Macrophage-mediated regulation of joint homeostasis

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ACADEMIC ABSTRACT

Osteoarthritis (OA) is the leading cause of musculoskeletal disability in people and horses, and is characterized by progressive joint degeneration. There is a critical need for a better understanding of disease processes leading to OA in order to develop more efficient therapies. A shared feature among different arthritic conditions is chronic synovitis. Macrophages are the main drivers of synovitis and can display pro-inflammatory (M1) or pro-resolving responses (M2). Macrophages promote joint health through phagocytic and secretory activities; however, when these functions are overwhelmed, macrophages upregulate inflammation, recruiting more cells to counteract damage. Once cell recruitment is efficiently accomplished, macrophages coordinate tissue repair and further resolution of inflammation. Bone marrow mononuclear cells (BMNC) are a source of macrophages used to treat inflammation and produce essential molecules for cartilage metabolism; however, little information exists regarding their use in joints. The studies presented in this dissertation focus on understanding the dual role of macrophages in driving and resolving synovitis and how to harness their therapeutic potential.

In the first study, patterns of macrophage phenotypes (M1:M2) in healthy and osteoarthritic equine synovium were compared and correlated with gross pathology, histology, and synovial fluid cytokines. M1 and M2 markers were co-expressed in normal and osteoarthritic joints, varying in intensity of expression according to degree of inflammation. Concentrations of synovial fluid IL-10, a macrophage-produced cytokine that is vital for

chondrocyte recovery from injury, was lower in OA joints. The combined findings of this study suggest homeostatic mechanisms from synovial macrophages in OA may be overwhelmed, preventing inflammation resolution.

In the second study we investigated the response of BMNC to normal (SF) and inflamed synovial fluid (ISF). BMNC cultured in autologous SF or ISF developed into macrophage cultures that were more confluent in ISF (~100%) than SF (~25%), and exhibited phenotypes that were ultimately similar to cells native to normal joints. BMNC cultured in SF or ISF were neither M1 nor M2, but exhibited aspects of both phenotypes and a regulatory response, characterized by increasing counts of IL-10$^+$ macrophages, decreasing concentrations of IL-1β, and progressively increasing concentrations of IL-10 and IGF-1, all more marked in ISF. These findings suggest that homeostatic mechanisms were preserved over time, and potentially favored by macrophage proliferation. Our data suggest that BMNC therapy could potentiate the macrophage- and IL-10-associated mechanisms of joint homeostasis lost in OA.

Finally, using an equine model of synovitis, the last study investigated the response of normal and inflamed joints to autologous BMNC injection. Inflamed joints treated with BMNC showed gross and analytical improvements in synovial fluid and synovial membrane, with increasing numbers of regulatory macrophages and synovial fluid concentrations of IL-10, not observed in saline-treated controls. Autologous BMNC are readily available, downregulate synovitis through macrophage-associated effects, and can benefit thousands of patients with OA.

Combined, the results of these studies support the role of macrophage-driven synovial homeostasis and identified a therapeutic way to recover homeostatic mechanisms of synovial macrophages lost during chronic inflammation. Our findings also uncover new research directions and methods for future studies targeting modulation of joint inflammation.

Macrophage-mediated regulation of joint homeostasis

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GENERAL AUDIENCE ABSTRACT

Osteoarthritis (OA) is a common cause of joint deterioration in people and horses. Current treatments provide limited recovery of joint function, creating an urgent need for more efficient therapies; however, development of new treatments requires better understanding of the mechanism causing OA. A shared characteristic among many arthritic conditions is long-standing inflammation. Cells called macrophages are the main drivers of joint inflammation and can exert pro- and anti-inflammatory effects. Macrophages promote joint health by clearing aggressor agents and secreting molecules required for optimal joint function. However, when these housekeeping functions are overwhelmed by damage, macrophages drive inflammation recruiting more cells to cope with increased demands for repair. If this process is efficiently accomplished, macrophages then resolve inflammation, recovering joint health. Macrophages in the bone marrow (BMNC - bone marrow mononuclear cells) are used to treat inflammation in several tissues and are known to produce molecules essential for joint health. Although little information exists regarding their use in joints, studies treating different organs suggest it can provide high rewards. The studies presented in this dissertation focused on understanding the dual function of macrophages in driving and controlling joint inflammation, and harnessed their therapeutic potential.

