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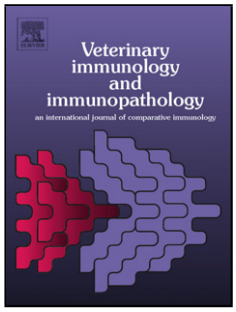

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Dynamics of local gene regulations in synovial fluid leukocytes from horses with lipopolysaccharide-induced arthritis

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Highlights

- Expression of inflammation-related genes is highly dynamic in equine synovial fluid leukocytes.
- The early leukocytic intra-articular response is dominated by elevated expression of pro-inflammatory cytokines (*IL1, Il6* and *TNF), COX2, TLR4* and chemokines.
- The early response is followed by increased gene expression of anti-inflammatory cytokines (*IL10* and *IL1RN*) and *SOD2* in synovial fluid leukocytes.
- Intra-articular leukocytes change their gene expression of apoptosis-related genes and genes involved in cartilage turnover during the course of joint inflammation.
- Considering the extensive changes in gene expression in the infiltrating leukocytes, the intra articular milieu is likely to be significantly affected by the changing gene expression in these cells during inflammatory arthritis.

Abstract

The role of resident cells such a synoviocytes and chondrocytes in intra-articular inflammation is well-characterized, however the *in vivo* gene expression patterns of cells (predominantly leukocytes) in the synovial fluid (SF) of an inflamed joint have never previously been investigated. The aim of this study was to investigate gene expression in SF leukocytes from the inflamed joint cavity after intra-articular lipopolysaccharide (LPS) injection in horses to improve our understanding of the temporal regulation of the intra-articular inflammatory response. Gene expression was investigated in SF samples available from six horses 2, 4, 8 16 and 24h after experimental induction of inflammation in the radiocarpal joint by lipopolysaccharide (LPS) injection. Leukocytic expression of 43 inflammation-related genes was studied using microfluidic high throughput qPCR (Fluidigm®). Expression of 26 genes changed significantly over the 24h study period, including pro- and anti-inflammatory genes such as interleukin (*IL)1*, *IL6*, tumor necrosis factor (*TNF*), cyclooxygenase 2 (*COX2*), IL1 receptor antagonist (*IL1RN*), *IL10*, and superoxide dismutase 2 (*SOD2*), chemokine genes, apoptosis-related genes, and genes related to cartilage turnover (matrix metalloproteinase 8 and tissue inhibitor of metalloproteinase 1). The inflammatory responses appeared to be regulated, as an early increase (at 2h) in expression of the pro-inflammatory genes *IL1*, *IL6*, *TNF* and *COX2* was rapidly followed by increased expression (at 4h) of several anti-inflammatory genes (*IL10*, *IL1RN* and *SOD2*). Similarly, both pro- and anti-apoptotic gene expression as well as expression of chondrodegenerative and chondroprotective genes were activated in SF leukocytes. Thus, the inflammatory response in leukocytes infiltrating the joint in the acute stage of arthritis was well orchestrated in this single-hit LPS-induced arthritis model. This study is the first to describe gene expression patterns in SF-derived leukocytes *in vivo* during severe joint inflammation, and the results thus expand our knowledge of basic inflammatory mechanisms in the early local response in an inflamed joint.

Abbreviations

ECM	Extracellular matrix
MMP	Matrix metalloproteinase
NTC	No template control
PGE_2	Prostaglandin E_2
OA	Osteoarthritis
RIN	RNA integrity number
ROS	Reactive oxygen species
-RT	Non-reverse transcriptase controls
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SF	Synovial fluid
WBC	White blood cell count

Keywords

Leukocytes, gene expression, joint, inflammation, arthritis, horses

Introduction

In arthritis, the responses of the resident cells of the joint, synoviocytes and chondrocytes, are well described. Moreover, it is well documented that severe arthritis cause a massive influx of leukocytes into the joint cavity (de Grauw et al., 2009; Jacobsen et al., 2006a), however the role of these infiltrating leukocytes is not fully elucidated. Synoviocytes and chondrocytes contribute to the accumulation of pro-inflammatory molecules in synovial fluid (SF), e.g. IL-1β, IL-6 (Ma et al., 2017), and prostaglandin E_2 (PGE_2) (Campbell et al., 1990). These in turn cause decreased hyaluronan concentration in cartilage (Guerne et al., 1990; Hardy et al., 1998), stimulate bone destruction, loss of proteoglycan, and breakdown of type II collagen (McCoy et al., 2002), and elicit pain (Kawabata, 2011). Furthermore, chondrocytes and synoviocytes release various matrix metalloproteinases (MMPs), which are involved in cartilage extracellular matrix (ECM) turnover and have a crucial role in degradation of articular cartilage (Clegg et al., 1997). During arthritis, leukocytes are attracted by chemokines, migrate across the synovial membrane, and accumulate in SF. Cell counts greater than 30 x 10^9/L (and up to 200 x 10^9/L) are commonly found in SF from horses with septic arthritis (Meijer et al., 2000). Neutrophil granulocytes dominate in septic arthritis, with more than 90% of the total SF cells being neutrophils and the remaining being lymphocytes, macrophages, and very few eosinophils, basophils and exfoliated cells (Denton, 2012). The infiltrating leukocytes are thought to play important roles in the inflamed joint cavity, where they may be involved in beneficial reactions such as phagocytosis and killing of invading microorganisms (Kumar and Sharma, 2010; Verdrengh and Tarkowski, 1997), as well as deleterious reactions such as release of cytokines and reactive oxygen species (ROS) that may cause degradation of the cartilage and synovial inflammation (Cedergren et al., 2007; Tiku et al., 1999). *In vitro* studies (Riedemann et al., 2003) and studies on gene expression in leukocytes from the

systemic circulation (Vinther et al., 2015) have shown expression of genes involved in inflammation in leukocytes but gene expression patterns have never been investigated in leukocytes isolated from the inflamed joint cavity. To obtain a more complete understanding of the activities and timing of gene expression in leukocytes migrating into the synovial cavity during arthritis, the aim of this study was to investigate gene expression in leukocytes isolated from SF of six horses 0, 2, 4, 8, 16 and 24 h after experimental induction of joint inflammation with LPS. It was hypothesized that expression pattern of inflammation-related genes would change over the course of intra articular inflammation.

Materials and Methods

Animals and study design

Synovial fluid samples were available from a previous study of experimental arthritis, where 6 horses (3 mares and 3 geldings, 3-15 years, 425-620 kg) had received an intra-articular injection of LPS resulting in severe joint inflammation as described previously as described in details by Andreassen et al. 2017 (Andreassen et al., 2017). Briefly, joint inflammation was induced by injection of 3 µg LPS (*Escherichia coli* strain 055:B5, catalogue number L2880, Sigma-Aldrich Denmark A/S, Søborg, Denmark) into one radiocarpal joint.

The study was carried out in accordance with the EU Directive 2010/63/EU for animal experiments. The protocol was approved by the Danish Animal Experiments Inspectorate, Ministry of Justice (license no. 2011/561-1996) and by the ethics and welfare committee of Department of Veterinary Clinical Sciences, University of Copenhagen. All procedures were carried out according to the Danish Animal Testing Act.

