuclear cells to subsequent microbial stimulation.

Our present and previous findings (Joubert et al., 2008; Lavoie-Lamoureux et al., 2010b) reveal that gene expression is not altered in resting peripheral blood neutrophils of asymptomatic heaves-susceptible horses compared to normal horses. Neutrophil aberrant activation in susceptible horses appears to be agonist-specific since it is observable using microbial products (LPS + fMLP), but not IL-4 (Lavoie-Lamoureux et al., 2010b), suggesting that altered

innate immune responses to PAMPS may be associated with heaves pathology. We found here that mononuclear cells also present an exaggerated response to these stimuli in horses with heaves. These results are in accordance with, and extend the previous findings by Laan et al. that alveolar macrophages of LPS-challenged heaves-affected horses expressed higher IL-8 and IL-1 β mRNA levels than those from normal horses (Laan et al., 2006). Taken together, these results suggest that in heaves, peripheral blood leukocytes are prone to have exaggerated responses following activation with microbial stimuli and therefore the differential cytokine expression of pulmonary leukocytes may not solely reflect the influence of the local inflammatory milieu. The contribution of innate immune stimuli to this phenomenon, as opposed to adaptative responses, is further demonstrated in Laan et al.'s study by the overexpression of inflammatory cytokine by heaves-susceptible horses in response to hay dust suspension, but not after stimulation with purified antigen extracts. It has been shown that LPS may possibly participate to the development of airway inflammation and obstruction in heaves-affected horses in response to moldy hay or purified allergens (Pirie et al., 2003a,b). Leukocyte exaggerated response to LPS in heaves may also contribute to increase the horses' sensitivity to LPS inhalational challenges, as reported by Pirie et al. (2001). In asthmatic individuals, the nature of the agonist is also pivotal in identifying dysregulated cell activation as aberrant cytokine profiles were found in LPS-stimulated but not phytohemagglutinin (PHA)-stimulated peripheral blood leukocytes (Bettiol et al., 2000).

Interestingly, in human asthmatics, a potentiation of LPS responses by peripheral blood neutrophils and peripheral blood mononuclear cells (PBMCs) is also described (Baines et al., 2010; Chun et al., 2010). This phenomenon was not associated with an increased expression of TLR4 by these cells. Similarly, we did not find difference between groups of horses in TLR4 mRNA expression by neutrophils and neutrophil-depleted leukocytes. However, we found that the expression of pro-inflammatory cytokines is more strongly associated with TLR4 expression in neutrophils than in neutrophil-depleted leukocytes (not shown). These differences are likely due to the lack of TLR4 and TNF up-regulation after microbial stimulation for 5 h in neutrophil-depleted leukocytes, as opposed to neutrophils. However it may imply that increase in TLR4 expression by stimulated neutrophils contribute to their enhanced pro-inflammatory cytokine expression induced by microbial products. These results also suggest that an increased proportion of neutrophil in sputum or BAL samples from asthmatics or heaves-affected horses, respectively, would contribute to the associated increased expression of innate immune molecules. In support for this hypothesis, Simpson et al. showed that pulmonary inflammation in patients with neutrophilic asthma was associated with increased mRNA expression of receptors related to innate immune responses, particularly to LPS (TLR4, TLR2 and CD14), as well as up-regulated expression of signature cytokines of innate immunity (IL-1 β , IL-8 and TNF) when compared with different inflammatory phenotypes (Simpson et al., 2007). Similar findings are reported in

heaves-affected horses as their bronchoalveolar lavage (BAL) cells expressed higher TLR4 and IL-8 mRNA levels compared to control horses exposed to similar environment (Ainsworth et al., 2006).

