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Comparative analysis of macrophage activation in the synovium of healthy and osteoarthritic equine joints

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Abstract

Background: Synovitis is a major component of osteoarthritis and is driven primarily by macrophages. Synovial macrophages are crucial keepers of joint health by driving joint homeostasis, tissue repair and inflammation resolution (M2-like phenotype), but can also induce an inflammatory response (M1-like phenotype) when these regulatory functions are overwhelmed. Macrophage phenotypes in synovium from osteoarthritic and healthy joints are poorly characterized. Defining the patterns of synovial macrophage phenotypes in normal and osteoarthritic joints is paramount for developing targeted therapeutic approaches. The objective of this study was to compare patterns of macrophage activation phenotypes in healthy and osteoarthritic equine joints. We hypothesized that synovium from osteoarthritic joints would have increased M1-like:M2-like ratios compared to normal joints.

Methods: Gross evaluation of the articular surfaces, histology (H&E) and immunohistochemistry for M1-like (CD86), M2-like (CD206, IL-10), and pan macrophage (CD14) markers was performed on synovial biopsies from healthy (n=29) and osteoarthritic equine joints (n=26). Cytokines (MCP-1, IL-10, PGE_2, IL-1β, IL-6, TNF-α, IL-1ra) and growth factors (GM-CSF, SDF-1α+β, IGF-1, and FGF-2) in synovial fluid were quantified.

Results: All macrophage markers were co-expressed in all joints with minimal differences between OA and normal joints. Intensity of expression varied with degree of synovial inflammation. CD14, CD86, CD206, and IL-10 were more highly expressed in grossly inflamed osteoarthritic synovium, with CD86 most highly expressed. Synovial fluid MCP-1 was higher in osteoarthritic joints while SDF-1 was lower. Overall, concentrations of synovial fluid IL-10 and PGE2 was not different between OA and normal joints. Increased CD14/CD86/CD206/Il-10

expression in the synovium was associated with synovial hyperplasia, consistent with macrophage recruitment and activation in response to higher demands for repair.

Conclusions: Macrophages are not as clearly defined *in vivo* as they are *in vitro*. The course of an effective response to injury in joints should start with inflammation and be followed inflammation resolution, both of which centrally driven by macrophages. Combined, our findings suggest that homeostatic mechanisms from synovial macrophages are impaired in OA, resulting in unresolved, chronic joint inflammation. Therapeutic approaches aimed at recovering mechanisms of macrophage-driven synovial homeostasis may be more effective in treating osteoarthritis than simply inhibiting inflammation.

Keywords: joint homeostasis, osteoarthritis, synovitis, inflammation, phenotype, polarization, endogenous resolution.

Background

Osteoarthritis (OA) is a leading cause of morbidity and presents significant treatment challenges (1, 2). The pathophysiology of OA is incompletely understood; however, there is increasing evidence that the innate immune system plays a central role in the synovial inflammation leading to disease development (3, 4). Macrophage depletion studies have established that macrophages drive synovial inflammation in experimental arthritis (5, 6), which is further confirmed in clinical cases of rheumatoid arthritis (7). Recently, the specific role of synovial macrophages as the main drivers of synovial inflammation was confirmed by the presence of dramatically decreased inflammatory markers in the absence of synovial macrophages (8, 9). Activation of macrophages in osteoarthritic synovium is directly related to disease activity, severity, and pain (10, 11).

During homeostatic conditions, macrophages are key regulators of synovial integrity through phagocytic activity (i.e., clearance of foreign material, tissue debris, and efferocytosis) and secretion of synovial fluid, cytokines, chemokines, and growth factors. These macrophage functions are centrally important for joint homeostasis and chondrogenesis (4, 12). When these homeostatic functions become overwhelmed, synovial macrophages upregulate inflammation, recruiting other immune cells to respond to the increased demands for repair and homeostasis (9, 13-15).

In vitro, macrophages activate into a spectrum of cell phenotypes, with the extremes represented by cells displaying classical inflammatory (M1) or pro-resolving/healing (M2) responses (16).

In vivo, macrophages activate in response to environmental stimuli with marked phenotype plasticity, and play a fundamental role in resolving inflammation and promoting tissue repair. Their depletion or exhaustion results in severely compromised wound healing or leads to chronic inflammation (17-20). The roles of different macrophage phenotypes during health, chronic inflammation, and disease have been described in a variety of tissues (21, 22). *Ex vivo* chondrogenesis of synovial progenitor cells is impeded by classically activated (M1-like) macrophages from the osteoarthritic synovium (23). Alternatively activated (M2-like) macrophages are required for efficient chondrogenesis (12). Inflammation in arthritic joints is dampened by M2-like macrophages, improving clinical and histological signs of joint disease (14, 24, 25). Collectively, these findings suggest that enhancing the M2-like response in diseased joints may provide a mechanism for resolving inflammation and restoring a healthy synovial environment with improved capacity for tissue repair.

Specific information regarding macrophage phenotypes in joint disease is limited to *in vitro* studies, experimental data, or end stage OA. Reports describing comparisons of diseased and healthy joints include extrapolations from other types of arthritides, such as rheumatoid arthritis or are limited to the low numbers of synovial fluid macrophages shedding from the synovium following hyperactivation (6, 9, 12, 23, 26, 27). Defining patterns of macrophage activation in normal and osteoarthritic synovium is paramount to enhancing the understanding of the roles of macrophages *in vivo,* and to optimize strategies targeting macrophage-driven joint homeostasis (25). This study was designed to compare the patterns of macrophage activation phenotypes in biopsies of healthy and osteoarthritic equine synovium, and to correlate macrophage phenotypes with gross pathology, histology, and synovial fluid cytokine, chemokine, and growth factor

concentrations. We hypothesized that synovial macrophages in osteoarthritic joints would exhibit increased ratios of M1:M2(-like) marker expression compared to healthy joints, and that differences in concentrations of pro- and anti-inflammatory cytokines in synovial fluid of osteoarthritic and normal joints would associate with differences in M1:M2(-like) macrophage ratios in synovium biopsies.

