Cellular and humoral immunity in chronic equine laminitis

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Chronic equine laminitis causes persistent pain and lameness in affected animals and often necessitates euthanasia when pain management strategies become ineffective. Published studies as well as anecdotal reports suggest that this chronic inflammatory disease is associated with systemic alterations in immune responsiveness, perhaps involving an autoimmune component. We investigated this broad hypothesis by measuring a variety of immune indicators in healthy control horses (CON) and horses with chronic laminitis (LMN). We found that white blood cells from LMN horses produced more IFN\(\gamma\) than did cells from CON horses when stimulated in vitro with polyinosinic-polycytidylic acid [poly(I:C)], possibly due to an elevated number of circulating monocytes. No differences between groups were observed in plasma concentrations of IgG, IgA, IgM, IgE, or rheumatoid factor. Laminar tissue from LMN horses expressed elevated levels of keratinocyte damage-related genes as well as inflammatory cytokines and chemokines, which corresponded with a modest amount of neutrophil infiltration as shown by histological staining of fixed tissue and accumulation of neutrophil elastase protein. Taken together, our results do not support the hypothesis of an autoimmune component in chronic laminitis, although the strong induction of neutrophil chemokines and the presence of tissue neutrophils suggests that this cell type is likely involved in perpetuating the inflammation and tissue damage associated with this disease.

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\textbf{1. Introduction}

Laminitis is a debilitating disease of the equine foot that often occurs secondary to gastrointestinal disease or metabolic syndrome (Slater et al., 1995; USDA, 2000). The acute phase of the disease is characterized by severe lameness, disintegration of the laminar tissue connecting the hoof wall to the underlying third phalanx (Pollitt, 1996), and in some cases, dorsopalmar rotation of the third phalanx. This condition is usually not reversible and horses that survive an episode of acute laminitis often experience persistent lameness for years. This stage of the disease is termed chronic laminitis or “founder” (Hood, 1999). Acute laminitis transitions to the chronic form of the disease in an estimated 75% of cases (Moore, 2010), leaving the majority of afflicted horses with some degree of continued lameness. Whereas much effort has been expended to identify the causes of acute laminitis, the chronic form of the disease remains relatively uncharacterized.

Existing publications regarding chronic laminitis primarily focus on management and shoeing strategies to reduce pain and improve quality of life (Hunt and Wharton,
218

2.1. Animals and tissue collection

All animal protocols were approved by the Texas A&M University College of Veterinary Medicine Clinical Research Review Committee and/or the Institutional Animal Care and Use Committee, as appropriate, and were performed with the consent of the owner or the owner’s agent. Details of the horses used for each experiment are included in Supplementary Table 1. Horses with chronic laminitis were diagnosed by a licensed veterinarian based on clinical presentation, case history, and radiographic evidence of dorsopalmar rotation of the distal phalanx. A detailed description of all laminitis cases, including Obel grade of lameness (Obel, 1948), is provided in Supplementary Table 2.

Blood samples (n = 35) were collected from horses and ponies admitted to the Texas A&M University Large Animal Clinic (College Station, TX), the Hoof Diagnostic and Rehabilitation Clinic (Bryan, TX), or that were owned by Texas A&M University. Blood samples were collected from the jugular vein into evacuated blood tubes containing heparin or EDTA. Samples were either centrifuged at 1200 x g for plasma collection, processed immediately for WBC RNA isolation, or used as described below for in vitro culture of WBCs. Complete blood counts were performed by the Texas A&M University Clinical Pathology service on a subset of samples.

Tissue samples were collected at necropsy from the dorsal aspect of the hoof of either forelimb of horses admitted to the Texas A&M University Large Animal Clinic, the Hoof Diagnostic and Rehabilitation Clinic, or the Louisiana State University Veterinary Teaching Hospital (Baton Rouge, LA). Horses in the control group were euthanized for reasons unrelated to laminitis or other lameness. Control samples were not collected from unaffected limbs of unilaterally lame horses, as it would be impossible to rule out potential effects of increased weight bearing on the unaffected limb.