Possible mechanisms implicated in differential intrinsic innate immune responses include aberrant receptors expression or signaling pathways. Polymorphisms in CD14 (Lachheb et al., 2008), TLR2 (Eder et al., 2004) and TLR4 (Fageras Bottcher et al., 2004) genes have been associated with the development of asthma in children or to LPS responsiveness (Arbour et al., 2000). Importantly, gene–environment interactions seem to be pivotal for the determination of outcomes related to asthma and allergic sensitization in humans (Eder et al., 2004; Vercelli, 2010) and in horses (Eder et al., 2001; Marti et al., 1991). Hence, it can be postulated that horses bearing alteration in genes related to innate immunity could be at higher risk for the development of heaves if housed in certain environments. Of note, TLR4 and CD14 SNPs were reported in certain breeds of horses (Vychodilova-Krenkova et al., 2005), but have not yet been associated with altered LPS responsiveness using equine whole blood leucocytes (Werners et al., 2006). Alternatively, as the expression of the anti-inflammatory cytokine IL-10 was not significantly higher in neutrophil-depleted leukocytes from heaves-affected horses compared to controls, we can infer that cellular activation by microbial stimuli in heaves is prone towards a global pro-inflammatory balance. This altered balance also characterized the LPS response of pulmonary cells from COPD patients, a human neutrophilic airway disease, mainly through a dysregulated TNF/IL-10 expression ratio (Hackett et al., 2008). Nonetheless, from our results, it may be hypothesized that heaves-susceptible horses possess a hyperresponsive phenotype concerning their inflammatory response to PAMPS and that this would allow antigen presenting cell activation upon inhalation of endotoxins whereas hyporesponsive phenotypes would not (Iwasaki and Medzhitov, 2004). The concomitant presence of allergens in inhaled air would favour the process of allergic sensitization in these horses.

One consequence of inappropriate neutrophil activation is the inhibition of spontaneous apoptosis and their prolonged release of toxic mediators at inflammatory sites. In heaves-affected horses, resolution of inflammation corresponds in time with pulmonary neutrophils apoptosis and their removal by macrophages (Brazil et al., 2005). Neutrophil apoptosis can be regulated by pro-inflammatory cytokines in autocrine and paracrine manners as well as by microbial-derived stimuli. Therefore, we explored the relationship between neutrophil cytokine expression and their survival as a consequence of dysregulated innate immune responses. We found that microbial-induced TNF expression by neutrophils from heaves-affected horses, unlike IL-8, was strongly associated with their survival *ex vivo*. Although both cytokines are up-regulated in the lungs of these horses after hay exposure (Franchini et al., 2000; Giguere et al., 2002), and may contribute to neutrophil activation and recruitment to the lungs, the autocrine TNF production by neutrophils may particularly contribute to sustain cellular activation and survival in the airways. This hypothesis is in agreement

with Bureau et al.'s proposal that high NF- κ B activity and release of IL-1 β and TNF by airway neutrophils prevent apoptosis and contribute to chronic airway inflammation in horses with heaves (Bureau et al., 2000). In human neutrophils, it has been shown that LPS induces early TNF expression which contributes to its anti-apoptotic effect (Keel et al., 1997). LPS induced-TNF also enhances the late IL-8 secretion by human neutrophils (Cassatella, 1995), which is associated with TNF-induced neutrophils survival (Cowburn et al., 2004), in contrast to the early IL-8 expression evaluated in the present study (5 h). Other mechanisms implicated in TNF-induced survival include activation of NF- κ B, phosphoinositide-3-kinase (PI3-K) and ERK1/2 (Cowburn et al., 2004; Kilpatrick et al., 2006) and the transcription of the survival factor myeloid cell leukemia-1 (Mcl-1) which inhibits in the intrinsic apoptosis pathway (Luo and Loison, 2008). Also, it is known that TNF delays apoptotic neutrophils phagocytosis by macrophages *in vivo* (Borges et al., 2009).