In the first study, macrophages were investigated in normal and OA-affected joints, and curiously exhibited a hybrid pro- and anti-inflammatory identity in both groups. The indicators of this mixed identity were more markedly expressed in arthritic joints showing gross

inflammation. Low levels of a macrophage-derived anti-inflammatory protein called IL-10 were detected in OA joints. The results of this study suggest that anti-inflammatory mechanisms from macrophages may be overwhelmed in OA-affected joints, preventing inflammation to be resolved, and that recovering this anti-inflammatory function may aid in the treatment of OA.

In the second study we investigated how the incubation of BMNC in fluid from normal and inflamed joints affects the response of macrophages. Similar to what we observed in the first study, BMNC incubated in both normal and inflamed joint fluid induced macrophages to develop a hybrid identity that was ultimately similar to native cells from normal joints. Macrophages proliferated more when incubated in fluid from inflamed joints. Macrophages in both groups produced anti-inflammatory effects with high levels of IL-10 that were highest in ISF cultures. These observations suggest that higher proliferation of macrophages in inflamed joint fluid helped preserve anti-inflammatory mechanisms. Therefore, our study suggests that joint injection with BMNC could maximize macrophage- and IL-10-associated mechanisms required to resolve joint inflammation.

The third and final study investigated the response of normal and inflamed joints to BMNC injection using a model of joint inflammation in horses. Inflamed joints treated with BMNC showed visual and laboratorial markers of improvement, with increasing numbers of macrophages and concentrations of IL-10 in the joint fluid, which remained lower in joints treated with placebo. BMNC provide means to recover macrophage-associated effects required to control joint inflammation and can benefit thousands of patients with OA.

Together, the results of these studies show that macrophages are biased promoters of joint health, leading to inflammation when their anti-inflammatory mechanisms are overwhelmed.

Replenishing inflamed joints with healthy macrophages maximizes their anti-inflammatory effects, favoring the recovery of a healthy articular environment.

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INTRODUCTION

Osteoarthritis (OA) is a common condition that affects joints and is characterized by progressive degeneration of articular tissues. Arthritic conditions of various etiologies, such as rheumatoid, psoriatic and post-traumatic arthritis, can all ultimately lead to the same cumulative pathology, OA. One shared feature among these different arthritic conditions is chronic inflammation of the synovial membrane. Chronic synovitis creates an environment unfavorable for a balanced metabolism of articular tissues, which having a poor capacity to counteract and repair damage eventually degenerate. Irreversible joint damage results in progressive loss of function and morbidity, considerable medical expenditures and reduced quality of life. Due to its progressively degenerative nature, the clinical presentation of OA increases with age, although high performance athletes face higher risk of disease at early life (1-3).

OA is the leading arthritic condition and the main cause of physical disability worldwide (1-3). Only in the United States, approximately 10% of the population suffers from some form of arthritis. Osteoarthritis is the second most costly health condition in the United States, costing approximately $300 billion each year (1, 8-10). In addition, approximately 40% of affected people experience physical limitations that incur further direct costs such as medical consultations, treatments and surgery, while morbidity and physical disabilities lowering employability and income, account for indirect costs (13). The negative impact of OA is even more evident among the elderly who often require assistance for daily tasks, adding to the financial burden (13). Several conditions including aging, obesity, conformation and trauma are associated to increased risk of developing OA, and managing those risk factors helps decrease the presentation, progression and burden of OA (1, 13-16).