Synovial fluid samples and clinical examination

Synovial fluid was collected before (0h) and after (2, 4, 8, 16, and 24h) LPS injection and directly transferred into PAXgene™ blood RNA tubes (BD Vacutainer, Becton Dickinson A/S, Albertslund, Denmark) for RNA analysis, and into tubes containing EDTA for assessment of white blood cell count (WBC) and cytology (BD Vacutainer, Becton Dickinson A/S, Albertslund, Denmark). Due to variability in obtained SF volumes sufficient sample material was not obtained from every horse at every time point yielding n = 4 at 0h (baseline), n = 5 at 2h, n = 6 at 4h, n = 5 at 8h, n = 5 at 16h, and n = 3 at 24h. According to the manufacturer's instruction, PAXgene tubes were gently inverted 8-10 times and kept at room temperature for 2-24h, before storage at -80°C. White blood cell count in SF was obtained within 24h by manual cell counting using a hematocytometer. Cytology was performed by manual counting of a cytospin of the SF sample. Extraction RNA extraction from PAX samples took place 5 years after samples were obtained

At each sampling point, the horses underwent a clinical examination, pain assessment and lameness evaluation as detailed in Andreassen et al. (2017) (Andreassen et al., 2017) .

As described in Andreassen et al. (2017) (Andreassen et al., 2017) intra-articular injection of LPS induced a severe, short lived arthritis with high degree lameness and pain score. Moreover all horses showed increased heart rate, respiratory rate and rectal temperature. The majority of the clinical parameters normalized within 24h of LPS injection.

Target genes and primers

Relative gene expression in SF derived cells, predominantly leukocytes, was measured by quantification of specific mRNA coding for the following genes *IL1B, IL1RN, IL2, IL4, IL6,*

IL6ST, IL8, IL10, IL15, IL17, IL18, TNF, TLR4, SELL, ITGAM, ITGAX, CD14, MAPK14, CASP3, BCL2L1, MMP8, TIMP1, CCL5, SOD2, TLR9, TGFB1, HMGB1, MIF, NFKB1, FAS, BID, MPO, CSF2, HP, COX2, NFKBIA, HIF1A, PLAUR, SLC16A3, SLC16A1 LDHA, LDHB, SDHB (a list of gene names and primer sequences are included in supplementary table 1). Specific gene primer pairs were designed as previously described (Vinther et al., 2015) and synthesized at TAG Copenhagen (Frederiksberg, Copenhagen, Denmark) or Sigma-Aldrich (Denmark A/S, Søborg, Denmark).

Total RNA extraction and initial characterization

Total cellular RNA was extracted from the SF samples using PAXgene miRNA kit (Qiagen Denmark, Vedbæk, Denmark) according to manufacturer's protocol. The RNA yield was determined by spectrophotometry (Nano Drop ND-1000 spectrophotometer, Saveen and Werner AB, Limhamn, Sweeden) at 260 nm and purity of RNA was evaluated from absorbance ratios at 260/280 and 260/230,with satisfactory ratios in all samples. RNA integrity (RIN, RNA Integrity Number) was assessed by on-chip gel electrophoresis in a Bioanalyzer (Agilent Technologies Denmark ApS, Glostrup Denmark) using the Agilent RNA 6000 Nano kit (Agilent Technologies Denmark ApS, Glostrup, Denmark) according to the manufacturer's protocol. In SF samples obtained before LPS injection (0h samples), RNA quantity was too low to perform further quality control and reverse transcription quantitative real-time PCR (RT-qPCR) (quantity mean 1.9 ng/µl, range from 0 to 3.4 ng/µl).The RNA quality of the remaining samples (2, 4, 8, 16, 24h after LPS injection) varied, as the RIN values (numbering system range from 1-10, where 1 is most degraded and 10 intact RNA) ranged from 2.70-8.50 (mean ± SEM = 6.26 ± 0.6). Extracted total RNA from the SF samples was stored at -20°C for less than 48h before converted into cDNA.

cDNA synthesis

After extraction of total RNA, samples were DNase treated to degrade genomic DNA using RNase free DNase sets (Qiagen Denmark, Vedbæk, Denmark). After DNase treatment 100 ng RNA was converted into single stranded cDNA by reverse transcription using a Thermocycler (TProfessional TRIO 3x48, Fisher Scientific, Denmark, Slangerup, Denmark) and QuantiTect Reverse Transcription Kits (Qiagen Denmark, Vedbæk, Denmark) according to the manufacturer's instructions. Three separate cDNA syntheses (technical replicates) were performed for each RNA sample and two non-reverse transcriptase controls (-RT) were included. Four cDNA samples from a previous study (Vinther et al., 2016) stored at -80°C were included as positives controls.

Pre-amplification and exonuclease treatment

cDNA was pre-amplified and exonuclease treated in order to ensure a sufficient amount of DNA for quantification and in order to degrade unincorporated primers, respectively. Stocks of 200 nM primer pairs containing equal amount of all the selected primers mixed with low TE-buffer (VWR-Bie & Berntsen, Herlev, Denmark) were prepared. A pre-amplification primer mix was prepared; this contained 5µLTaqMan PreAmp Master Mix (Applied Biosystems, Foster City, CA, USA,), 2.5µL primer mix and 2.5µL cDNA; it was incubated at 95°C for 10 min followed by 20 cycles of 15s at 95°C and 4 min at 60°C. Afterwards, pre-amplified cDNA was incubated with 4 U/µl exonuclease (New England Biolabs, Ipswich, Massachusetts, USA) at 37°C for 30 min followed by 80°C for 15 min. An aliquot of the pre-amplified cDNA was saved undiluted for preparation of dilution series. Finally, the pre-amplified exonuclease treated cDNA was diluted 1:10 with low TE-buffer (VWR-Bie & Berntsen, Denmark) and stored at -20°C until qPCR was performed.

High throughput qPCR

High throughput qPCR was performed in a 96.96 Dynamic Array Integrated Fluidic Circuit chip (Fluidigm, San Francisco, CA, USA). This high throughput qPCR platform combines 96 pre-amplified samples with 96 primer sets for 9216 individual simultaneous qPCR reactions. The primer mix consisted of 3µL 2X Assay Loading Reagent (Fluigdim, San Francisco, CA, USA) and 3µL 20µM forward and reverse primer. Sample mix was made of following reagents: 3µL TaqMan Gene Expression Master mix (Applied Biosystems, Foster City, CA, USA), 0.3µL 20X DNA Binding Dye Sample Loading Reagent (Fluidigm, San Francisco, CA, USA), 0.3µL 20 X EvaGreen (Biotium; VWR- Bie & Berntsen, Herlev Denmark), 0.9µL low TE-buffer (VWR-Bie & Berntsen, Herlev, Denmark) and 1.5µL pre-amplified cDNA. Samples and primers were loaded in the integrated fluidic circuit chip according to the manufactures' instructions using a HX IFC controller (Fluidigm, San Francisco, CA, USA). The integrated fluidic circuit chip was placed in the real-time PCR instrument (BioMark, Fluidigm, San Francisco, CA, USA). The following cycle conditions were used: 2 min at 50°C and 10 min at 95°C for thermal mix, followed by 35 cycles with denaturation for 15 s at 95°C and annealing/elongation for 1 min at 60°C. After each run, melting curves were generated to confirm a single PCR product (increasing 1 °C/3s).