We hypothesized that systemic inflammation could account for the priming of peripheral blood leukocytes in asymptomatic heaves-susceptible horses. Systemic inflammation is indeed a feature of chronic inflammatory diseases including obesity (Park et al., 2005), cancer (Deans and Wigmore, 2005), rheumatoid arthritis (RA) (Snow and Mikuls, 2005), inflammatory bowel diseases (Niederau et al., 1997), asthma and chronic obstructive pulmonary disease (COPD) (Gan et al., 2004; Higashimoto et al., 2008). The presence of inflammatory molecules such as cytokines or acute phase proteins (APP) in the systemic circulation may directly modulate leukocytes activation state. TNF is a cytokine found to be up-regulated in the blood of patients with asthma (Barnes, 2003; Higashimoto et al., 2008) along with other acute phase proteins. We previously showed that serum TNF concentrations were high in heaves-affected horses during exacerbation (Lavoie-Lamoureux et al., 2010a). Here we report for the first time that markers of systemic inflammation persist in asymptomatic heaves-affected horses. Activated PBMCs could represent a source of TNF as its mRNA expression was highly expressed at baseline in heaves-susceptible horses. However, we did not find agreement between the mRNA expression and protein content of PBMCs culture supernatants suggesting that the secretion TNF is tightly regulated. Post-transcriptional regulation of TNF is indeed well described in other species (Lai et al., 1999; Seko et al., 2006) and occurs in peripheral blood leukocytes in horses (Vick et al., 2007). Interestingly, in a murine model of thermal injury, systemic priming of neutrophils involves p38 mitogen-activated protein kinase (MAPK) phosphorylation and activation of both NF- κ B and activator-protein (AP)-1 through TNF-dependant and -independent pathways (Chen et al., 2006). It remains to be determined whether similar mechanisms are involved in systemic leukocytes priming in heaves-affected horses and contribute to enhance their pro-inflammatory cytokine expression in response to microbial stimuli.

5. Conclusion

We found that peripheral blood leukocytes from heaves-susceptible horses in clinical remission of the

disease show exaggerated pro-inflammatory cytokine responses to common bacterial-derived products. These altered innate immune responses are associated with systemic inflammation in these horses. It may be postulated that horses with heaves have a susceptible genotype/phenotype for enhanced responses to PAMPs and that low levels of microbial products inhalation in low dust environments may contribute to sustain low-grade local and systemic inflammation despite the absence of airway obstruction. Alternatively, heaves may underlie a systemic disease component which may modulate leukocytes innate immune responses and contribute to disease exacerbation. Systemic inflammation could also be a consequence of airway remodeling as the latter is a stable process in heaves (Leclere et al., 2011) and involves inflammatory mediator release by proliferating cells. Future studies are required to identify the mechanisms involved in systemic priming of leukocytes and other systemic inflammation markers in heaves-affected horses.

Conflict of interest

The author declares that there is no conflict of interest.

Acknowledgements

The authors would like to acknowledge Mathilde Leclère, Marie-Laure Cortes, Josiane Lefebvre-Lavoie and Julie Dauvillier for their help related to blood sample collection and processing as well as Annie Desrosiers for her help related to flow cytometry. This study was supported by the Natural Sciences and Engineering Research Council of Canada and the Frederick Banting and Charles Best Canada Graduate Scholarships (A.L.-L.).