Methods

Experimental design

Synovial fluid and synovial membrane biopsies were collected from 26 osteoarthritic joints (16 metacarpophalangeal [MCP] joints and 10 radiocarpal/middle carpal joints) of horses undergoing arthroscopy or following euthanasia at the Hagyard Equine Medical Institute (Lexington, KY) or the Virginia-Maryland College of Veterinary Medicine (Blacksburg, VA). Control samples from normal joints (15 MCP and 14 carpal joints) were collected at the same hospitals from horses without history and evidence of lameness referable to the harvested joints and with grossly normal articular surfaces at euthanasia. All procedures were approved by the Institutional Animal Care and Use Committee and written owner consent. Synovial inflammation and concentrations of pro- and anti-inflammatory cytokines and growth factors were assessed by gross pathology, synovial membrane histology, synovial fluid cytology, and immunoassays for cytokine, chemokine and growth factor quantification. Synovial macrophage phenotype activation *in situ* was defined by immunohistochemistry.

Inclusion criteria

A total of 29 horses 3-15 years old (skeletally mature, but not aged) were recruited and lameness exams performed, including response to joint manipulation, joint flexion, gait analysis at the trot and radiography. Diagnostic analgesia was performed at the discretion of the referring veterinarian, and therefore not in all horses. Inclusion was based on arthroscopic or post mortem findings of cartilage abnormalities according to the OARSI scale (0-3) (28). Only moderate OA (OARSI grade 2) joints were included, as representative of those most commonly treated clinically and when synovial cellularity is highest (29). As per the OARSI guideline, carpal joints were selected according to degree of macroscopic cartilage erosion, and MCP joints were included if presenting a score 2 of one of the three macroscopic diagnostic parameters (wear lines, erosion or palmar arthroses). Horses with a history of septic arthritis, non-steroidal anti-inflammatory therapy or intra-articular diagnostic anesthesia within 2 weeks, intra-articular corticosteroids within 2 months prior to sample collection, or evidence of osteochondrosis were excluded. Only healthy horses with body score condition between 4 and 6 were considered.

Sample collection

Synovial fluid (2 mL) was aseptically collected and aliquoted (EDTA and Protein LoBind microfuge tubes, Eppendorf®, Westbury, CT). Anticoagulant-free synovial fluid was immediately centrifuged (12,000xg; 10 min; 4°C) and the supernatant stored at -20°C for cytokine and growth factor quantification. Two synovial membrane biopsies were obtained from each OA joint adjacent to the major cartilage alterations (30). Control samples were harvested at sites where each joint is traditionally most commonly affected (28). Samples were fixed (AZF Fixative®; Newcomer Supply, WI) at room temperature for 24 hours, rinsed and stored in PBS at 4°C until processing.

197

198 *Synovial fluid analysis*

199 Synovial fluid cytology was processed for total nucleated cell count (TNCC) by hemocytometer
200 and total protein (TP) by refractometer. Differential cell counts were performed following
201 Romanowski stain (Microscopy Hemacolor®, Merck, Germany). Pro- (IL-1β, IL-6, GM-CSF,
202 TNF-α) and anti-inflammatory cytokines (IL-10, IL-1ra), chemokines (MCP-1, SDF-1), growth
203 factors (IGF-1, FGF-2), and PGE_2 in synovial fluid were quantified. Thawed samples (200 µL)
204 were hyaluronidase-digested (10 µL of 100 IU hyaluronidase/mL acetate buffer; Worthington
205 Biochemical Corporation, Lakewood, NJ) for 30 minutes at 37°C, centrifuged (12,000xg; 10
206 min; 4°C), and the supernatant recovered. Based on previous experience and interfering factors
207 in cytokine detection in synovial fluid (31, 32), spike-and-recovery assays were performed for
208 the PGE_2 ELISA and 4 representative serially-diluted targets in the multiplex assay (IL-1β, IL-6,
209 IL-10, TNF-α). Based on the results, a dilution of 1:2 was selected for PGE_2 quantification and
210 no dilution was deemed necessary for the multiplex assay.

211

212 PGE_2 was quantified by ELISA (R&D Systems, Minneapolis, MN). Hyaluronidase-digested
213 samples were solid-phase extracted (500 µL synovial fluid in 490 µL 100% ethanol and 10 µL
214 glacial acetic acid incubated at 23°C for 5 minutes), centrifuged (2,500xg; 8 min; RT), and the
215 supernatant collected. Remaining analytes were quantified by bead-based multiplex assay
216 (MILLIPLEX MAP Equine Cytokine/Chemokine Multiplex Assay with manufacturer
217 modification to include IGF-1, SDF-1, and IL-1ra; Luminex 200 plate reader Millipore Sigma,
218 Burlington, MA).

219

Synovial membrane histology and immunohistochemistry

Fixed synovial membrane biopsies were paraffin-embedded, sectioned at 5 µm, and H&E-stained. Synovitis was scored based on the OARSI histopathology guide and included cell infiltration, vascularity, hyperplasia, edema, and fibrosis (28). For immunohistochemistry, tissue sections were baked at 66°C overnight, deparaffinized, and incubated in antigen recovery solution (Antigen Retrieval Citra Plus, BioGenex, Fremont, CA) at 95°C for 10 minutes. Slides were stained (Super Sensitive™ Polymer-HRP IHC Detection System, BioGenex) using antibodies targeting the following markers: pan macrophage (equine CD14, Wagner Lab, Cornell University); M1 (mouse anti-human CD86 [clone 2331(FUN-1), BD Biosciences, San Jose, CA]; M2 (CD 206 [clone ab64693, Abcam, Cambridge, UK]); and IL-10 (equine IL-10, Wagner Lab). Markers were selected based on the extensive literature review (CD14 (33-37), CD 86 (38-41), CD206 (42-47), and IL-10 (22, 48-50)) and specificity or validated cross-reactivity to equine samples (51-54). Cell staining was assessed in a semi-quantitative fashion as previously described (55): absent (0); mild (1); moderate (2); or intense (3). Staining distribution across synovial villi was also scored: base of the synovial villus only (1); reaching portions of the synovial villus tip (2); and throughout the entire synovial villus (3). Staining patterns were scored on 3 tissue sections and averaged. Composite scores for each marker were compared between groups for both histology and immunohistochemistry (cell staining + staining distribution).