2.2. Blood cell culture

Heparinized blood samples from control (CON, n = 10) and chronic laminitis (LMN, n = 10) horses were thoroughly mixed by inversion and the total number of WBCs was counted for each sample. Sterile aliquots of whole blood were then treated with 1 μg/ml lipopolysaccharide (LPS; from E. coli 0111:B4, Sigma-Aldrich, St. Louis, MO), 50 μg/ml polyinosinic:polycytidylic acid (poly I:C; Sigma-Aldrich), or vehicle control (PBS) for 6 h at 37 °C. Upon completion of the culture, RNA was isolated immediately.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>TACCTATTACTGCGGCGGCCG</td>
<td>ATCCAGGAAAAGAGGCCCCA</td>
</tr>
<tr>
<td>IL-6</td>
<td>ACTGTTCCTTCAAGAGAACCG</td>
<td>AAGGTGTTGAGGAAAAAGACT</td>
</tr>
<tr>
<td>IL-1</td>
<td>CAGACGGCGGCCGCGCATACAC</td>
<td>GGAGAACGCACTGGCATAGTATT</td>
</tr>
<tr>
<td>TNFs</td>
<td>CTGGGCACAGACTCAATGCATCT</td>
<td>CATTGACACCCACATACG</td>
</tr>
<tr>
<td>ACTB</td>
<td>CACGACCGATGAAGTACGAAG</td>
<td>GTGGACAAATAGGCGGAAAT</td>
</tr>
<tr>
<td>CD3</td>
<td>AATGCAGCAGCTGCTGGGGC</td>
<td>TCTGAAGCTTCAGCTGGGACAG</td>
</tr>
<tr>
<td>CD14</td>
<td>TGGCCGCCATGTCTCCTCGT</td>
<td>CCGACACAGCTGGCATAGTATT</td>
</tr>
<tr>
<td>CD19</td>
<td>AGACCTCTTCCTCAAGCGTAA</td>
<td>CACCCCTTCTCCATCAATT</td>
</tr>
<tr>
<td>CCL2</td>
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<td>ATTTCCGTGTTTGGACTAG</td>
</tr>
<tr>
<td>CCL4</td>
<td>AGAGACCAGGGAAAAATCAA</td>
<td>GTTCAAGATAGGAAACAAAG</td>
</tr>
<tr>
<td>CCL7</td>
<td>TCAATAGAAGAAGATCCATCC</td>
<td>TTCTGTCAAGTAGTGTGGA</td>
</tr>
<tr>
<td>IL-8</td>
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<td>ACAAGCCGACGTCCACACAGA</td>
</tr>
<tr>
<td>DEFB4</td>
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<td>AGCCAGCTTCAGGTTCTAT</td>
</tr>
<tr>
<td>PI3</td>
<td>CTGCTTGTACCTCTGCGTTT</td>
<td>ACTGAACTTCGGCCATGACT</td>
</tr>
<tr>
<td>SOD2</td>
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<td>CAGGGAGTAAAGACCTGTTT</td>
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<tr>
<td>TLR4</td>
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<td>AGGCTGTACGGTGTACATC</td>
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<tr>
<td>S100A8</td>
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<td>TCGATGCTAAGCTTTGAAACC</td>
</tr>
<tr>
<td>S100A9</td>
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<td>TCGGTGTCTCATTAGTGTTC</td>
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<tr>
<td>MMP13</td>
<td>CTGACGCTGAGCTCTGTTT</td>
<td>CAGGAGATTAAAGGGGATAGT</td>
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</table>
Table 2
Summary of complete blood counts performed on healthy control horses (n=5) and horses with chronic laminitis (n=12). Lymphos: total lymphocytes, Monos: monocytes, Eosin: eosinophils, Total WBC: white blood cell count, RBC morph: red blood cell morphology.