Dilution curves were constructed from three individual dilution series (1:3, 1:30, 1:300, 1:3000) of cDNA from a sample pool made up of equal amounts of all samples in order to obtain dynamic range and PCR efficacy for all and primer sets. No template controls (NTC) were included to monitor non-specific amplification or sample contaminations, and -RT controls were included to assess potential DNA contamination. Data were acquired using the Fluidigm Real-Time PCR Analysis software 3.0.2 (Fluidigm, San Francisco, CA, USA). A PCR efficiency between 95 and

105% and a separation by more than five Cq-values between –RTs and sample signal were required for acceptance of results from each primer set, and only reactions yielding products with a single melting peak at the expected melting temperature were accepted for further data analyses.

Data pre-processing and statistical analysis

Data was corrected for PCR efficiency for each primer pair as inferred by the standard curves. The two most optimally performing reference genes *(B-ACTIN* and *B2M*) were identified from a panel of 6 reference genes (*18S, GAPDH, B-ACTIN, B2M, RPL32, TBP*) using NormFinder (Andersen et al., 2004) and GeNorm (Vandesompele et al., 2002) and used for normalization of all expression values in order to reduce non-biological variation between samples. To insure data reproducibility of each primer set cDNA technical replicates were compared, and excluded if the deviation was too high (>1.5 Cq between triple determinations for more than 20 % of all replicates). cDNA technical replicates were averaged for each gene. Gene expression data never reaching the fluorescence threshold (missing data points, 13 out of 1452, 0.9 %) were assigned a Cq value corresponding to the highest Cq value measured (= lowest expression) for the gene in question in any sample +1. Cq data were subsequently transformed into relative quantities and relative expression levels were established for each gene as the sample with the lowest expression was assigned the value 1 and all other samples were scaled according to this. Expression data were log2 transformed to approach normal distribution. Statistically significant changes in gene expression were identified using linear mixed model with "time" as fixed effect and "horse" as random effect using R (version 3.6.2 (2019-12-12)).

Differential gene expression was defined as significant if 1) expression level changed at least three fold (maximal expression level ≥ 3) over time, and 2) P-value < 0.05. Relative expression levels for

selected genes meeting these P-value and maximal expression level criteria were visualized in GraphPad Prism version 8.4.3 (GraphPad Software Inc., La Jolla, CA, USA).

Synovial fluid WBCs are depicted as mean and range.

During preprocessing of the qPCR results, 10 of the 43 genes and two samples (Horse C 2h after LPS injection and Horse E 8h after LPS injection) were excluded due to poor assay efficiency or high variation between cDNA technical replicates. Thus, 33 genes were evaluated for differential expression over time.

Results

Leukocytosis with profound neutrophilia developed in response to the intra-articular LPS injection (Table 1).

RNA content in the 0h samples was too low to allow assessment of gene expression levels, probably reflecting the low SF WBC at this time point. Of the 33 genes analyzed, 26 displayed statistically significant differential expression over time (Table 2). Gene expression of pro-inflammatory cytokines peaked within the first 2h after LPS injection and subsequently declined throughout the 24h study period (Figure 1A) and included the three prototypic pro-inflammatory cytokines *IL1*, *IL6* and *TNF*. Expression levels of these genes changed more than 10 (and up to 200) times (Table 2). The gene expression of anti-inflammatory cytokines *IL10* and IL1 receptor antagonist (*IL1RN)* changed more than 13 and 51 times respectively and peaked 4h after LPS injection (Figure 1B). Expression of cyclooxygenase 2 (*COX2*), followed an expression pattern similar to the pro-inflammatory cytokines (Figure 2).

Expression of the oxidative stress inhibitor and anti-apoptotic gene superoxide dismutase 2 (*SOD2*) peaked 4h after LPS injection (Table 2, Figure 3).

Two chemokine genes were differently expressed over time (Table 2, Figure 4). They showed opposing expression patterns with *IL8* expression peaking within the first 2h after LPS injection, while C-C motif chemokine ligand 5 (*CCL5*) expression increased steadily over the 24h study period (Figure 4).

Two genes (*MMP8* and tissue inhibitor of MMP 1 (*TIMP1*)) involved in turnover of ECM were differentially expressed over timer (Table 2, Figure 5), expression of both genes peaked 8h after LPS injection (Figure 5).

Two genes were classified as receptors involved in pattern recognition of LPS (Table 2, Figure 6). Expression of the pattern recognition receptor genes showed opposing patterns with high toll-like receptor 4 (*TLR4*) expression 2h after LPS injection followed by a decline in expression over the 24h study period, whereas cluster of differentiation 14 (*CD14*) expression increased throughout the study period (Figure 6).

Two genes involved in apoptosis were differentially expressed over time and changed more than 14 times (Table 2, Figure 7). Expression of the pro-apoptotic caspase 3 (*CASP3*) peaked before expression of the anti-apoptotic Bcl-2-like protein 1 (*BCL2L1*) gene did (Figure 7).

The remainder of the differentially expressed genes were classified as being involved in cell metabolism (n = 4) and miscellaneous innate immune functions (n = 7) including adhesion and signal transduction (Table 2). One of these genes (solute carrier family 16 member 1, *SLC16A1*) was strongly regulated, with a 130 times change of relative expression level.

Discussion

This study is, to the authors' knowledge, the first to describe *in vivo* gene expression in leukocytes isolated from inflamed SF. By reporting gene expression relative to reference gene expression, changes in gene expression reflected individual cellular responses and were not affected by the fluctuations in SF WBC. Overall, the early phase (2-4h after injection) of the LPS-induced joint inflammation was dominated by pro-inflammatory responses with increased gene expression of pro-inflammatory cytokines, *COX2, TLR4* and chemokines. This was followed by an anti-inflammatory response (4-8h after injection), where gene expression of anti-inflammatory cytokines and *SOD2* was increased in the leukocytes.

The pro-inflammatory cytokines IL-1, IL-6 and TNFα are major inducers of inflammation and have essential roles in the pathogenesis of inflammatory joint diseases in horses, e.g., osteoarthritis (OA) (de Souza, 2016; Ma et al., 2017) and septic arthritis (Morton, 2005). These inflammatory cytokines elicit destruction of articular cartilage through their stimulation of degradation of proteoglycan and type II collagen (Tortorella et al., 1999). The harmful intra-articular effects of pro-inflammatory cytokines have been studied in experimental arthritis in laboratory rodents, where pro-inflammatory cytokine blockage or knock-out retarded onset of disease, reduced disease severity, and substantially reduced the risk of developing arthritis (Houssiau et al., 1988; Hovdenes et al., 1990; Kay and Calabrese, 2004; Pascual et al., 2005; Saijo et al., 2002; van den Berg, 2001).

Elevated expression of *COX2* and its product PGE_2 has been demonstrated in cultured equine chondrocytes (Byron et al., 2008), synoviocytes (Byron et al., 2008) and equine SF (van Loon et al., 2010) under inflammatory conditions. In humans, increased COX2 and PGE2 expression is present in cartilage and synovial membrane in OA and rheumatoid arthritis (Amin et al., 1999; Amin et al.,

1997; Crofford et al., 1994). A previous study found that *COX2* mRNA was present in varying degree in cells derived from SF from humans with acute and chronic arthritis (Iniguez et al., 1998). Moreover rapid upregulation of *COX2* mRNA has been demonstrated in cytokine-stimulated equine chondrocytes (Farley et al., 2005). Similarly, our results demonstrate that equine SF leukocytes express *COX2* and that expression changed more than 12-fold over time and peaked rapidly (within the first 2h) after intra-articular LPS injection. Leukocytic *COX2* expression may thus contribute to the increased PGE_2 concentrations found in inflamed equine SF (Bertone et al., 2001).