Statistical analysis

Data analysis was performed using SAS version 9.4 (SAS Institute, Inc, Cary, NC). Effects of different joints (carpi vs. MCP) sampled and effects of disease (normal vs. OA) on outcomes

were assessed using linear General Estimating Equations (GEE) in an incomplete block design. Each of the linear models specified joint, disease, and the interaction between joint and disease as fixed effects. Correlation between observations within horse (the blocking factor) were modeled by specifying a compound symmetry covariance matrix. The interaction between joint and disease was further analyzed (sliced) to extract comparisons between disease conditions within joint. Scatterplots and analysis of covariance models were used to determine associations between synovial fluid cytology, synovial membrane histology, and synovial membrane immunohistochemistry parameters with joint condition (normal vs. OA). For the analysis of covariance models, immunohistochemistry parameters (macrophage markers) were specified as covariates (one parameter at a time) while disease was the design effect. Statistical significance was set to $p < 0.05$.

Results

Characterization of study population

The experimental population was composed of 11 females and 18 castrated males. Normal and OA samples were harvested from 13 horses, OA samples only from 8, and only normal samples from 7 horses. The mean age of horses used to harvest normal samples (7.4 years) was similar to those with OA (6.0 years) and comparable to horses used for both purposes (8 years). OA joints were from horses exhibiting grade 1-3 out of 5 lameness (51), localized to the selected joint.

Synovial fluid cytology

Overall, TP ($p = 0.0331$) and TNCC ($p = 0.0532$) were significantly higher in OA compared to normal joints (Table 1). No overall differences were detected between normal and OA joints for differential cell counts, and monocytes were the predominant cell type in all groups. No significant differences were observed between normal and OA carpal or MCP joints for any of the cytological parameters.

Cytokine/chemokine and growth factor quantification

GM-CSF was below detectable limits (3.7 pg/mL) for all samples. Detection of MCP-1, SDF-1α+β, IL-10, and PGE_2, was possible in the majority of samples. The remaining analytes (IL-1β, IL-6, TNF-α, IL-1ra, IGF-1, and FGF-2) were detected in only a minority of samples, precluding statistical analysis (Table 2). Overall, MCP-1 concentrations were significantly higher in OA than normal joints ($p = 0.0443$). SDF-1 concentrations were significantly lower in the overall comparison of OA to normal joints ($p = 0.0243$) and within normal and OA MCP joints ($p = 0.0378$). Overall, there was no difference in IL-10 concentrations between normal and OA joints; however, IL-10 was significantly lower in OA compared to normal MCP joints ($p = 0.0462$). Concentrations of PGE_2 did not vary overall or when examined by joint.

Synovial membrane histology

Overall scores for intimal hyperplasia ($p = 0.0076$) and subintimal edema ($p = 0.0514$) were significantly higher in OA compared to normal joints (Figure 1), while those for cell infiltration ($p = 0.0818$), vascularity ($p = 0.1398$), and fibrosis ($p = 0.3053$) were higher for OA joints, but not significantly (Table 3). The composite of these individual scores was significantly higher overall in OA compared to normal joints ($p = 0.0122$). Within MCP joints, only subintimal

edema was significantly higher in OA joints compared to normal ($p = 0.0158$). Within carpal joints, intimal hyperplasia ($p = 0.0103$) and composite scores ($p = 0.0420$) were significantly higher in OA joints. In a subset of OA joints with gross signs of inflammation, histological parameters were increased, with a notable pattern of increased synovial vascularity and shedding of markedly hyperplastic cells in the outermost layer of the intima, where cell nuclei were often decondensed with decreased hematoxylin uptake (Figure 2).

Synovial membrane immunohistochemistry

Overall, composite values of expression for all markers (CD14, CD86, CD206, and IL-10) was higher in OA compared to normal joints, yet only significantly for CD14 ($p = 0.0157$; Table 4). These differences were most apparent in the synovium from MCP joints, and again, only significant for CD14 when comparing normal and OA MCP joints ($p = 0.0279$). In both MCP and carpal joints, expression of CD206 was higher in OA joints, although not significant. Staining for all markers was most intense around blood vessels, especially over endothelial cells (Figure 3). Overall, staining for macrophage markers (CD14, CD86, and CD206) was limited primarily to the area immediately adjacent to cells and cell aggregates within the synovial intima and sub intima, whereas staining for IL-10 was diffuse throughout the synovial tissue in both normal and OA joints. The distribution of macrophage markers across the synovial villi differed between normal and OA joints. In normal joints, staining was largely limited to the base of synovial villi, while in OA joints the tip of villi was also frequently stained (Figure 4). When observed in normal joints, staining for macrophage markers at the tip of villi was subtle and primarily located at scattered areas of the synovial lining around cell nuclei. In contrast, staining patterns in OA joints were more diffusely distributed in the synovial lining around cell nuclei.

In the subset of OA joints with gross signs of synovitis, staining for CD86 was intense. A similar, but less consistent pattern was observed for CD14, IL-10, and CD206. For 8 horses, it was possible to compare normal and OA carpal or MCP joints from the same individual. Gross signs of synovitis were present in half of these OA joints (Figure 4). While increased expression of all markers in OA joints varied in intensity, CD86 expression was the most intense and consistently increased, distributed throughout the synovium and with intense staining in the intima and subintima. Three of these samples represented the highest CD86 staining scores among all samples of our study.