<table>
<thead>
<tr>
<th>Case #</th>
<th>Group</th>
<th>Neutrophils</th>
<th>Lymphos</th>
<th>Monos</th>
<th>Eosin</th>
<th>Total WBC</th>
<th>RBC morph</th>
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<tbody>
<tr>
<td>21</td>
<td>CON</td>
<td>5934</td>
<td>2494</td>
<td>86</td>
<td>86</td>
<td>8.6</td>
<td>rouleaux</td>
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<tr>
<td>22</td>
<td>CON</td>
<td>4392</td>
<td>2376</td>
<td>–</td>
<td>432</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>CON</td>
<td>5610</td>
<td>2635</td>
<td>85</td>
<td>170</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>CON</td>
<td>6175</td>
<td>3040</td>
<td>95</td>
<td>95</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>CON</td>
<td>4160</td>
<td>1920</td>
<td>128</td>
<td>128</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>LMN</td>
<td>4160</td>
<td>2015</td>
<td>325</td>
<td>–</td>
<td>6.5</td>
<td>rouleaux</td>
</tr>
<tr>
<td>29</td>
<td>LMN</td>
<td>4745</td>
<td>2409</td>
<td>73</td>
<td>73</td>
<td>7.3</td>
<td>rouleaux</td>
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<tr>
<td>30</td>
<td>LMN</td>
<td>5251</td>
<td>3204</td>
<td>267</td>
<td>89</td>
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</tr>
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<td>31</td>
<td>LMN</td>
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<td>2520</td>
<td>216</td>
<td>216</td>
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<tr>
<td>33</td>
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<td>8284</td>
<td>2180</td>
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<td>rouleaux</td>
</tr>
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<td>34</td>
<td>LMN</td>
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<td>1426</td>
<td>248</td>
<td>434</td>
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</tr>
<tr>
<td>35</td>
<td>LMN</td>
<td>8510</td>
<td>2530</td>
<td>345</td>
<td>115</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>LMN</td>
<td>5002</td>
<td>2788</td>
<td>–</td>
<td>410</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>LMN</td>
<td>3596</td>
<td>2170</td>
<td>186</td>
<td>186</td>
<td>6.2</td>
<td>rouleaux</td>
</tr>
<tr>
<td>38</td>
<td>LMN</td>
<td>4686</td>
<td>1775</td>
<td>284</td>
<td>355</td>
<td>7.1</td>
<td>rouleaux</td>
</tr>
<tr>
<td>39</td>
<td>LMN</td>
<td>5369</td>
<td>3367</td>
<td>273</td>
<td>91</td>
<td>9.1</td>
<td>rouleaux</td>
</tr>
<tr>
<td>40</td>
<td>LMN</td>
<td>5355</td>
<td>2720</td>
<td>255</td>
<td>170</td>
<td>8.5</td>
<td></td>
</tr>
</tbody>
</table>

2.3. Gene expression

RNA was isolated from WBCs using the RNeasy Blood Mini Kit (Qiagen, Valencia, CA) and from laminar tissue using Qiazol Lysis Buffer (Qiagen) followed by the RNeasy Cleanup Kit (Qiagen). RNA was reverse transcribed (SuperScript III First Strand Synthesis Kit or Superscript VILO cDNA Synthesis Kit, Invitrogen, Carlsbad, CA) and the subsequent cDNA was used to determine gene expression levels using real time PCR. Twenty-five nanograms of laminar tissue cDNA or 2.5 ng of WBC RNA per PCR reaction was added to LightCycler 480 SYBR Green I master mix (Roche Applied Science, Indianapolis, IN) and 0.6 μM forward and reverse primers. Primer sequences are listed in Table 1. PCR reactions were performed on a LightCycler 480 (Roche) and the data was analyzed using the Pfaffl method (Pfaffl, 2001). Negative controls included samples in which the reverse transcriptase was omitted as well as PCR reactions containing no template. Melting curves were examined for each reaction to ensure that the fluorescence detected came from a single product of the expected size.