References

- Ainsworth, D.M., Grunig, G., Matychak, M.B., Young, J., Wagner, B., Erb, H.N., Antczak, D.F., 2003. Recurrent airway obstruction (RAO) in horses is characterized by IFN- γ and IL-8 production in bronchoalveolar lavage cells. *Vet. Immunol. Immunopathol.* 96, 83–91.
- Ainsworth, D.M., Wagner, B., Franchini, M., Grunig, G., Erb, H.N., Tan, J.Y., 2006. 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.
- Arbour, N.C., Lorenz, E., Schutte, B.C., Zabner, J., Kline, J.N., Jones, M., Frees, K., Watt, J.L., Schwartz, D.A., 2000. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat. Genet.* 25, 187–191.
- Baines, K.J., Simpson, J.L., Bowden, N.A., Scott, R.J., Gibson, P.G., 2010. Differential gene expression and cytokine production from neutrophils in asthma phenotypes. *Eur. Respir. J.* 35, 522–531.
- Barnes, P.J., 2003. Cytokine-directed therapies for the treatment of chronic airway diseases. *Cytokine Growth Factor Rev.* 14, 511–522.
- Bettl, J., Bartsch, P., Louis, R., De Groot, D., Gevaerts, Y., Louis, E., Malaise, M., 2000. Cytokine production from peripheral whole blood in atopic and nonatopic asthmatics: relationship with blood and sputum eosinophilia and serum IgE levels. *Allergy* 55, 1134–1141.
- Borges, V.M., Vandivier, R.W., McPhillips, K.A., Kench, J.A., Morimoto, K., Groshong, S.D., Richens, T.R., Graham, B.B., Muldrow, A.M., Van Heule, L., Henson, P.M., Janssen, W.J., 2009. TNF α inhibits apoptotic cell clearance in the lung, exacerbating acute inflammation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 297, L586–L595.
- Brazil, T.J., Dagleish, M.P., McGorum, B.C., Dixon, P.M., Haslett, C., Chilvers, E.R., 2005. Kinetics of pulmonary neutrophil recruitment and clearance in a natural and spontaneously resolving model of airway inflammation. *Clin. Exp. Allergy* 35, 854–865.
- Brazil, T.J., Rossi, A.G., Haslett, C., McGorum, B., Dixon, P.M., Chilvers, E.R., 1998. Priming induces functional coupling of N-formyl-methionyl-leucyl-phenylalanine receptors in equine neutrophils. *J. Leukoc. Biol.* 63, 380–388.
- Bureau, F., Delhalle, S., Bonizzi, G., Fievez, L., Dogne, S., Kirschvink, N., Vanderplassen, A., Merville, M.P., Bours, V., Lekeux, P., 2000. Mechanisms of persistent NF- κ B activity in the bronchi of an animal model of asthma. *J. Immunol.* 165, 5822–5830.
- Cassatella, M.A., 1995. The production of cytokines by polymorphonuclear neutrophils. *Immunol. Today* 16, 21–26.
- Chen, L.W., Huang, H.L., Lee, I.T., Hsu, C.M., Lu, P.J., 2006. Thermal injury-induced priming effect of neutrophil is TNF- α and P38 dependent. *Shock* 26, 69–76.
- Chun, E., Lee, S.H., Lee, S.Y., Shim, E.J., Cho, S.H., Min, K.U., Kim, Y.Y., Park, H.W., 2010. Toll-like receptor expression on peripheral blood mononuclear cells in asthmatics; implications for asthma management. *J. Clin. Immunol.* 30, 459–464.
- Colotta, F., Re, F., Polentarutti, N., Sozzani, S., Mantovani, A., 1992. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* 80, 2012–2020.
- Cowburn, A.S., Deighton, J., Walmsley, S.R., Chilvers, E.R., 2004. The survival effect of TNF- α in human neutrophils is mediated via NF- κ B-dependent IL-8 release. *Eur. J. Immunol.* 34, 1733–1743.