Discussion

This is the first study comparing macrophage phenotypes in the synovium from normal joints to those with naturally occurring OA. Markers widely used to define M1- (CD86) and M2-like (CD206 and IL-10) macrophages were co-expressed in both groups with mild differences. Intensity of expression did vary with degree of synovial inflammation. While expression of all markers was mildly increased in OA joints with low-grade inflammation (majority), it was markedly increased in grossly inflamed OA joints, with CD86 most highly expressed. Combined expression of M1- and M2-like macrophage markers (6, 23, 56) and their increased expression proportionate to inflammation *in vivo* (9, 25) are reported. Although none of the parameters analyzed revealed significant or marginal associations with joint condition, histochemical and histologic findings were consistent with lower concentrations of synovial fluid IL-10 and SDF-1, and higher concentrations of MCP-1 in OA. Our findings, along with previous reports (24, 57-

62), suggest that, *in vivo,* macrophages are by default homeostatic cells that, following injury, drive inflammation with the purpose of counteracting tissue aggressors and guiding tissue repair. In the chronic inflammation of OA, higher synovial macrophage recruitment and activation, combined with decreased synovial fluid IL-10 and SDF-1 concentrations, as observed in our study, suggests that regulatory functions of macrophages are impaired. As such, macrophage-derived pro-resolving cytokines were insufficient, preventing recovery of joint homeostasis (17, 18, 25, 58-61, 63)

The concept of macrophage activation as either inflammatory (M1) or regulatory (M2) originated from monocyte-derived macrophages treated *in vitro* with defined and overwhelming cytokine stimuli (16, 46, 64). Clear identification of macrophage phenotypes *in vivo* is significantly more complex than proposed by *in vitro* models (6, 11, 17, 27). Increased CD14 expression during OA, as observed in our study, combined with increased macrophage recruitment and activation corroborated by histology, is consistent with previous reports of increased soluble CD14 in the synovial fluid of OA joints, and correlates with disease activity and clinical signs (65) Although CD86 and CD206 expression have historically been considered markers of M1- and M2-like macrophages (9, 23, 46, 64), this is an oversimplification of events that occur *in vivo* (11, 17, 66). CD86 is constitutively expressed by early myeloid cells and resting macrophages, and increased CD86 expression is part of the cellular checkpoints required for monocytic lineage commitment, activation, and survival (59). Therefore, as observed in the control joints in this study and additional reports (17, 25), CD86 is poorly associated with an inflammatory phenotype *in vivo*.

Similar to CD86, the mannose receptor (CD206) has a pivotal function in host defenses during inflammation, clearance of debris, wound healing and remodeling, and resolution of inflammation. CD206 is also constitutively expressed in mature mononuclear phagocytes and the intensity of its expression is proportionate to demands for anabolic cytokine secretion, efferocytosis, and sensing of damage-associated molecular patterns (61). Thus, the expression of both CD86 and CD206 increase with inflammatory stimuli, as a result of increased macrophage recruitment and response to injury (6), and therefore should be carefully analyzed in conjunction with clinical and analytical indicators of disease. Although expression of CD86 and CD206 was reported to associate to M1- and M2-like macrophages in the synovial fluid from normal and OA joints (26), this observation is in disagreement with the profiles of macrophages in the synovium in this and other experimental studies (6, 23, 25).

Like CD86 and CD206, expression of IL-10 in the synovial membrane in our study was directly associated with the degree of synovial inflammation. After injury, macrophage activation leads to increased expression of IL-1, IL6, and TNF-α, which is followed by proportional increases in expression of IL-10 as a compensatory, negative feedback (67-69). Consequently, the production of these pro-inflammatory cytokines decreases (67). However, if the injurious challenge persists, this cytokine feedback loop is sustained, as shown by increased synovium expression of IL-10 in our OA joints compared to normal, especially grossly inflamed joints (68, 70). Therefore, marked staining in grossly inflamed joints suggests that the dynamics of cell recruitment and activation during inflammation (increased CD14, CD206, and CD86), and compensatory negative feedback (IL-10) are being persistently triggered in the vicious cycle of inflammation seen in OA (6, 11, 59, 60, 67, 68, 70-72).

The overall lack of difference in synovial fluid IL-10 concentrations between normal and OA joints is not surprising (68, 72, 73). Intra- and peri-articular IL-10 oscillates and increases after exercise as a homeostatic response following physiologic mechanical stress (73), and could explain why IL-10 concentrations are increased in highly inflamed joints with rheumatoid arthritis (68, 72). During chronic low-grade inflammation, concentrations of synovial fluid IL-10 regress close to baseline (72, 73). An *in vitro* study challenging monocytes from OA and healthy people reported that patients with no significant IL-10 increase following challenge were three times more likely to develop OA compared to those responding with a significant increase (69). This could explain the significantly lower concentrations of IL-10 in the synovial fluid of OA MCP joints in our study, and suggests that in OA joint mechanisms compensating for tissue damage may be impaired or overwhelmed. As a matter of fact, injection of inflamed joints with autologous bone marrow-derived macrophages results in marked clinical improvement, decreased markers of inflammation, and increased synovial fluid concentrations of IL-10 and IL-10^+ macrophages (25, 74). Inflamed joints treated with IL-10-expressing macrophages were comparable to healthy joints histologically, whereas PBS-treated controls remained severely inflamed. Combined, these studies reinforce the important role of IL-10-producing macrophages in driving resolution of inflammation and promoting joint homeostasis (25, 74).

In response to injury, synovial macrophages form a protective immunological barrier in the synovial lining, protecting intra-articular structures. Exchange of solutes and cells from the sub-synovial to intra-articular space (15) is restricted and could explain higher IL-10 staining in the synovium from OA fetlocks, which had lower synovial fluid IL-10 concentrations than normal

joints. During overwhelming inflammation, this tight-junction barrier is lost (75), allowing free exchange of cellular and molecular components between intra-articular and sub-synovial spaces and could explain the overall similar IL-10 staining patterns in normal and OA carpi (15). Importantly, each of these mechanisms can be affected by the stage of the inflammatory response (acute-chronic / mild-severe), which was not part of our study design.