In inflammatory conditions regulation of immune cell trafficking by chemokines such as IL-8 and CCL-5 is of great importance to attract leukocytes to the site of inflammation. Elevated IL-8 and CCL-5 concentrations have been demonstrated in SF from humans with OA (Pierzchala et al., 2011). Interleukin-8 synthesis has been demonstrated in cultured equine chondrocytes, and it was suggested that this contribute to the migration of inflammatory cells including neutrophils into the inflamed joint (David et al., 2007). Leukocytes isolated from systemic circulation of horses with LPS-induced systemic inflammation expressed *IL8* and *CCL5* in a pattern similar to that demonstrated in this study (Vinther et al., 2015). Chemokines seem to have a multifaceted role in arthritis. Not only do they orchestrate leukocyte recruitment, activation and retention, they also stimulate angiogenesis, osteoclast differentiation, synoviocyte proliferation and activation and hyperalgesia (Bernardini et al., 2017). Moreover they regulate cartilage and bone metabolism, e.g. by stimulating MMP release from chondrocytes (Borzi et al., 2000).

The pro-inflammatory responses were followed by expression of the anti-inflammatory genes *IL10, IL1RN*, and the reactive oxygen species scavenger *SOD2*. These molecules serve to mitigate some of the deleterious effects of the pro-inflammatory cascade on intra-articular tissues. The chondroprotective effects of IL-10 has been established in murine models of inflammatory arthritis

(Finnegan et al., 2003), and a recent study demonstrated the pivotal role of IL-10 in equine chondrocytes, where its overexpression reduced expression of pro-inflammatory cytokines and PGE_2 significantly (Ortved et al., 2018). Interleukin-1 receptor antagonist is a potent anti-inflammatory protein that binds IL-1 receptors and inhibits IL-1α and IL-1β signalling, and thereby their NF-κB activated pro-inflammatory cascade in OA (Granowitz et al., 1991). Gene therapy with IL-1Ra reduced clinical signs and pathological changes in synovial membrane and cartilage in murine and equine models of arthritis (Nixon et al., 2018). The upregulation of *IL10*, *IL1RN* gene expression showed in this study could thus serve to counteract the deleterious effects of the pro-inflammatory cytokines on the cartilage.

Reactive oxygen species has been linked to a variety of deleterious pathways in arthritis including cartilage senescence (Brandl et al., 2011), cartilage damage, synovial inflammation and subchondral dysfunction (Lepetsos and Papavassiliou, 2016). Superoxide dismutase 2 serves as a scavenger of ROS. Koike et al. (2015) demonstrated *in vitro* (in murine chondrocytes) and *in vivo* (in murine models of OA) that *SOD2* is downregulated in OA and that *SOD2* deficits result in cartilage damage (Koike et al., 2015). The upregulation of *SOD2* gene expression demonstrated in our study may thus serve to balance ROS production, thereby preventing cartilage damage.

Taken together, these pro- and anti-inflammatory, chemokine, *COX2*, and *SOD2* responses of the infiltrating leukocytes represent a set of tightly regulated events designed to first clear the inflammatory trigger (e.g. bacteria) and then dampen the inflammation to reduce the deleterious effects of the inflammatory molecules in the joint. This correspond with the clinical course of disease in the horses (Andreassen et al., 2017), where intra-articular LPS injection caused severe arthritis with lameness and pain scores peaking at 2-4h, followed by normalization of the clinical parameters within 24h. Also on protein level, fast changes in cytokine concentrations in SF have recently been shown to occur in horses after LPS injection into the tibiotarsal joint (Sotelo et al.,

2020), suggesting that the mRNA is translated into protein. It is, however, important to remember that SF protein content represents the combined contributions from both infiltrating leukocytes and cytokine synthesis in the resident cells within the joint including synoviocytes and chondrocytes.

Expression of the cartilage-degrading genes *MMP8* and the inhibitor, *TIMP1* increased in the early phase of joint inflammation and peaked 8h after LPS injection. Matrix metalloproteinases degrade ECM components. In healthy articular cartilage, a tightly balanced turnover (degradation and synthesis) of ECM exists. Matrix metalloproteinases induce degradation, and their activity is largely controlled by tissue inhibitors of metalloproteinase (Clutterbuck et al., 2010). Inflammatory mediators such as pro-inflammatory cytokines, chemokines, ROS and PGE_2 disturb this balance by activating MMPs, thereby favouring degradation of the ECM. Increased MMP activities has been demonstrated in equine cartilage and subchondral bone explants stimulated with IL-1 (Byron and Trahan, 2017). Matrix metalloproteinase activity was elevated in SF from horses with OA (Brama et al., 2004) and correlated with severity of cartilage damage (Van Den Boom et al., 2005). Tissue inhibitor of matrix metalloproteinase activity has previously been identified in equine synovial fibroblasts and chondrocytes as well as in equine SF (Clegg et al., 1998). Our results suggest that TIMPs may also be produced by leukocytes infiltrating the joint. Expression of *TIMP1* as well as *MMP8* peaked simultaneously 8h after intra-articular LPS injection, and these concurrent responses may serve to maintain the ECM turnover in balance and prevent degradation of the articular cartilage.

The pattern recognition molecules CD14 and TLR4 are a part of the receptor complex that recognises LPS, thereby activating intracellular signalling pathways and initiating the inflammatory response (Aderem and Ulevitch, 2000). Toll-like receptor 4 was found to be upregulated in cultured chondrocytes from humans with OA (Kim et al., 2006) and synovial tissue from humans with rheumatoid arthritis (Ospelt et al., 2009; Radstake et al., 2004). Our results demonstrate that the

infiltrating leukocytes also are a source of *TLR4* and that the gene expression peaks in the early phase after LPS injection. Moreover, there is evidence suggesting that toll-like receptors are mediators of degradation of cartilage and increased production of MMPs in cartilage implants (Liu-Bryan and Terkeltaub, 2010). The gene expression patterns of *TLR4* and *CD14* demonstrated in the SF leukocytes is very similar to what has been demonstrated in equine blood leukocytes after LPS stimulus (Vinther et al., 2015).

Neutrophil apoptosis is thought to be an important mechanism of resolving the inflammation (Schett and Neurath, 2018). Our results indicate that leukocyte gene expression of genes involved in apoptosis is tightly regulated, as increased expression of the pro-apoptotic *CASP3* was followed by increased expression of the caspase activation inhibitor *BCL2L1*. Decreased apoptosis in SF leukocytes has been detected in the early phases of rheumatoid arthritis in humans, and this was suggested to result in accumulation of leukocytes in the SF and in development of persistent rheumatoid arthritis (Raza et al., 2006). How apoptotic signals influence development of inflammatory joint disease in horses needs further investigation.