- Deans, C., Wigmore, S.J., 2005. Systemic inflammation, cachexia and prognosis in patients with cancer. *Curr. Opin. Clin. Nutr. Metab. Care* 8, 265–269.
- Debrue, M., Hamilton, E., Joubert, P., Lajoie-Kadoch, S., Lavoie, J.P., 2005. 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.
- Eder, C., Curik, I., Brem, G., Cramer, R., Bodo, I., Habe, F., Lazary, S., Solkner, J., Marti, E., 2001. Influence of environmental and genetic factors on allergen-specific immunoglobulin-E levels in sera from Lipizzan horses. *Equine Vet. J.* 33, 714–720.
- Eder, W., Klimecki, W., Yu, L., von Mutius, E., Riedler, J., Braun-Fahrlander, C., Nowak, D., Martinez, F.D., 2004. Toll-like receptor 2 as a major gene for asthma in children of European farmers. *J. Allergy Clin. Immunol.* 113, 482–488.
- Fageras Botcher, M., Hmani-Aifa, M., Lindstrom, A., Jenmalm, M.C., Mai, X.M., Nilsson, L., Zdolsek, H.A., Bjorksten, B., Soderkvist, P., Vaarala, O., 2004. A TLR4 polymorphism is associated with asthma and reduced lipopolysaccharide-induced interleukin-12(p70) responses in Swedish children. *J. Allergy Clin. Immunol.* 114, 561–567.
- Franchini, M., Gill, U., von Fellenberg, R., Bracher, V.D., 2000. 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.
- Gan, W.Q., Man, S.F., Senthilselvan, A., Sin, D.D., 2004. Association between chronic obstructive pulmonary disease and systemic inflammation: a systematic review and a meta-analysis. *Thorax* 59, 574–580.
- Giguere, S., Prescott, J.F., 1999. Quantitation of equine cytokine mRNA expression by reverse transcription-competitive polymerase chain reaction. *Vet. Immunol. Immunopathol.* 67, 1–15.
- Giguere, S., Viel, L., Lee, E., MacKay, R.J., Hernandez, J., Franchini, M., 2002. Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. *Vet. Immunol. Immunopathol.* 85, 147–158.
- Hackett, T.L., Holloway, R., Holgate, S.T., Warner, J.A., 2008. Dynamics of pro-inflammatory and anti-inflammatory cytokine release during acute inflammation in chronic obstructive pulmonary disease: an *in vivo* study. *Respir. Res.* 9, 47.
- Halliwell, R.E., McGorum, B.C., Irving, P., Dixon, P.M., 1993. Local and systemic antibody production in horses affected with chronic obstructive pulmonary disease. *Vet. Immunol. Immunopathol.* 38, 201–215.
- Haslett, C., 1999. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am. J. Respir. Crit. Care Med.* 160, S5–S11.
- Higashimoto, Y., Yamagata, Y., Taya, S., Iwata, T., Okada, M., Ishiguchi, T., Sato, H., Itoh, H., 2008. Systemic inflammation in chronic obstructive pulmonary disease and asthma: similarities and differences. *Respirology* 13, 128–133.
- Horohov, D.W., Beadle, R.E., Mouch, S., Pourciau, S.S., 2005. Temporal regulation of cytokine mRNA expression in equine recurrent airway obstruction. *Vet. Immunol. Immunopathol.* 108, 237–245.
- Huang, H., Lavoie-Lamoureux, A., Moran, K., Lavoie, J.P., 2007. IL-4 stimulates the expression of CXCL-8, E-selectin, VEGF, and inducible nitric oxide synthase mRNA by equine pulmonary artery endothelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* 292, L1147–L1154.