Just as OA is widespread in people, OA is a common medical problem in horses and results in lameness and poor athletic performance (17). Musculoskeletal conditions causing lameness are the most common medical issue in horses and an important cause of horse wastage and financial losses in the equine industry (18). With an estimated population of 9.5 million horses, the equine industry is a relevant business in the US economy. The direct financial impact oscillates around US$ 39 billion, generating approximately 460,000 full-time jobs and US$ 1.9 billion in taxes (19, 20) annually (19, 20). The negative impact of lameness on the equine industry is estimated at US$ 678 million to 1 billion annually (21). Amongst the many conditions leading to lameness, OA is the primary diagnosis in about 60% of cases and results in a high financial impact due to treatment-associated costs and early retirement of equine athletes due to loss of function (22, 23).

Based on the significant quality of life and financial concerns related to OA in both horses and people, there is a critical need for a better understanding of the disease processes leading to OA and the development of more successful approaches aimed at prevention and treatment. Horses and people share substantial biological similarities in the development and progression of OA. As a result, horses have become a valuable and validated model for studying the comparative aspects of OA (15). Veterinarians, physicians and researchers have combined substantial efforts to identify ways of preventing and treating OA, with the ultimate goal of improving the health, ability to remain physically active and quality of life for affected patients. The additional goal of ensuring permanence in the workforce and therefore access to health care and a viable income is uniquely important in people (8, 15).

OA has long been a poorly defined entity, in both people (24) and horses (15). In human medicine, pathologists define OA based on structural changes to the joint (25), whereas

clinicians define it based on symptoms (16), and epidemiologists use pain as the basis of their

definition of OA (26). One current consensus defines OA as "a group of different conditions

with overlapping biological, morphological and clinical features that affect all intra- and peri-

articular tissues. Ultimately the articular cartilage degenerates with fibrillation, fissures,

ulceration, and full-thickness loss of the joint surface" (27-29). A study from the United States

Department of Agriculture (USDA) emphasized the importance of OA in horses for the

associated presentation of lameness and poor athletic performance (18). Lameness resulting

from repetitive joint trauma is the most commonly considered etiology of equine OA, and the

presence of cartilage lesions was considered a central criterion for its diagnosis. Although the

definition of OA has commonly centered on clinical measures, several studies have focused on

the associated gross pathology and histological parameters reported in clinical and experimental

OA (25, 30-34). Currently, equine OA is defined as a group of disorders that share a common

end stage: progressive articular cartilage deterioration combined with subchondral bone and soft

tissues changes (34). Recently, it was recognized by consensus that the disease process of

equine OA can initiate in the synovium, peri- and intra-articular ligaments, subchondral bone,

and/or articular cartilage (15, 35, 36). Ultimately, all articular tissues degenerate, with

fibrillation, fissures and ulceration of cartilage resulting in full thickness loss of the joint surface

(27, 36).

The same poor understanding of the etiology of OA that prevented its clear definition has

also precluded development of efficient therapies to control its clinical signs and progression.

Many years of studies, in both horses and people, have been collectively analyzed and effective

long-term treatments yet to be identified, further defining the urgent need for therapies with the

potential to provide lasting effects (16, 19). Modern concepts of OA are constantly changing as

a consequence of current clinical and research techniques. These technological resources have evidenced some features of the complex biology of tissues involved in OA, revealing increased awareness of the role of synovial inflammation (3, 37). Synovitis is associated with both early and late symptoms of OA (12, 38). Several studies using current imaging and histological techniques have reported that, during all stages of OA, there is a high prevalence of synovitis associated with pain and disability, and that synovitis is often the single driver of catabolic processes in OA (3, 39-41). Therefore, advancing our understanding of synovitis is a paramount step in the pathway to develop disease-modifying therapies capable of preserving joint structure and resulting in reduced joint pain (3).