Some methodical difficulties where encountered during design and data collection. Firstly, PAXgene tubes were used for collection of SF, as they contain a proprietary solution preventing degradation of cellular RNA. The tubes are, however, not marketed for this purpose, but are intended for use with whole blood. Others have used PAXgene blood tubes to study cellular gene expression in bronchoalveolar lavage fluid in horses (Hansen et al., 2020) and SF samples from humans (Li et al., 2011) without encountering analysis issues. Secondly, RNA integrity was found to vary. To accommodate for thus qPCR analysis was made more robust by 1) using three separate cDNA syntheses per sample, 2) robust primer design, 3) using up to five different primers targeting different areas of the same gene transcript, and 4) adding the same primer more than once to the qPCR platform. For samples with RIN value between 1 and 4 it is recommended to use PCR

platforms with short regions of amplification like the Fluidigm platform (Le Page et al., 2013). Similarly, shorter RT-qPCR products have been shown to be less dependent or even independent on RNA quality (Fleige and Pfaffl, 2006). So to obtain robust primers, these were designed to amplify short templates of 100 or less base pairs. The high reproducibility of qPCR data for the three cDNA replicates demonstrated during data preprocessing signified a strong assay design and adequate RNA integrity. Thirdly, the WBC and hence the amount of RNA in pre-injection SF samples (0h) was too low to permit gene expression analysis at baseline conditions, changes in expression profiles taking place within the first 2h after intra-articular LPS injection thus remain undescribed.

A major limitation of this study is the lack of control group. Previous studies have shown that repeated arthrocenteses cause a small inflammatory activation in the joint similar with modest increase in SF WBC (Jacobsen et al., 2006b; Sanchez Teran et al., 2012), and with lack of control the contribution of this procedure to the observed gene expression changes cannot be gauged. However, given the magnitude of the inflammatory response after LPS injection, the effect of repeated arthrocentesis was hypothesized to be negligible.

Understanding the inflammatory mechanisms in the pathogeneses of arthritis is of great importance, as inflammation is thought to have a key role in both OA (Kamm et al., 2010) and septic arthritis (Morton, 2005) in horses. We have demonstrated that the SF leukocytes are an important source of pro- and anti-inflammatory, pro- and anti-apoptotic, and chondrodegenerative and chondroprotective molecules, as well as chemokines – similar to what has been shown for the resident cells of the joint (Otero and Goldring, 2007; Sellam and Berenbaum, 2010). The observed gene expression patterns of SF leukocytes were qualitatively very similar to what has been

demonstrated in LPS-stimulated equine blood leukocytes *in vitro* and *in vivo* (after intravenous LPS injection) (Jacobs et al., 2013; Nieto et al., 2009; Vinther et al., 2015).

The tightly regulated gene expression demonstrated in infiltrating leukocytes after a single intra-articular LPS injection may be disrupted in clinical cases of septic arthritis, where ongoing active inflammation with constant influx of leukocytes may cause saturation or destruction of the natural inhibitors (Harris et al., 1975). Based on our results the recommended treatment with aggressive endoscopic lavage of the synovial cavity in horses with septic arthritis to remove leukocytes and inflammatory molecules from the infected synovial cavity by endoscopic lavage and debridement thus seem justified (Morton, 2005).

In conclusion, this *in vivo* study in a single-hit LPS arthritis model expands our knowledge about the role of the infiltrating leukocytes in acute joint inflammation in horses. In vivo assessment of the local temporal gene regulation over time provides a deeper understanding of how gene expression in SF leukocytes is orchestrated. Very significant changes in gene expression occurred in these cells within the first 24h after induction of inflammation concurrently with their increased presence in the joint cavity. Considering the clear leukocyte influx in SF, the intra-articular milieu is prone to be dramatically affected by the changing gene expression not only in the resident cells of the joint, but also in infiltrating leukocytes.

Author Contributions

All authors have made substantial contributions; to the conception and design of the study (S.J., P.H.A., P.M.H.H and K.S.), acquisition of data (M.W., K.S., S.J. and P.H.A.), interpretation of data (M.W., K.S., P.M.H.H and S.J.) and drafting the article or revising it critically for important intellectual content (M.W., S.J., K.S., P.M.H.H and P.H.A.). All authors have approved the final manuscript (M.W., S.J., K.S., P.M.H.H and P.H.A.). SJ (stj@sund.ku.dk) takes responsibility for the integrity of the work as a whole, from inception to finished article.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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Figure Captions

Figure 1. Changes in expression level of pro-inflammatory (A) and anti-inflammatory (B) genes in leukocytes isolated from equine synovial fluid (SF) after injection of LPS in one radiocarpal joint. Data points represent mean relative expression of the genes in SF from 3-6 horses (n = 4 at 2h, n = 6 at 4h, n = 4 at 8h, n = 5 at 16h, and n = 3 at 24h) ± SEM (error bars).

TNF = tumor necrosis factor, *IL1RN*= IL1 receptor antagonist

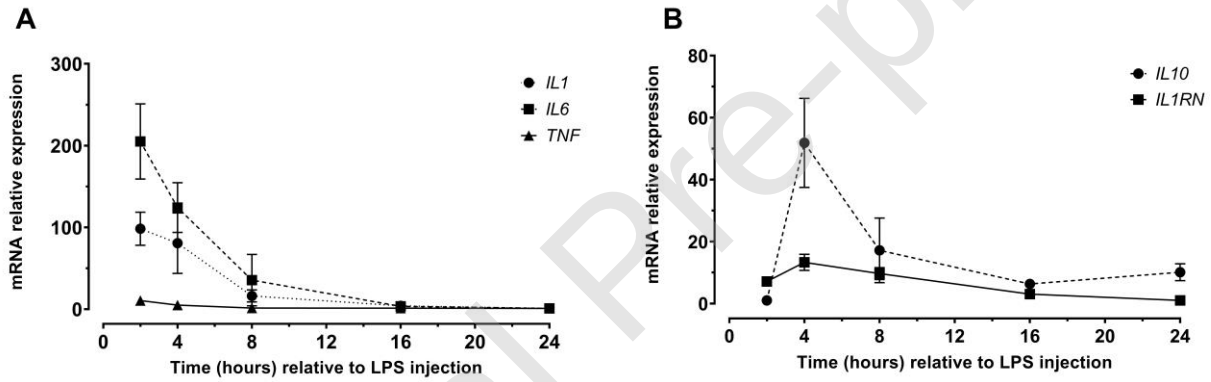

Figure 2. Changes in expression level of cyclooxygenase 2 (*COX2*) in leukocytes isolated from equine synovial fluid (SF) after injection of LPS in one radiocarpal joint. Data points represent mean relative expression of the gene in SF from 3-6 horses (n = 4 at 2h, n = 6 at 4h, n = 4 at 8h, n = 5 at 16h, and n = 3 at 24h) ± SEM (error bars).