- Iwasaki, A., Medzhitov, R., 2004. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 5, 987–995.
- Joubert, P., Cordeau, M.E., Boyer, A., Silversides, D.W., Lavoie, J.P., 2008. Cytokine expression by peripheral blood neutrophils from heaves-affected horses before and after allergen challenge. *Vet. J.* 178, 227–232.
- Joubert, P., Silversides, D.W., Lavoie, J.P., 2001. Equine neutrophils express mRNA for tumour necrosis factor- α , interleukin (IL)-1 β , IL-6, IL-8, macrophage-inflammatory-protein-2 but not for IL-4, IL-5 and interferon- γ . *Equine Vet. J.* 33, 730–733.
- Keel, M., Ungeth, U., Steckholzer, U., Niederer, E., Hartung, T., Trentz, O., Ertel, W., 1997. Interleukin-10 counter regulates proinflammatory cytokine-induced inhibition of neutrophil apoptosis during severe sepsis. *Blood* 90, 3356–3363.
- Kilpatrick, L.E., Sun, S., Mackie, D., Baik, F., Li, H., Korchak, H.M., 2006. Regulation of TNF mediated antiapoptotic signaling in human neutrophils: role of delta- PKC and ERK1/2. *J. Leukoc. Biol.* 80, 1512–1521.
- Laan, T.T., Bull, S., Pirie, R., Fink-Gremmels, J., 2006. The role of alveolar macrophages in the pathogenesis of recurrent airway obstruction in horses. *J. Vet. Intern. Med.* 20, 167–174.
- Lachheb, J., Dhifallah, I.B., Chelbi, H., Hamzaoui, K., Hamzaoui, A., 2008. Toll-like receptors and CD14 genes polymorphisms and susceptibility to asthma in Tunisian children. *Tissue Antigens* 71, 417–425.
- Lai, W.S., Carballo, E., Strum, J.R., Kennington, E.A., Phillips, R.S., Blackshear, P.J., 1999. Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor α mRNA. *Mol. Cell. Biol.* 19, 4311–4323.
- Lavoie-Lamoureux, A., Maghni, K., Lavoie, J.P., 2010a. Optimization of a procedure to accurately detect equine TNF α in serum samples. *Vet. Immunol. Immunopathol.* 138, 118–123.
- Lavoie-Lamoureux, A., Moran, K., Beauchamp, G., Mauel, S., Steinbach, F., Lefebvre-Lavoie, J., Martin, J.G., Lavoie, J.P., 2010b. IL-4 activates equine neutrophils and induces a mixed inflammatory cytokine expression profile with enhanced neutrophil chemotactic mediator release ex vivo. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 299, 472–482.
- Lavoie, J.P., Maghni, K., Desnoyers, M., Taha, R., Martin, J.G., Hamid, Q.A., 2001. Neutrophilic airway inflammation in horses with heaves is characterized by a Th2-type cytokine profile. *Am. J. Respir. Crit. Care Med.* 164, 1410–1413.
- Leclere, M., Lavoie-Lamoureux, A., Gelinas-Lymburner, E., David, F., Martin, J.G., Lavoie, J.P., 2011. Effect of antigenic exposure on airway smooth muscle remodeling in an equine model of chronic asthma. *Am. J. Respir. Cell Mol. Biol.* 45, 181–187.
- Luo, H.R., Loison, F., 2008. Constitutive neutrophil apoptosis: mechanisms and regulation. *Am. J. Hematol.* 83, 288–295.
- Marr, K.A., Foster, A.P., Lees, P., Cunningham, F.M., Page, C.P., 1997. Effect of antigen challenge on the activation of peripheral blood neutrophils from horses with chronic obstructive pulmonary disease. *Res. Vet. Sci.* 62, 253–260.
- Marr, K.A., Lees, P., Cunningham, F.M., 2002. Antigen challenge increases adherence of circulating neutrophils in horses with chronic obstructive pulmonary disease. *Equine Vet. J.* 34, 65–70.
- Marti, E., Gerber, H., Essich, G., Oulehla, J., Lazary, S., 1991. The genetic basis of equine allergic diseases. 1. Chronic hypersensitivity bronchitis. *Equine Vet. J.* 23, 457–460.
- McGorum, B.C., Dixon, P.M., Halliwell, R.E., 1993. Phenotypic analysis of peripheral blood and bronchoalveolar lavage fluid lymphocytes in control and chronic obstructive pulmonary disease affected horses, before and after 'natural (hay and straw) challenges'. *Vet. Immunol. Immunopathol.* 36, 207–222.