Increased overall synovial fluid MCP-1 in OA concomitant with gross signs of inflammation and clinical signs is consistent with the literature (76-78). During synovial inflammation, MCP-1 contributes to recruitment, homing, and accumulation of mononuclear cells in the synovial fluid and membrane (68, 79), as part of the homeostatic response to joint damage (15). As such, MCP-1-deficient mice are unable to home macrophages to sites of injury and are prone to infection and chronic inflammation (68, 80). Higher SDF-1 in synovial fluid from normal versus OA joints in our study is inconsistent with previous studies (81-83). SDF-1 has multifaceted roles in synovial tissue biology, including homeostatic and pro-inflammatory functions (81, 83). SDF-1 is reportedly expressed proportionate to disease activity, with higher concentrations in inflamed joints (81-83). Our results showing lower synovial fluid SDF-1 concentrations in OA joints is comparable to two other studies from our lab, where inflammation decreased synovial fluid SDF-1 (25).

Traditionally, IL-1β and TNF-α have been considered the main drivers of disease processes in OA (84-87). However, these two classic inflammatory cytokines were detected in less than half of our samples with no significant differences between normal and OA samples, similar to previous reports (88, 89). Limitations in the detection of IL-1 in synovial fluid are widely

426 reported, even in samples from patients experiencing marked inflammation (31, 32, 90, 91).
427 Recent proteomic analysis of synovial fluid and genome-wide transcriptomic analysis of
428 cartilage comparing samples from OA and healthy joints did not identify IL-1 or TNF-α as
429 central targets (92, 93). PGE_2 has also been used as an important marker of joint inflammation
430 (8, 94, 95). However, PGE_2 also plays anti-inflammatory and anabolic roles such as inhibition of
431 inflammatory cytokines and neutrophil infiltration to the site of injury, chondrocyte protection,
432 and activation of pro-resolving macrophages (18, 46, 96, 97). PGE_2 generated during the early
433 inflammatory response can induce inflammation resolution by upregulating the synthesis of
434 potent mediators of resolution (18). Therefore, PGE_2 is involved in both inciting and resolving
435 inflammation and concentrations in synovial fluid vary with the stage of response to injury, and
436 may explain the lack of differences between normal and OA joints in our study.
437
438 Limitations of this study include the reduced cohort size, particularly considering the number of
439 outcomes measured. This study was not designed to infer causality of our findings in the
440 development and progression of OA, and therefore the meaning of our observations are
441 interpreted based on the literature and additional studies from our lab. Quantifying soluble
442 CD14 in the synovial fluid could have reinforced the role of macrophage activation in joint
443 inflammation and disease progression, yet such observations have already been reported, and
444 similar to our study, associated with increased MCP-1 concentrations in synovial fluid (78).
445 Immunoblots comparing the activity of the TLR-4 – NFκB-IL-10 axis between the synovium of
446 normal and OA joints, as well as quantification of other pro-resolving mediators in the samples
447 of this study, would have provided additional information for understanding the mechanism by
448 which drivers of joint homeostasis become overwhelmed.

Conclusions

Horses are an established translational model to study OA (1). The majority of parameters investigated in our study, pragmatically called pro- or anti-inflammatory, are building blocks of a complex immune response and must be carefully interpreted, with attention to the phases of the inflammatory response, including its resolution. Secretion of pro- and anti-inflammatory mediators increase simultaneously after injury, decreasing when inflammation is efficiently resolved (18, 60, 71). Inflammation resolution is an active process, largely orchestrated by macrophages, and requires lipid mediators produced during the acute inflammatory response. Thus, the idea of inhibiting inflammation as a therapy may need to be revisited (18). An alternative way of thinking about the treatment of OA is to stimulate endogenous resolution of inflammation by increasing the innate homeostatic mechanisms of the joint, rather than simply blocking inflammation through the use of non-steroidal anti-inflammatory drugs and corticosteroids. Developing approaches to improve the homeostatic response by healthy macrophages in OA joints has the potential to resolve joint inflammation and re-establish an anabolic synovial environment and overall joint health.

Abbreviations

M1, classically activated/proinflammatory; M2, suppressive/healing; MCP-1, macrophage chemoattractant protein 1; OA, osteoarthritis; PGE_2, prostaglandin E_2; SDF-1, stromal cell–derived factor 1; TNCC, total nucleated cell count; TP, total protein; CD14, Cluster of differentiation 14/LPS coreceptor along with Toll-like receptor 4— proposed mature

macrophage marker; CD86, Cluster of differentiation 86/T cell costimulatory receptor (B7.2)—proposed M1 marker; CD206, Cluster of differentiation 206/Mannose receptor 1 (MRC1)—proposed M2 marker.

Declarations

Ethics approval and consent to participate

This study was conducted in compliance with the Animal Welfare Act and the approval of the Virginia Tech Institutional Animal Care and Use Committee. Written informed consent was received from owners prior to horse inclusion in the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

BCM, DHR, SRW, and LAD contributed substantially to study conception and design. BCM and DHR collected samples. BCM and KG was primarily responsible for data acquisition, analysis, and interpretation. KG, AO, and YN assisted BCM with data collection and assembly. SHB supervised the synovial fluid cytology performed by BCM and KG. SRW performed statistical analysis and consulted on its interpretation. BCM and LAD were responsible for manuscript preparation. All authors reviewed the final manuscript.

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Tables

Table 1: Synovial fluid cytology from normal and OA equine metacarpophalangeal and carpal joints (median, 95% CI).