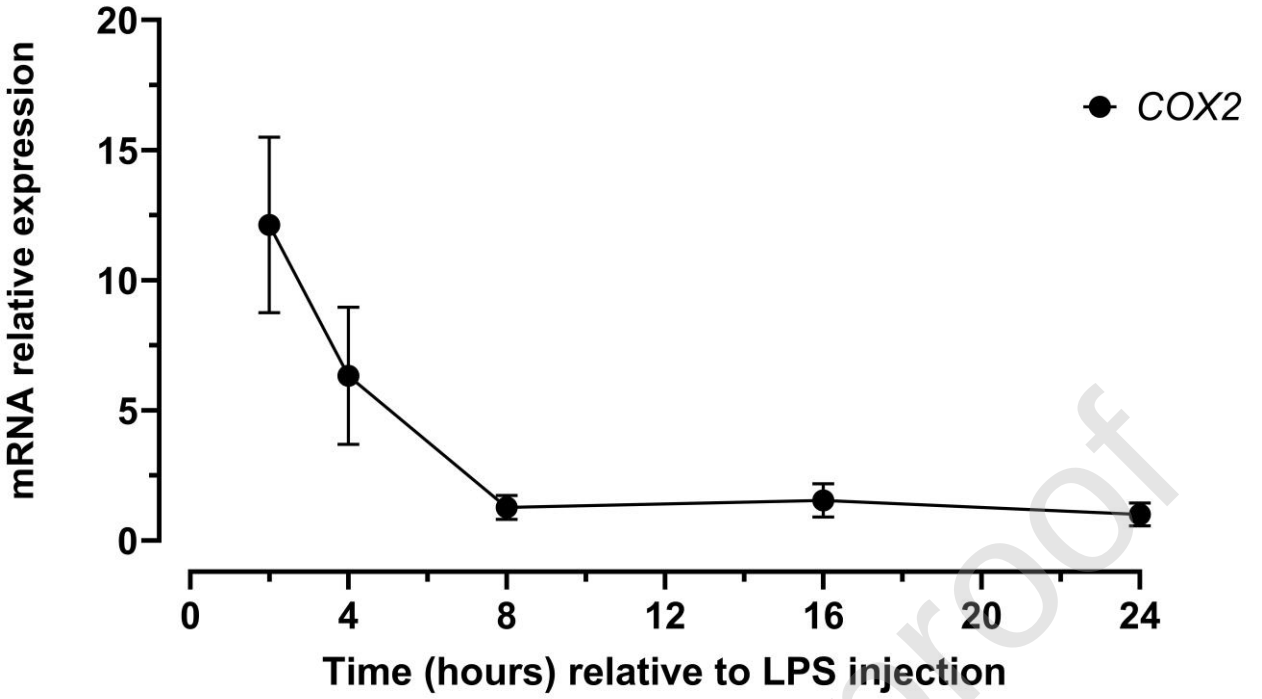

Figure 3. Changes in expression level of superoxide dismutase 2 (*SOD2*) in leukocytes isolated from equine synovial fluid (SF) after injection of LPS in one radiocarpal joint. Data points represent mean relative expression of the gene in SF from 3-6 horses (n = 4 at 2h, n = 6 at 4h, n = 4 at 8h, n = 5 at 16h, and n = 3 at 24h) ± SEM (error bars).

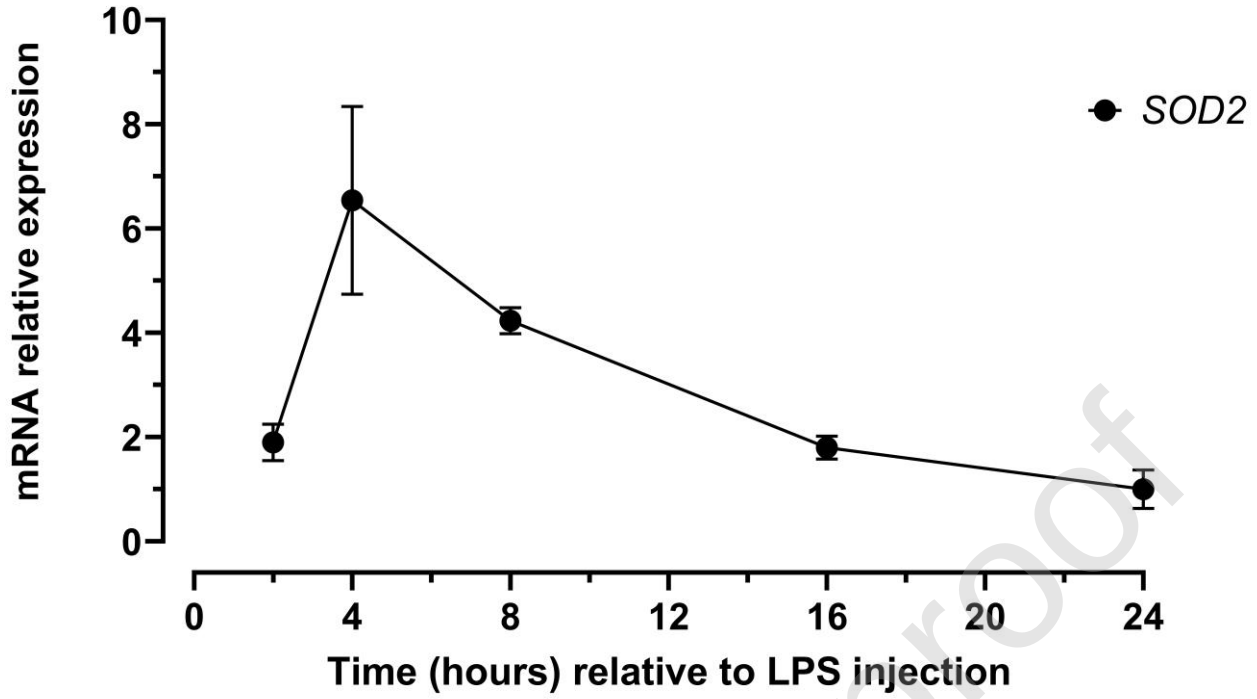

Figure 4. Changes in expression level of the two chemokines, *IL8* and C-C motif chemokine ligand 5 (*CCL5*), in leukocytes isolated from equine synovial fluid (SF) after injection of LPS in one radiocarpal joint. Data points represent mean relative expression of the genes in SF from 3-6 horses (n = 4 at 2h, n = 6 at 4h, n = 4 at 8h, n = 5 at 16h, and n = 3 at 24h) ± SEM (error bars).