The normal synovial membrane is populated by synovial fibroblasts, macrophages and a minority of lymphocytes (39). Synovial inflammation is characterized by increases in synovial membrane thickness and cell infiltration, mainly consisting of macrophages (42, 43). Macrophages work in conjunction with synovial fibroblasts to promote synovial health by performing phagocytosis of cartilage breakdown products, cellular debris and foreign material, but also through secretion of regulatory cytokines and growth factors (38). When these phagocytic and homeostatic functions are overwhelmed, synovial macrophages function as signaling cells to recruit other immune cells (e.g., more macrophages, lymphocytes and neutrophils) and upregulate inflammation to counteract the increased demands for repair. However, the uncontrolled production of inflammatory cytokines and stromelysins by the overall inflammatory response ultimately results in joint degeneration (38). Synovial macrophages are therefore the main instigators of inflammation in the synovial membrane (37, 43, 44), and a precise marker of joint inflammation and response to therapy (37, 41, 45).

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In the face of a growing identification of the molecular drivers of synovitis (3, 12, 37, 38, 40, 43, 46) there is an increasing awareness that the resolution of inflammation and recovery of homeostasis is an active process that also intrinsically depends on macrophages (47-51). Therefore, understanding the dual role of macrophages in driving and resolving joint inflammation is a mandatory step in advancing in the medical management of OA (37, 44) and is the inspiration for the research presented in this thesis.

CHAPTER 1: LITERATURE REVIEW

Joint anatomy and physiology

Synovial joints are a connective tissue unit with a fluid-filled cavity that allows free movement of two adjacent bone ends. Also called diarthrosis, this type of joints is the most common type of joint in the body. Musculoskeletal movement is made possible by muscle contraction and translation of these forces through their associated tendons that span the joints and lead to flexion and extension (5). The almost frictionless nature of these movements results in superior efficiency to any artificially produced material (52), but absolutely requires that all components of the joint are intact and functional (**Fig. 1-1**) (53).
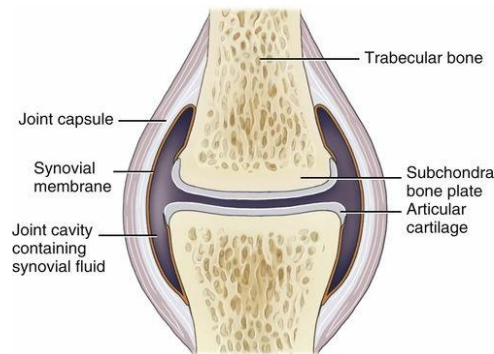
Figure 1-1: Schematic representation of a diarthrodial joint. From P. R. van Weeren, 2016, "Joint Disease in the Horse" (4). Reprinted with permission of Elsevier, 2019.

On each side of a joint, smooth cartilage covers the bone extremities providing almost frictionless movement between the articular surfaces (5). The joint capsule is a fibrous connective tissue that attaches to bone on either side of the joint delimitating the joint space providing boundaries to the joint and stabilizing it. In addition, the joint is also stabilized by surrounding muscles, and intra- and peri-articular ligaments (23). In the inner side of the joint

capsule is the synovial membrane. Each of these structures exhibit particularities that vary

according to function and location of the joint, and altogether this assembly should be regarded

as a continuum (54). Therefore, synovial joints should be considered an organ which's health

depends on the combined function of each of its components. Malfunction of one or more

articular components culminates in joint pathology (4, 23).

Synovial membrane and synovial fluid

The synovial membrane, also called the synovium, refers to the mobile, non-adherent soft

tissue lining on the inner side of the joint capsule. The normal synovium provides a surface area

for immune and homeostatic functions of the joint. It controls the production of synovial fluid

(volume and composition), providing nutrition for chondrocytes in the articular cartilage and

lubrication of the joint surfaces (39). The synovium is composed of a combination of flat

surfaces and loose collections of finger-like villi (areolar synovium). These two different

presentations are an effect of biomechanical stimuli to which the synovium is exposed in

different areas in the joint (23). The areolar synovium is often crimped into elastic folds that

deform when stretched, thus providing viscoelastic properties necessary to adjust to the

mechanical forces to which the synovium is exposed during physiologic joint movement (23,

39).