2.4. ELISAs

Capture ELISAs were performed to determine plasma concentrations of IgA and IgM using validated antibody pairs (Bethyl Labs, Montgomery, TX) and standard protocols. Briefly, high protein-binding 96 well plates (Nunc MaxiSorp, Thermo Scientific, Rockford, IL) were coated with 100 μg of unlabelled capture antibody overnight at 4 °C. Plates were blocked with SuperBlock buffer (Thermo Scientific, Waltham, MA) for 30 min at room temperature. Whole plasma samples were diluted 1:4000 in assay buffer (10% SuperBlock in PBS) and 100 μL of each diluted sample was pipetted in duplicate onto the plates, which were then incubated at 37 °C for 90 min. HRP-conjugated detection antibodies were diluted 1:50,000 (IgM) or 1:150,000 (IgA) in assay buffer and incubated on the plates for 2 h at room temperature. SigmaFast OPD (Sigma-Aldrich) was used as a substrate for HRP. Sample concentrations were calculated using dilutions of a reference serum of known concentration. Plasma concentrations of IgG were determined using a similar protocol adapted for use with a direct ELISA. Plates were coated overnight at 4 °C with plasma samples diluted 1:80,000 in PBS and the HRP-conjugated detection antibody was used at a dilution of 1:50,000; the remainder of the protocol was unchanged. IgE was measured as described previously (Wagner et al., 2003a).

Rheumatoid factor (RF) assays were also performed by ELISA using heat-aggregated equine IgG (10 min at 70 °C) diluted 1:100 in PBS as the capture antibody. Detection antibodies were anti-IgA or anti-IgM (Bethyl Laboratories) diluted 1:5000 in assay buffer. Plasma samples were serially diluted from 1:31.25 to 1:8000 (IgM-RF) or 1:125 to 1:2000 (IgA-RF) in order to determine titer.

2.5. Western blots

Western blotting was performed according to published protocols (Steelman and Chowdhary, 2012). Antibodies used are as follows: polyclonal rabbit anti-human neutrophil elastase (ab88672, Abcam, Cambridge, UK) and goat anti-rabbit secondary antibody (ab97080, Abcam); monoclonal rabbit anti-human beta actin (clone 13E5, Cell Signaling Technology, Danvers, MA) and goat anti-rabbit secondary antibody (#7074, Cell Signaling Technology). Resulting bands were quantified using ImageJ software (Rasband, 1997–2011).

2.6. Histology

Laminar tissue was fixed in neutral buffered formalin for at least 48 h before being embedded, sectioned to a thickness of 10 μm, and stained with H&E. Observation of neutrophils was performed by manual inspection of 10–20 40x fields of view per animal.

2.7. Statistics

Comparisons between CON and LMN groups were made by Student’s t test or two-way ANOVA, as appropriate.
As previous studies (Wang, 2010) suggested that inflammatory gene expression would be upregulated in laminar tissue from horses with laminitis, a one-tailed t-test was used to evaluate these data. A one-tailed t-test was also used to assess differences in IgA and IgM concentrations between groups, as previous proteomics experiments in our lab suggested that these immunoglobulins were elevated in horses with chronic laminitis (SMS, unpublished data; Steelman and Chowdhary, 2012). Calculations were performed using Prism (GraphPad Software Inc., La Jolla CA) or a variation of the S programming language called R, which is commonly used for statistical analysis (http://www.r-project.org). A P value < 0.05 was considered sufficient to reject the null hypothesis.

3. Results

3.1. Cellular immunity

Complete blood counts (CBCs) were performed on 5 healthy control horses and 12 horses with chronic laminitis. Absolute counts of all cell types fell within normal ranges, although the number of monocytes was higher in LMN horses than in controls (99 ± 10 CON vs. 254 ± 38 LMN, P < 0.01). Rouleaux were observed in 8 LMN horses (75%) and 1 CON horse (20%). Total WBC number did not differ between groups.