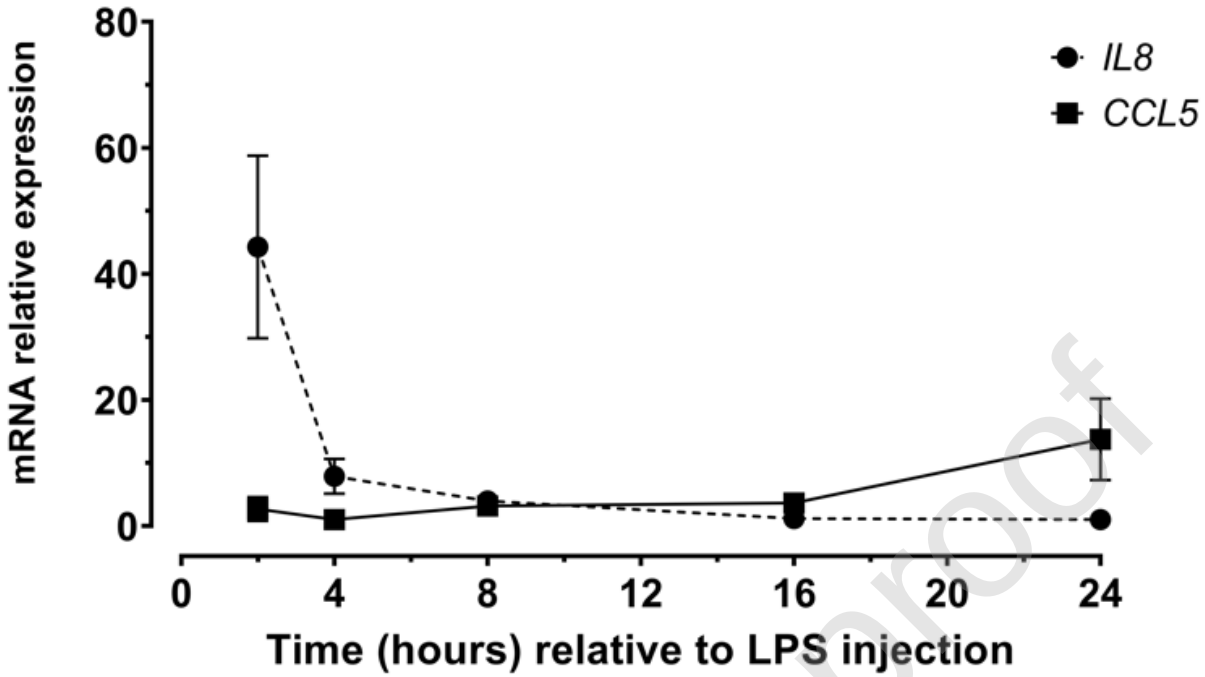

Figure 5. Changes in expression level of the two genes involved in extracellular matrix turnover, matrix metalloproteinase 8 (*MMP8*) and tissue inhibitor of matrix metalloproteinase 1 (*TIMP1*), in leukocytes isolated from equine synovial fluid (SF) after injection of LPS in one radiocarpal joint. Data points represent mean relative expression of the genes in SF from 3-6 horses (n = 4 at 2h, n = 6 at 4h, n = 4 at 8h, n = 5 at 16h, and n = 3 at 24h) ± SEM (error bars).

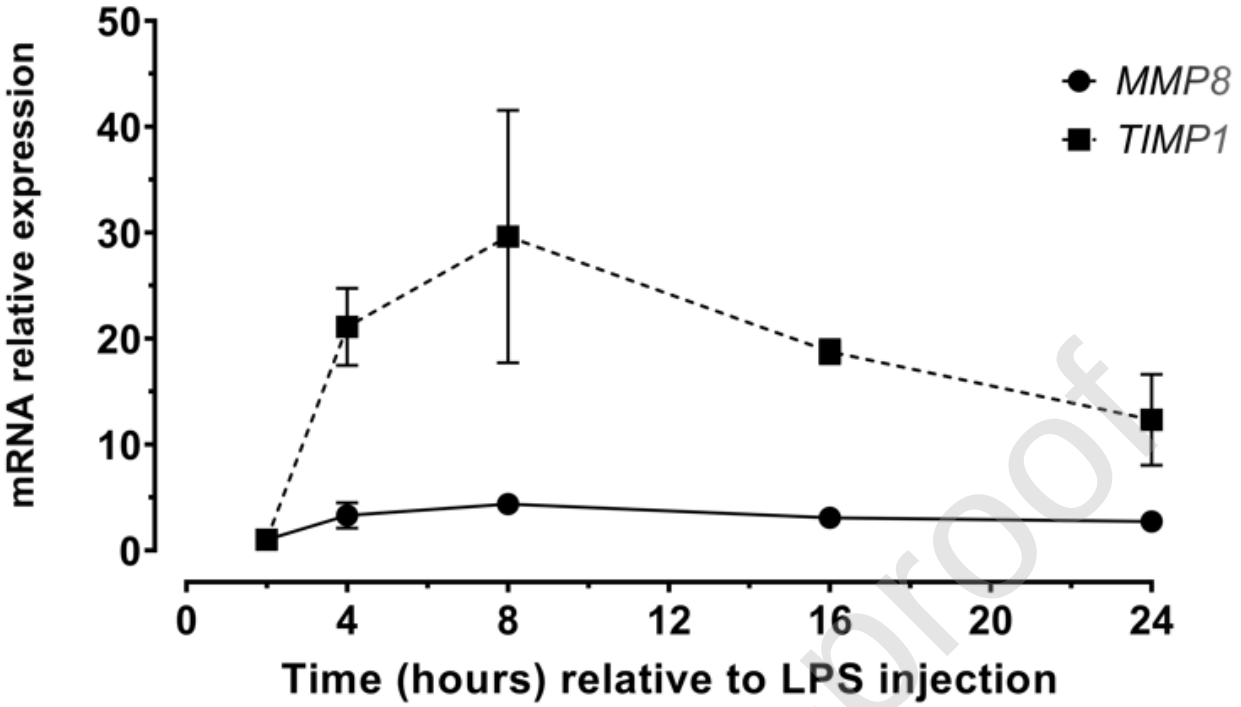

Figure 6. Changes in expression level of the two pattern recognition receptor genes, cluster of differentiation 14 (*CD14*) and toll-like receptor 4 (*TLR4*), in leukocytes isolated from equine synovial fluid (SF) after injection of LPS in one radiocarpal joint. Data points represent mean relative expression of the genes in SF from 3-6 horses (n = 4 at 2h, n = 6 at 4h, n = 4 at 8h, n = 5 at 16h, and n = 3 at 24h) ± SEM (error bars).

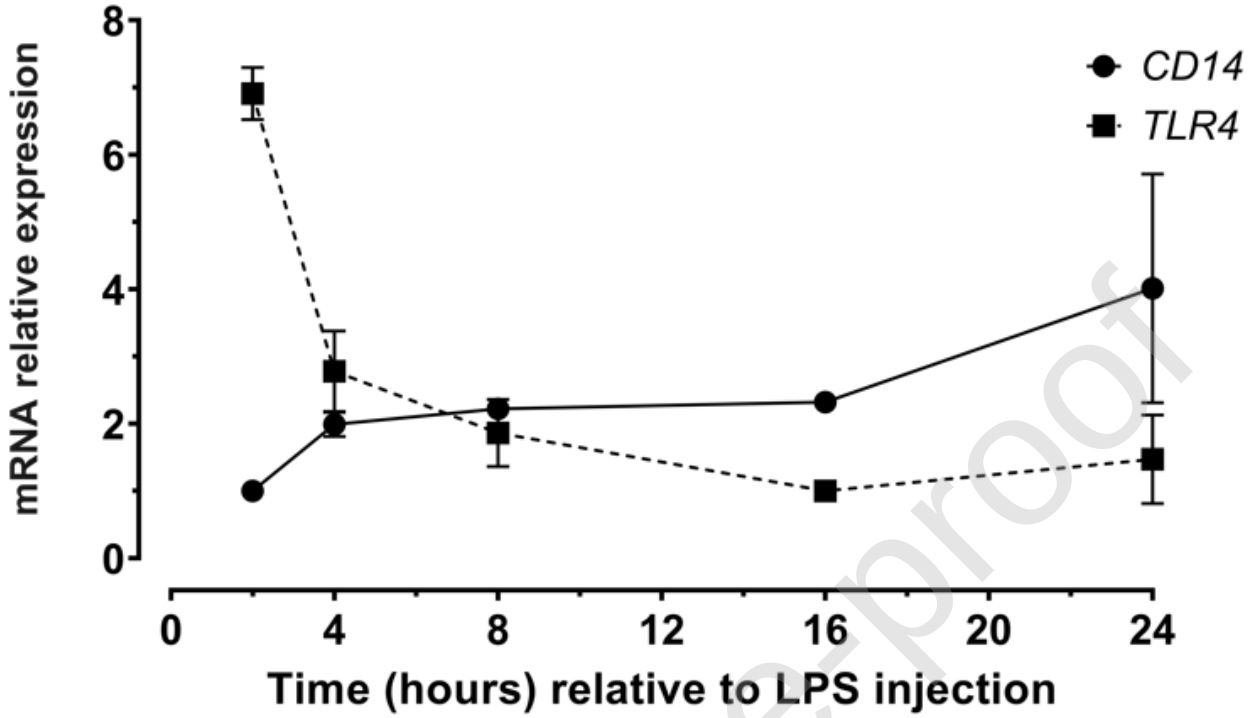

Figure 7. Changes in expression level of two apoptosis-related genes, Bcl-2-like 1 (*BCL2L1*) and caspase 3 (*CASP3*), in leukocytes isolated from equine synovial fluid (SF) after injection of LPS in one radiocarpal joint. Data points represent mean relative expression of the genes in SF from 3-6 horses (n = 4 at 2h, n = 6 at 4h, n = 4 at 8h, n = 5 at 16h, and n = 3 at 24h) ± SEM (error bars).