The synovium is stratified in two continuous layers of cells: the most superficial layer

called the intima and the underlying, subintimal layer. The intima is a composite of synovial

macrophages and fibroblasts (1-4 cell layers thick) located within a very loose collagenous

assembly of extracellular matrix proteins (4, 39). The subintima is composed of fibrous, areolar,

and adipose tissues (55) and contains blood vessels and lymphatics and a lower density of

synovial fibroblasts, macrophages and lymphocytes. The subintima is also the site of joint

innervation originating from muscles and peripheral nervous branches (4, 23, 39). In the healthy

synovium, the intimal layer is 20-40 µm thick and the adjacent subintima is up to 5 mm thick.

The subintima can be acellular in places, especially where it is composed of fatty or fibrous

tissue (39). The virtual space between the intima and subintima is a reservoir of hyaluronan-rich

fluid, which makes this fluid-tissue assembly a non-adherent surface between joint elements (3,

39). Unlike other tissues with non-adherent properties, such as serosal or mucosal epithelia, the

synovium does not contain a basal lamina (39). Lack of a basal lamina facilitates the passage of

plasma components from the blood to the subintimal space and then the synovial cavity, defining

the composition of the synovial fluid (23, 39).

The cells within the intimal layer have been traditionally considered to have two main

roles: phagocytic or secretory activity (4, 56-58). Based on their function and ultrastructural

features, cells with phagocytic activity were termed type A synoviocytes, or synovial

macrophages, while those with protein secretion activity have been denominated type B, or

synovial fibroblasts. Also, cells sharing common features of type A and type B cells have been

reported and were described as type C synoviocytes and seem to be in a transitional stage from

one phenotype to the other (23, 56-58). Even though C type cells were described over 50 years

ago, only more recently this plasticity between phenotypes has been given attention (23).

The classic characterization of synoviocytes as types A, B and C was limited by the

techniques available at the time. Modern technologies, such as precise cell sorting, modern

immunostaining, next generation sequencing, proteomics and single cell analysis, have enabled

more accurate characterization of the specific features and identity of cells within the synovium.

Although early studies from the 1960's proposed that most of these cells were synovial

macrophages (type A) (56, 58), it has been confirmed that most cells in the synovium are synovial fibroblasts (type B, 55-70%) (12). Synovial macrophages are the second most predominant cell in normal synovium and are in higher density in the sub-intima as compared to the intima (39, 59). Even during inflammation, when relative counts of macrophages increase (from ~25% to up to ~40%), they are still found in lower numbers than fibroblasts. T cells can be found in normal and inflamed synovium in OA at relatively low counts (<15%) (12, 37, 59). Modern concepts of the identity and function of cells in the synovium will be further explored in detail in this review.

Cross talk between synovial macrophages and fibroblasts is required for them to perform fundamental "housekeeping" functions during joint health and disease. One of these functions is the coordinated secretion of signaling and structural molecules (i.e., cytokines, chemokines, growth factors, hyaluronan, lubricin, prostaglandin E_2 and matrix pro-metalloproteinases (proMMP)), which participate both in the anabolic and the catabolic metabolism of the joint (37, 43, 44). This cellular cross-talk is negatively affected by sustained joint inflammation, which dysregulates both anabolic and catabolic pathways resulting in altered turnover of cartilage components and development of disease (3, 4, 23, 37, 43, 44).

One central function of the synovium is to regulate the composition and rate of production of synovial fluid (4), a dynamic process that is largely related to the discontinuous arrangement of cells within the synovium. The organization of synovium cells creates intercellular gaps containing collagen (I, III, and V), hyaluronan, lubricin, chondroitin sulphate, biglycan, as well as decorin and fibronectin, forming a permeable barrier that allows plasma molecules ≤10 kDa (e.g., glucose, oxygen, carbon dioxide and some small proteins) to cross the synovial vessels into the joint cavity, and prevents larger solutes from entering the synovial fluid