WBC expression of the inflammatory cytokines interleukin-1β (IL-1), interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), and interferon gamma (IFNγ) was assessed in horses with chronic laminitis (n = 9) and in healthy controls (n = 9). As the 18 horses used for this experiment had not had CBCs performed, the total number of WBCs was counted manually; no differences were seen between groups. In RNA isolated from freshly collected blood samples, we found no significant differences between the two groups in any of the cytokines tested (Fig. 1).

To assess the response of WBCs to exposure to pathogen-associated molecular patterns (PAMPs), whole blood samples from control (n = 9) and foundered (n = 9) horses were exposed to LPS, poly(I:C), or a vehicle control for 6h. LPS and poly(I:C), a double-stranded RNA molecule, were chosen as they are often associated with pathogenic bacteria and viruses, respectively. LPS and poly(I:C) induced expression of IL-1, TNFα, IL-6, and IFNγ (P < 0.01) in both groups; induction of IFNγ was higher in the LMN group than in controls (P < 0.05; Fig. 2). Post hoc testing revealed a significant elevation of IFNγ in LMN samples treated with poly(I:C) and a trend toward elevation in samples treated with LPS. No differences were observed in IL-1, TNFα, or IL-6 between CON and LMN groups.

3.2. Humoral immunity

Total plasma concentrations of IgM, IgA, IgE, and IgG in healthy horses and those with chronic laminitis were measured by ELISA. No differences were found between the LMN group (n = 8) and controls (n = 14), with the exception of IgA, which was significantly elevated in the LMN group (P = 0.025, Fig. 3).

As chronic laminitis shares several similarities with autoimmune diseases such as rheumatoid arthritis, we looked for the presence of IgM and IgA rheumatoid factors in the plasma of LMN horses, young controls (YC), and mature, age-matched controls (MC). The YC group uniformly tested negative for both types of rheumatoid factor; we thus used the YC values to establish the minimum value for a positive titer, indicated by the dashed horizontal lines in Fig. 4. Two MC horses and two LMN horses exceeded this limit for IgA rheumatoid factor, although the titers for the MC horses were 500 and 1000, whereas the titers for the LMN horses were 62 and 250 (Fig. 4). Similar results were seen with IgM rheumatoid factor, with 3 and 2 horses testing positive in the MC and LMN groups, respectively (Fig. 4). No significant differences were seen between the MC and LMN groups for either rheumatoid factor.

3.3. Laminar inflammation

RNA expression of innate inflammatory cytokines, antimicrobial and damage-related genes, chemokines, and WBC markers was assessed in laminar tissue of horses with chronic laminitis (n = 6) as well as in control horses (n = 5). Chemokine and WBC marker genes were chosen to screen for the presence of infiltrating inflammatory cells in the laminar tissue. Other genes of interest were chosen based on microarray data describing transcriptional changes in laminar tissue in three different experimental models of acute laminitis (Budak et al., 2009; Noschka et al., 2008; Wang, 2010). The genes investigated herein thus represent a small group of consistently upregulated transcripts that we believe to be integral to the pathology of acute laminitis and possibly involved in chronic laminitis.

We found significant upregulation of pro-inflammatory cytokine genes, antimicrobial/damage-related genes, and chemokine genes in laminar tissue from foundered horses (Fig. 5). IL-1 and IL-6 were both significantly increased (P < 0.05) in chronic laminitis, although TNFα and IFNγ were not (Fig. 5). The antimicrobial peptides beta-defensin 4 (DEFB4) and peptidase inhibitor 3 (PI3) were elevated 22- and 132-fold, respectively (P < 0.05), although no change
Fig. 2. Cytokine expression in PAMP-stimulated equine white blood cells. Whole blood of healthy controls (CON, n = 9) and horses with chronic laminitis (LMN, n = 9) was cultured for 6 h with vehicle (VEH), LPS (1 μg/ml), or poly(I:C) (50 ng/ml). RNA was isolated from whole blood and pro-inflammatory cytokine expression was determined by real time PCR. *P<0.05.