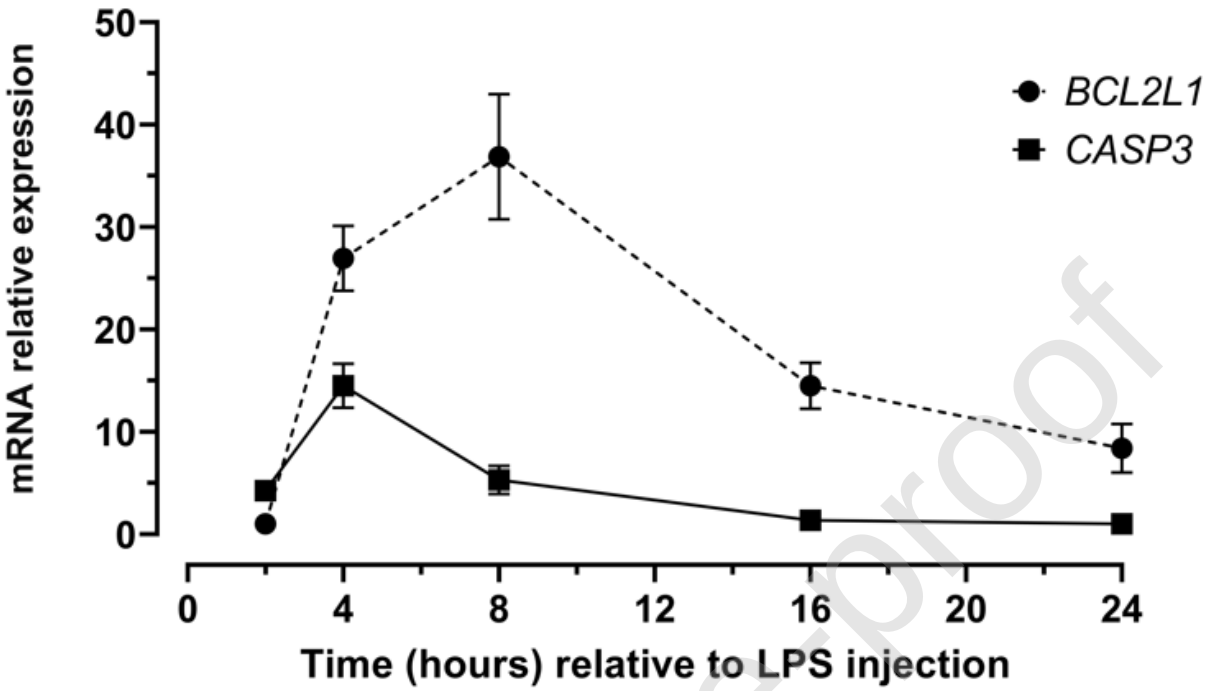

Table 1

Synovial fluid white blood cell count and differential counts (mean and range) in 3-6 horses before (0h) and 2, 4, 8, 16 and 24h after injection of LPS into one radiocarpal joint.

Outcome	**Hours relative to LPS injection**	**Mean**	**Range**
White blood cell count $x10^9$/L	0	4.2	0.2-12.0
	2	63.7	28.9-100.25
	4	117.8	25.3-207.5
	8	192.1	148.8-287.0
	16	92.1	58-114.0
	24	55.1	31.2-76.0
Neutrophils %	0	48.9	13.5-68.5
	2	96.9	95.0-98.5
	4	90.3	81.5-97.0
	8	81.6	78.5-84.5
	16	80.3	72.0-89.0
	24	78.2	73.0-83.5
Macrophages %	0	17.0	12.0-18.5
	2	1.0	0.5-1.5
	4	3.6	1.5-7.5
	8	9.0	0.5-17.0
	16	12.1	1.0-18.5

	24	13.0	2.5-25.0
Lymphocytes %	0	34.0	16.0-68
	2	1.8	0.5-4.5
	4	4.9	1.5-10.5
	8	9.3	2.0-15.0
	16	7.6	4.0-12.0
	24	7.9	2.0-14.0

Table 2. Genes differently expressed over 24h period in equine synovial fluid infiltrating leukocytes after injection of LPS in one radio carpal joint. P-values describe significant change of expression change of expression level over the 24h study period.

Gene symbol	Selected function	Maximal relative expression	Time point for lowest expression level (hours after LPS injection)	Time point for maximal expression level (hours after LPS injection)	P-value
IL1	Pro-inflammatory cytokine	98.35	24	2	<0.0001
IL6	Pro-inflammatory cytokine	205.10	24	2	0.0002

TNF	Pro-inflammatory cytokine	10.57	24	2	0.0039
IL1RN	Anti-inflammatory cytokine	13.31	24	4	<0.0001
IL10	Anti-inflammatory cytokine	51.85	2	4	0.00045
COX2 (PTGS2)	Prostaglandin synthesis	12.12	24	2	0.0008
SOD2	Oxidative stress inhibitor (scavenger)	6.54	24	4	0.0003
IL8 (CXCL8)	Chemokine	44.26	24	2	<0.0001
CCL5	Chemokine	13.73	4	24	0.0061
MMP8	Collagen degradation	4.37	2	8	0.0227
TIMP1	Metalloproteinase inhibitor	29.65	2	8	<0.0001
TLR4	Pattern-recognition receptor	6.91	16	2	<0.0001
CD14	Pattern-recognition receptor	4.01	2	24	0.001
CASP3	Apoptosis	14.50	24	4	<0.0001
BCL2L1	Apoptosis inhibitor	36.87	2	8	<0.0001
IL15	Activation of T and NK cells	6.88	16	4	0.0005

IL18	Induction of cell-mediated immunity	14.64	2	16	0.0018
ITAX	Adhesion	3.14	2	8	<0.0001
NFKBIA	Inhibitor of transcription factor (NFKB)	18.88	24	2	0.0001
IL6ST	Signal transducer	4.88	2	24	<0.0001
PLAUR	Plasminogen activation inhibitor	3.67	24	4	0.0024
HMGB1	Damage associated molecular pattern	3.27	8	24	0.0347
HIF-1A	Cell metabolism	3.89	2	4	0.0047
SLC16A	Cell metabolism	130.11	2	24	<0.0001
LDHA	Cell metabolism	4.24	2	24	<0.0001
LDHB	Cell metabolism	23.16	2	24	<0.0001