		Synovial Fluid Cytology				
		Total Protein g/dL	**TNCC** Cells/µL	**Monocytes** %	**Lymphocytes** %	**Neutrophils%** %
Metacarpo-phalangeal	**Control**	2.1 (1.5-2.4)	91 (24-256)	65 (55-73)	28 (24-43)	0 (0-3)
	OA	2.7 (1.1-3.9)	68 (21-607)	68 (49-79)	27 (4-44)	0 (0-3)
	P-value	*P=0.1402*	*P=0.6253*	*P=0.6278*	*P=5702*	*P=0.5805*
Carpi	**Control**	2.4 (1.6-2.8)	24 (19-221)	58 (50-67)	33 (28-48)	2 (0-3)
	OA	3.1 (1.7-3.8)	124 (14-204)	61 (46-77)	31 (16-39)	2 (0-19)
	P-value	*P=0.0595*	*P=0.3370*	*P=0.8715*	*P=2167*	*P=0.2251*
Combined	**Control**	2.1 (1.9-2.4)	91 (24-156)	64 (55-71)	30 (25-43)	1 (0-3)
	OA	2.7 (1.8-3.6)	110 (36-173)	65 (54-73)	29 (18-39)	0 (0-3)
	P-value	***P=0.0331***	***P=0.0532***	*P=0.8780*	*P=2699*	*P=0.1995*

Significant differences between samples from control and OA joints were not detected in this comparison; Abbreviations: CI = Confidence Interval; TNCC = Total Nucleated Cells

Table 2: Synovial fluid cytokines in normal and OA equine metacarpophalangeal and carpal joints (median, 95% CI).

		Analytes									
		FGF-2	**IGF-1**	**IL-1β**	**IL-6**	**IL1-ra**	**MCP-1**	**SDF-1**	**IL-10**	**PGE_2**	**TNF-α**
Min. D.C.		11.5 pg/mL	0.3 pg/mL	15.5 pg/mL	2.3 pg/mL	0.02 pg/mL	9 pg/mL	20.5 pg/mL	23.2 pg/mL	39 pg/mL	1.5 pg/mL
Metacarpophalangeal	**Control** N=15	N= 4		N=4	N=5		N=10	N=14	N=15	N=14	N=5
		44* (23-137)	U	335* (55-617)	6* (3-19)	U	799 (128-1508)	241 (129-292)	86 (55-97)	69 (53-73)	3.5* (2-10)
	OA N=16	N=3	N=1	N=5	N=5	N=1	N=13	N=13	N=11	N=13	N=3
		20* (13-53)	506*	44 (28-4014)	25 (3-65)	3*	773 (128-1463)	137 (89-208)	68 (40-96)	71 (53-75)	5* (3-24)
	P-value	-	-	-	-	-	*P=0.0803*	***P=0.0378***	***P=0.0462***	*P=0.7206*	-
Carpi	**Control** N= 14	N=2	N=2	N=7	N=6	N=4	N=14	N=14	N=14	N=14	N=5
		78* (16-141)	1917* (196-3639)	199 (21-5501)	13 (3-89)	6* (1-219)	786 (230-1867)	334 (152-467)	64 (41-98)	67 (53-73)	9* (6-35)
	OA N=10	N=3	N=4	N=6	N=5	N=1	N=10	N=10	N=10	N=10	N=5
		18* (16-42)	270* (83-863)	169 (18-695)	11 (3-172)	15*	933 (260-2526)	267 (73-498)	64 (57-108)	73 (62-83)	5 (3-41)
	P-value	-	-	-	-	-	*P=0.1360*	*P=0.1943*	*P=0.7362*	*P=0.3740*	-
Combined	**Control** N= 14	N=2	N=2	N=11	N=11	N=4	N=31	N=30	N=31	N=29	N=10
		44 (16-141)	1917 (196-3639)	283 (42-1014)	8 (3-22)	6* (1-219)	799 (240-1508)	276 (188-320)	80 (55-92)	68 (58-72)	6 (3-11)
	OA N=10	N=5	N=5	N=11	N=12	N=2	N=24	N=24	N=22	N=23	N=5
		19 (12-53)	407 (83-863)	64 (18-4014)	18 (4-40)	9* (3-14)	880 (442-1096)	150 (109-278)	66 (57-92)	71 (64-75)	5 (3-25)
	P-value	-	-	-	-	-	***P=0.0443***	***P=0.0243***	*P=0.2052*	*P=0.5159*	-

Significant differences were only detected for SDF1 and IL-10 concentrations, between samples from control and OA fetlocks, and for MCP-1 between control and OA carpi; Abbreviations: CI

798 = Confidence Interval; U = Undetected; - = *p*-values could not be determined due to small

799 number of samples in which the analyte was detected; * = the actual confidence level is < 95%.

Table 3: Individual and composite histological parameters for H&E-stained equine synovial membrane (median, 95% CI).

Synovial Membrane Histology							
		Cell Infiltration	Vascularity	Intimal Hyperplasia	Subintimal Edema	Fibrosis	Composite Scores
Metacarpo-phalangeal	Control	2 (1-2)	2 (1-3)	1 (0-1)	1 (0-2)	2 (2-3)	7 (4-11)
	OA	2 (1-3)	3 (1-4)	1 (0-3)	1.5 (1-3)	2 (1-3)	9.5 (6-14)
	P-value	*P=0.3084*	*P=0.1099*	*P=0.1747*	***P=0.0158***	*P=0.8501*	*P=0.0711*
Carpi	Control	2 (1-2)	2 (1-2)	0.5 (0-1)	1 (1-2)	2 (1-3)	8.5 (5-9)
	OA	2 (1-3)	2 (0-3)	1 (1-2)	2 (0-3)	3 (1-3)	9.5 (7-12)
	P-value	*P=0.1195*	*P=0.7087*	***P=0.0103***	*P=0.5231*	*P=0.1973*	***P=0.0420***
Combined	Control	2 (1-2)	2 (1-2)	1[ǂ] (0-1)	1 (1-2)	2 (1-3)	8 (6-9)
	OA	2 (1-3)	2.5 (0-4)	1[ǂ] (0-2)	2 (1-3)	2.5 (2-3)	9.5 (7-12)
	P-value	*P=0.0818*	*P=0.1398*	***P=0.0076***	***P=0.0514***	*P=0.3053*	***P=0.0122***

Synovial Hyperplasia was significantly higher in OA carpi, while Subintimal Edema was significantly higher in OA metacarpophalangeal (MCP) joints; Abbreviation: CI = Confidence Interval; Observation: [ǂ] = the categorical nature of the data produces median values that are equal between groups. However, the frequency of scores differ between groups, where OA joints have overall higher scores of intimal hyperplasia.