Fig. 3. Plasma immunoglobulin concentrations. Immunoglobulin concentrations were measured by ELISA in control horses (CON, n = 14) and horses with chronic laminitis (LMN, n = 8). *P<0.05.
**Fig. 4.** IgA and IgM rheumatoid factor titration curves. Titers were measured by ELISA in mature (> 5 y.o., n = 8) control horses [CON(M)] and horses with chronic laminitis (LMN, n = 8).

**Fig. 5.** Laminar expression of inflammatory cytokines, damage-related genes, chemokines, and cell surface markers. RNA was isolated from laminar tissue of control horses (CON, n = 5) and horses with chronic laminitis (LMN, n = 6). Gene expression was determined by real time PCR. *P < 0.05.
was seen in toll-like receptor 4 (TLR4). The keratinocyte damage-related genes superoxide dismutase 2 (SOD2), S100A9, and matrix metalloproteinase 13 (MMP13) were all significantly upregulated in the LMN group, although S100A8 was not. The macrophage chemokine CCL2 was upregulated approximately 10-fold \( (P < 0.05) \); the lymphocyte chemokines CCL4 and CCL7 did not differ between groups. IL-8, a neutrophil chemokine, was also increased in the LMN group (Fig. 5). The cell markers CD3, CD14, and CD19, which are used to identify T cells, macrophages, and B cells, respectively, did not differ in expression levels between the two groups (Fig. 5). Attempts to quantify RNA expression of myeloperoxidase in laminar tissue were unsuccessful, likely because neutrophils stop transcription of this gene shortly after leaving the bone marrow (Fouret et al., 1989). Nevertheless, western blot of laminar tissue showed a significant increase in neutrophil elastase protein in horses with chronic laminitis (Fig. 6).

In addition, scattered neutrophils (average < 1 cell per 40x field) were visible in H&E stained laminar tissues of horses with chronic laminitis (4/4, 100%), whereas none were observed in tissues from control horses. Neutrophils were found exclusively in the lamellae rather than in the deep dermis, often among the laminar epithelial cells (Fig. 7). Supplemental Fig. 1 shows a low magnification image of Fig. 7 and illustrates the nature of the structural changes seen in the laminar tissues.

4. Discussion

4.1. Cellular immunity

Circulating WBCs are a convenient, non-invasive means by which to evaluate the immune status of an animal. In the present study, we profiled both the differential counts and cytokine production of WBCs in order to gain insight into the immune responsiveness of horses with chronic laminitis. We found an ~2.5 fold elevation of monocytes in LMN horses, although all horses were well within the normal range for this cell type (0–1000 cells/µl). The clinical relevance of this finding remains unknown, as is the underlying physiological mechanism causing this difference. We also found evidence of rouleaux in 75% of LMN horses, but only 20% of controls. A previous study suggested elevated concentrations of fibrinogen in horses with chronic laminitis (Steelman and Chowdhary, 2012); fibrinogen is known to induce red blood cell aggregation and may underlie the rouleaux formation seen in foundered horses. As the presence of rouleaux has the potential to affect microvascular blood flow, further investigation of rheological parameters in chronic laminitis is warranted.

The in vivo environment of WBCs is the sum of factors secreted throughout the body; they are thus in a unique position to monitor and respond to peripheral infection or injury. Profiling cytokine expression in these cells
thus provides a static assessment of the animal’s overall health and well-being. In this study, however, we found no change in basal production of IL-1, IL-6, TNFα, or IFNγ. In vitro antigen-stimulated cytokine production, on the other hand, has been shown to be an excellent predictor of some disease states (Davis et al., 2010). In particular, gene expression of WBCs cultured in their in vivo environment (i.e., whole blood) is thought to be more representative of the true physiology of the animal than WBCs cultured in artificial media (Chen et al., 2010; Hodge et al., 2000). We found that, while WBCs from both groups responded to exposure to LPS or poly[I:C], foundered horses expressed significantly more IFNγ than did controls. It is possible that this is due to the elevated number of circulating monocytes in LMN horses, although unpublished data from our laboratory suggests that highly purified neutrophils isolated from LMN horses produce elevated amounts of IFNγ (S.M. Steelman, unpublished observations). Although neutrophils are not generally known for expression of interferons, some evidence suggests that IFNγ can be induced by stimulation with IL-12 (Ethuin et al., 2004). Importantly, the differential response of LMN horses to perceived pathogens, although conducted in vitro with mixed WBCs rather than tissue-resident antigen presenting cells, might underly the adverse reactions to vaccination in some LMN horses. This hypothesis was not specifically tested in the present study, but is currently under investigation in our laboratory.