- Nevalainen, M., Raulo, S.M., Brazil, T.J., Pirie, R.S., Sorsa, T., McGorum, B.C., Maisi, P., 2002. Inhalation of organic dusts and lipopolysaccharide increases gelatinolytic matrix metalloproteinases (MMPs) in the lungs of heaves horses. *Equine Vet. J.* 34, 150–155.
- Niederer, C., Backmerhoff, F., Schumacher, B., 1997. Inflammatory mediators and acute phase proteins in patients with Crohn's disease and ulcerative colitis. *Hepatogastroenterology* 44, 90–107.
- Park, H.S., Park, J.Y., Yu, R., 2005. Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF- α and IL-6. *Diabetes Res. Clin. Pract.* 69, 29–35.
- Pirie, R.S., Collie, D.D., Dixon, P.M., McGorum, B.C., 2003a. Inhaled endotoxin and organic dust particulates have synergistic proinflammatory effects in equine heaves (organic dust-induced asthma). *Clin. Exp. Allergy* 33, 676–683.
- Pirie, R.S., Dixon, P.M., Collie, D.D., McGorum, B.C., 2001. Pulmonary and systemic effects of inhaled endotoxin in control and heaves horses. *Equine Vet. J.* 33, 311–318.
- Pirie, R.S., Dixon, P.M., McGorum, B.C., 2003b. Endotoxin contamination contributes to the pulmonary inflammatory and functional response to *Aspergillus fumigatus* extract inhalation in heaves horses. *Clin. Exp. Allergy* 33, 1289–1296.
- Robinson, N., 2001. International workshop on equine chronic airway disease. Michigan State University 16–18 June 2000. *Equine Vet. J.* 33, 5–19.
- Robinson, N.E., Olszewski, M.A., Boehler, D., Berney, C., Hakala, J., Matson, C., Derksen, F.J., 2000. Relationship between clinical signs and lung function in horses with recurrent airway obstruction (heaves) during a bronchodilator trial. *Equine Vet. J.* 32, 393–400.
- Schmallenbach, K.H., Rahman, I., Sasse, H.H., Dixon, P.M., Halliwell, R.E., McGorum, B.C., Cramer, R., Miller, H.R., 1998. 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.
- Seko, Y., Cole, S., Kasprzak, W., Shapiro, B.A., Ragheb, J.A., 2006. The role of cytokine mRNA stability in the pathogenesis of autoimmune disease. *Autoimmun. Rev.* 5, 299–305.
- Simonen-Jokinen, T., Pirie, R.S., McGorum, B., Maisi, P., 2005. 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.
- Simpson, J.L., Grissell, T.V., Douwes, J., Scott, R.J., Boyle, M.J., Gibson, P.G., 2007. Innate immune activation in neutrophilic asthma and bronchiectasis. *Thorax* 62, 211–218.
- Snow, M.H., Mikuls, T.R., 2005. Rheumatoid arthritis and cardiovascular disease: the role of systemic inflammation and evolving strategies of prevention. *Curr. Opin. Rheumatol.* 17, 234–241.
- Vercelli, D., 2010. Gene-environment interactions in asthma and allergy: the end of the beginning? *Curr. Opin. Allergy Clin. Immunol.* 10, 145–148.
- Vick, M.M., Adams, A.A., Murphy, B.A., Sessions, D.R., Horohov, D.W., Cook, R.F., Shelton, B.J., Fitzgerald, B.P., 2007. Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. *J. Anim. Sci.* 85, 1144–1155.
- Vychodilova-Krenkova, L., Matiasovic, J., Horin, P., 2005. Single nucleotide polymorphisms in four functionally related immune response genes in the horse: CD14, TLR4, Cepsilon, and Fepsilon R1 alpha. *Int. J. Immunogenet.* 32, 277–283.
- Weiss, D.J., Evanson, O.A., 2002. Evaluation of lipopolysaccharide-induced activation of equine neutrophils. *Am. J. Vet. Res.* 63, 811–815.
- Werners, A.H., Bull, S., Vendrig, J.C., Smyth, T., Bosch, R.R., Fink-Gremmels, J., Bryant, C.E., 2006. 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- α response in equine whole blood. *Vet. Immunol. Immunopathol.* 111, 165–173.