Table 4: Composite immunohistochemical scores macrophage marker in normal and osteoarthritic joints (median, 95% CI).

Synovial Membrane Immunostaining					
		CD14	**CD86**	**CD206**	**IL-10**
Metacarpo-phalangeal	**Control**	4 (0-5)	4 (0-6)	4 (4-6)	5 (4-6)
	OA	5 (0-7)	5 (0-7)	5 (0-6)	6 (4-6)
	P-value	**P=0.0279**	P=0.8593	P=0.2987	P=0.1551
Carpi	**Control**	5 (4-6)	6 (4-6)	4 (0-6)	6 (5-6)
	OA	5 (0-6)	6 (5-8)	5.5 (4-7)	6 (5-7)
	P-value	P=0.1135	P=0.2099	P=0.1161	P=0.8826
Combined	**Control**	5 (0-5)	5 (4-6)	5 (0-6)	5 (5-6)
	OA	6 (4-6)	6 (5-7)	5 (4-6)	6 (5-6)
	P-value	**P=0.0157**	P=0.3677	P=0.5943	P=0.3651

Osteoarthritic metacarpophalangeal joints (MCP) exhibited increased expression of all markers, while only CD206 expression was higher in OA carpi; Abbreviation: CI = Confidence Interval

Figure Legends

Figure 1: Representative images demonstrating (arrowheads) the differences between normal and osteoarthritic (OA) joints for Intimal Hyperplasia and Subintimal Edema.

Figure 2: Compared to osteoarthritic joints with no or minimal signs of inflammation (A), OA joints exhibiting gross signs of synovitis (D; black arrow), exhibited increased histological changes such as severe cell infiltration and hyperplasia of the synovial intima with shedding of its outermost layer (B; black arrowhead), markedly increased vascularization (C; white arrowheads), or a combination of both (E). Marked synovial and sub-synovial edema were also frequent findings (B & F; white arrows).

Figure 3: Representative immunohistochemistry sections from normal and OA equine synovial membrane at low (top 2 rows; scale bar=100 μm) and high magnification (bottom 2 rows; scale bar = 50 μm) from the same histological section and demonstrating the median staining scores for macrophage markers (CD14, CD206 [M2], CD86 [M1], and IL-10 [M2]).

Figure 4: Sets of representative immunohistochemistry sections from normal and grossly inflamed OA equine synovial membrane from the same horse (2 different horses; scale bar = 150 μm) demonstrating increased staining intensity and distribution for all selected markers in OA joints, denoting more consistently marked increases for CD86 staining.

Figures

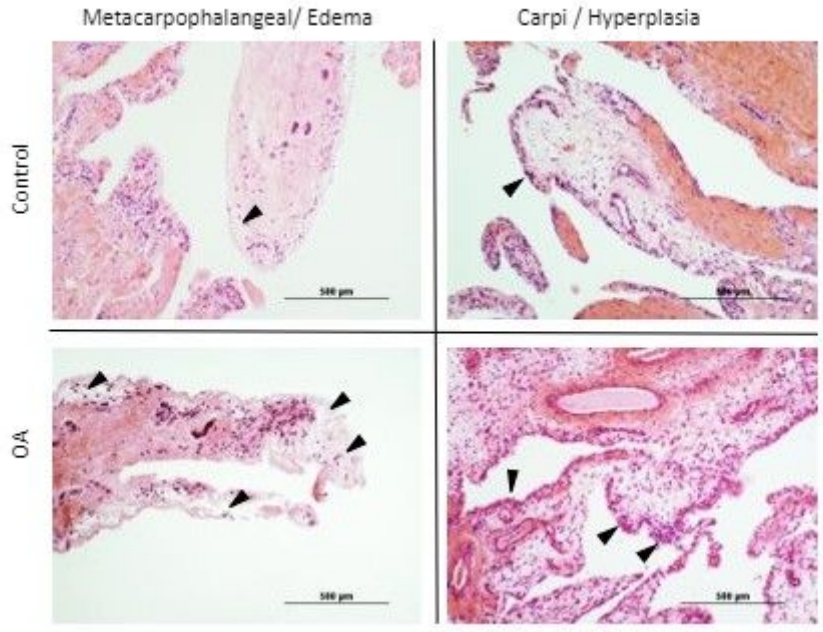

Figure 2

Representative images demonstrating (arrowheads) the differences between normal and osteoarthritic (OA) joints for Intimal Hyperplasia and Subintimal Edema.

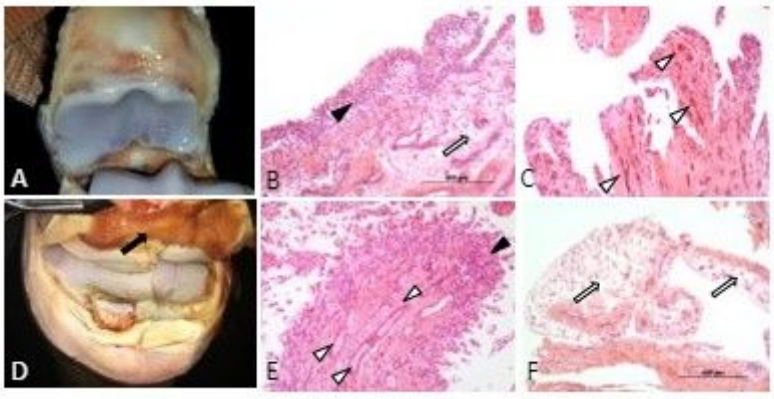

Figure 4

Compared to osteoarthritic joints with no or minimal signs of inflammation (A), OA joints exhibiting gross signs of synovitis (D; black arrow), exhibited increased histological changes such as severe cell infiltration and hyperplasia of the synovial intima with shedding of its outermost layer (B; black arrowhead), markedly increased vascularization (C; white arrowheads), or a combination of both (E). Marked synovial and sub-synovial edema were also frequent findings (B & F; white arrows).