4.2. Humoral immunity

Elevated immunoglobulin levels are a hallmark of a number of chronic inflammatory and autoimmune diseases, including hepatitis, asthma, and rheumatoid arthritis (Aho et al., 1997; Mieli-Vergani and Vergani, 2011; Platts-Mills, 2001). Previous work in our laboratory suggested differences in plasma immunoglobulin concentrations in a limited number of animals (Steelman and Chowdhary, 2012), so we sought to confirm and expand upon these data in the present study. Herein, we found that IgA was significantly elevated in horses with chronic laminitis. Whereas the functions of secretory IgA, primarily found at mucosal surfaces, are well described (Pabst, 2012), the immunological role of serum IgA is not completely understood. Serum IgA, which exists predominantly in the monomeric form, is elevated in several chronic inflammatory diseases such as ankylosing spondylitis and alcoholic liver disease and has been shown to have anti-inflammatory properties (reviewed in Monteiro, 2010). Specifically, IgA inhibits the respiratory burst in human neutrophils and monocytes (Wolf et al., 1994b), decreases monocyte production of TNFα and IL-6 (Wolf et al., 1994a), and impairs neutrophil phagocytosis (Wilton, 1978) and chemotaxis (Van Epps and Williams, 1976). Notably, our previous work uncovered a similar increase in the anti-inflammatory protein apolipoprotein A-IV (APOA-IV) in the plasma of horses with chronic laminitis (Steelman and Chowdhary, 2012). The elevation of plasma IgA and APOA-IV might represent a compensatory effort of the immune system to downregulate inflammation in chronic laminitis. Further investigation of the anti-inflammatory response in chronic laminitis is warranted.

It is also interesting to note that some older horses exhibited positive rheumatoid factor titers, whereas the younger ones did not; the reason for the occurrence of rheumatoid factor in otherwise healthy horses is unknown. A previous study found no increase in C1q-binding immune complexes in the serum of horses with chronic inflammatory diseases, including osteoarthritis and osteochondrosis disseccans (Osborne et al., 1995), although Stanek et al. (1984) found synovial fluid immune complexes in horses with laminitis [as reported by Osborne et al. (1995)]. Our data, however, do not support the hypothesis of immune complexes as contributors to the pathology of chronic laminitis.