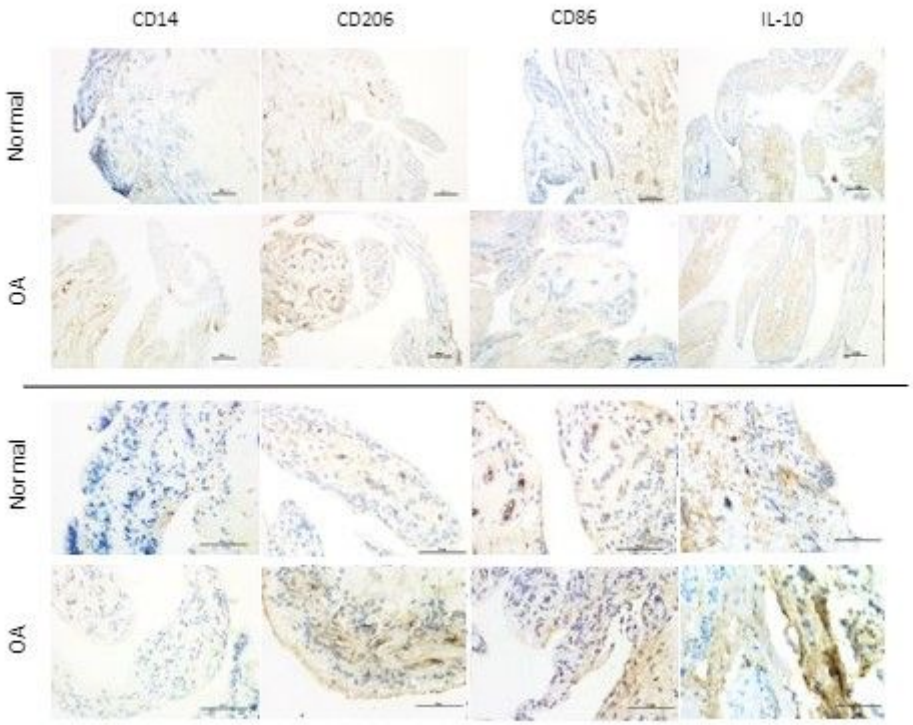

Figure 6

Representative immunohistochemistry sections from normal and OA equine synovial membrane at low (top 2 rows; scale bar=100 μm) and high magnification (bottom 2 rows; scale bar = 50 μm) from the same histological section and demonstrating the median staining scores for macrophage markers (CD14, CD206 [M2], CD86 [M1], and IL-10 [M2]).

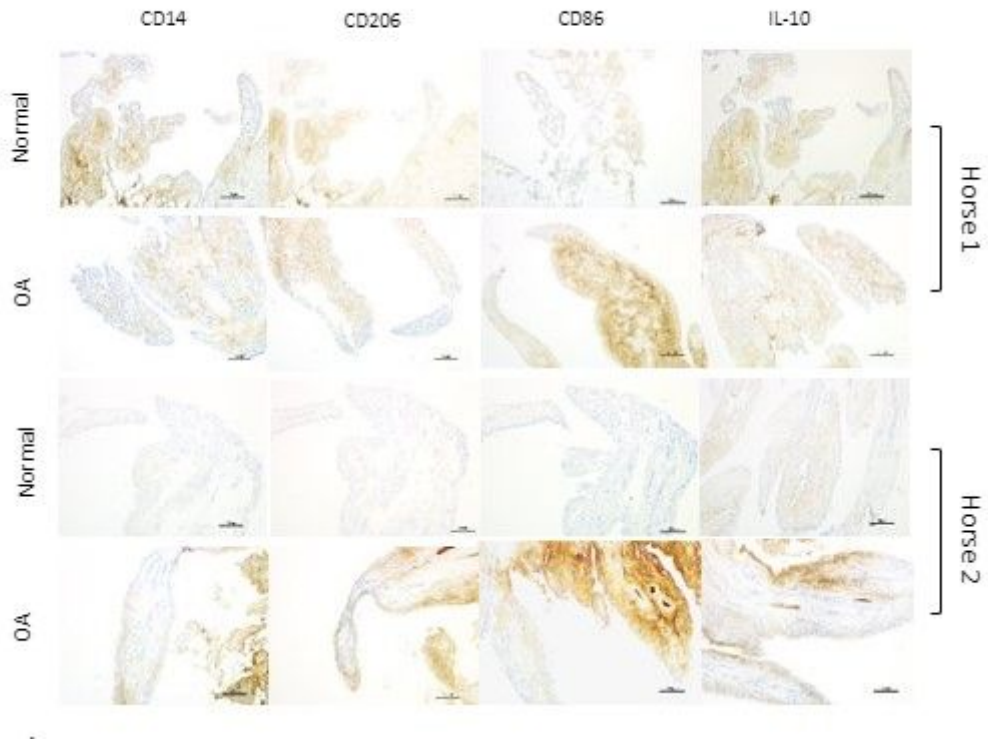

Figure 8

Sets of representative immunohistochemistry sections from normal and grossly inflamed OA equine synovial membrane from the same horse (2 different horses; scale bar = 150 μm) demonstrating increased staining intensity and distribution for all selected markers in OA joints, denoting more consistently marked increases for CD86 staining.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Menarim2020ARTTables.ppt
- Menarim2020ARTTables.ppt