4.3. Laminar inflammation

Although it is generally accepted that some level of inflammation is ongoing in the laminar tissue of founder horses, there is a lack of data describing the nature of this inflammation. For this reason, we measured the expression levels of several inflammation-related genes as well as a “core” group of genes that we and others have found to be consistently upregulated in several experimental models of acute laminitis. We believe that these core genes are integral to the pathology of laminitis and are possible candidates for targeted intervention. Although not consistently upregulated in our previous studies in acute laminitis (Wang, 2010), the innate cytokines IL-1, IL-6, IFNγ, and TNFα are potentially important given their major regulatory roles in initiating and sustaining the immune response. Similar to results seen in acute, carbohydrate-induced laminitis (Leise et al., 2011), we found that IL-1 and IL-6, but not TNFα, were overexpressed in the laminar tissue of founder horses. The source of these cytokines is unknown, but could be laminar keratinocytes or infiltrating phagocytes. No increase was found in laminar tissue IFNγ, despite the elevation seen in circulating WBCs. However, we also found no indication of increased numbers of antigen presenting cells (macrophages, dendritic cells), T cells, or B cells during chronic laminitis, as evidenced by the expression levels of CD14, CD3, and CD19, respectively. RNA expression of cell markers is often used to assess tissue infiltration of inflammatory cells (Michalak et al., 2000; Ponnuswamy et al., 2012), although the possibility remains that RNA level might not necessarily correlate with cell number. In the present study, however, the lack of change in cell marker expression was supported by an absence of inflammatory cells visible in histological sections, with the exception of neutrophils. This is interesting in light of the fact that the monocyte/macrophage chemokine CCL2 was overexpressed in founder horses, although the lymphocyte chemokines CCL4 and CCL7 did not differ between groups. IL-8, the prototypical neutrophil chemokine, was highly upregulated and, in this study, was accompanied by the presence of neutrophils within the laminar tissue, as evidenced by both histology and neutrophil elastase protein levels. With the exception of a single horse, neutrophil infiltration appeared to be minimal, although similarly low numbers of laminar neutrophils have been found in both the developmental and Obel grade I stages of experimental laminitis (Black
et al., 2006). Given the ability of neutrophils to release proteases, reactive oxygen species, and inflammatory cytokines, it is likely that these cells contribute to laminar tissue damage during chronic laminitis. Indeed, some groups have hypothesized that neutrophils drive both systemic and laminar inflammation during acute laminitis, particularly in the black walnut extract induction model, where they are likely the source of IL-1 (Belknap, 2010; de la Rebier de Pouvaye and Serteyn, 2011). As neutrophils are generally the first cell type to respond to injury or infection, it is tempting to speculate that their presence in chronic laminitis is the result of repeated trauma to the tissue from everyday weight-bearing activity.

The last class of genes examined, the damage-related genes, is an essential component of the epithelial response to infection or trauma, particularly the antimicrobial peptides (AMPs). The major keratinocyte AMPs include the defensins (such as DEF84) and PI3 (also known as SKALP or elafin), which defend against microbes by disrupting bacterial cell membrane integrity (Wiesner and Vilcinskas, 2010). Interestingly, PI3 is also a serine protease inhibitor that protects against excessive tissue damage by neutrophil degranulation. Both molecules are produced in response to inflammation resulting from infection or trauma and are chemotactic for several types of immune cells (Wiesner and Vilcinskas, 2010; Wilkinson et al., 2009). The overexpression of these peptides could be a link between the mechanical damage to the laminar tissue resulting from the instability of the decompensated foot and the chronic inflammation characteristic of this disease. The dramatic upregulation of MMP13, a collagenase enzyme, could also promote mechanical instability of the foot via degradation of laminar collagen. We also found a modest upregulation of the antioxidant enzyme SOD2, suggesting the occurrence of oxidative injury, perhaps from the neutrophil respiratory burst. Finally, the calcium-binding protein S100A9, but not its binding partner S100A8, was dramatically overexpressed in laminar tissue. S100A8 and S100A9 are generally co-regulated in laminar keratinocytes as part of the pro-inflammatory calprotectin complex (Faleiros et al., 2009), although there is some evidence that activated neutrophils express S100A9 in the absence of S100A8 (Kumar et al., 2001). Although immunohistochemistry would be necessary to definitively localize expression of S100A9 to neutrophils, it is possible that the upregulation of this gene in chronic laminitis is due to neutrophil infiltration.

5. Conclusion

Despite previous speculation that chronic laminitis might have an autoimmune component (Hood et al., 1990), we found no evidence to support this hypothesis, either in the form of circulating immune complexes or expression of the T cell marker CD3 within laminar tissue. Instead, we suggest a working hypothesis in which laminar inflammation results from the keratinocytes themselves and infiltrating neutrophils, likely in response to continual subclinical injury sustained during everyday weight-bearing activity. The current study provides evidence that specific targeting of keratinocyte mediators of inflammation or blockade of neutrophil infiltration might provide a more effective approach to limiting pain and inflammation in fondered horses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2013.03.001.

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