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(23, 60). The regulation of solute exchanges depends on the degree of synovial inflammation, which affects vascular permeability, lymphatic drainage, and intra-articular pressure, thus altering the size and concentration of molecules passing into the synovial fluid (4). Pressure within the joint is primarily subatmospheric (-2 to -6 cm H_2O), favoring synovial ultrafiltration. Inflammation leading to joint effusion and increased intra-articular pressure negatively affect this pressure gradient. Importantly, joint movement leads to constant changes in joint pressure, which is also affected by joint position through the effects of gravity on limbs extremities (4). Together, the appropriate lubricant secretion by synovial cells and its drainage by the synovium lymphatics are necessary to maintain a synovial fluid composition that is chemically and mechanically functional (60). Such balance among synovial fluid components is required for the adequate steric exclusion of larger molecules from the synovial cavity (4, 23, 60).

Hyaluronan is an important component of both synovial fluid and articular cartilage, and it is mainly produced by synovial fibroblasts together with other molecules that confer viscoelasticity and frictionless movement between joints surfaces, such as lubricin, surface-active phospholipids and synovial fluid proteoglycans (23, 52). The mechanisms regulating the concentration and molecular weight of synovial fluid hyaluronan are not exactly known, but are at least partially regulated by the reactive oxygen species (ROS) produced by cells within the joint (60). The normal concentration and molecular weight of hyaluronan ranges from 0.33-1.5 mg/mL and 6-10,000 kDa (23, 60, 61). Higher concentrations of hyaluronan are found in fluid from diseased joints (4 mg/mL) (60). In healthy joints, endogenous hyaluronan has an estimated turnover of 27 hours while it decreases to 11.5 hours in acutely inflamed joints as a result of increased clearance through lymphatics within the synovium for further degradation in the synovium, lymph nodes and liver (23, 60-62).

The cellular content within the synovial fluid also contributes importantly to joint homeostasis. Cell counts in normal synovial fluid are generally <500 cells/µL; however, ranges within normality can be as high as 1,350 cells/µL (63). Monocytes/macrophages represents the majority of the cells with a smaller percentage of lymphocytes (23, 64, 65). All of these cell types produce a range of different cytokines, chemokines and growth factors, whose concentrations in healthy equine synovial fluid have been minimally documented and their roles *in vivo* have been poorly characterized (4, 66-68).

Joint capsule and ligaments

The fibrous joint capsule provides passive stability to restrict joint displacement and retains synovial fluid within the joint space. The joint capsule varies in thickness according to the stresses to which the joint is subjected. In addition, proprioceptive nerve endings found within the fibrous joint capsule transfer information about joint position to the brain, providing active stability. The joint capsule is in intimal connection to extra-articular structures such as intra- and periarticular ligaments and may also incorporate tendons (4, 69). The way the joint capsule and adjacent ligaments contribute to joint stability varies according to anatomic location. Joints located more proximally have a higher support from regional muscles. Those located distally in the limbs have less muscular stabilization and rely more on the joint capsule and ligaments for stability. The stiffness of ligamentous and capsular structures are determined by the fibrous tissue deposition, which differ according to the joint's range of motion and other biomechanical considerations (23).

Subchondral bone

The subchondral bone is a thin layer of compact bone that is located between the more malleable trabecular bone in the epiphysis and the articular cartilage. The transitional layer of calcified cartilage serves as the point of integration between the subchondral bone and the cartilage. Subchondral bone lends both the contour of the epiphyseal bone and mechanical stability to the overlying articular cartilage (4). Although similar in composition to other types of bone, the haversian system of subchondral bone is oriented parallel to the joint surface rather than perpendicular as in diaphyseal cortical bone. This histological organization confers mechanical resistance to the subchondral bone plate, making it 10 times more capable to deform than the diaphyseal cortical shaft (23, 70). The subchondral bone is a well-vascularized tissue with high metabolic activity, and remarkable ability to adapt to exercise through quick changes in matrix turnover and mineral density in response to load (4, 71-73). These adaptations create mechanical and chemical cross-talk with the articular cartilage and intra-articular environment, however, excessive remodeling followed by sclerosis of the subchondral bone plate decrease its capacity of deformation and cushioning becoming more prone to sub-chondral bone and cartilage (23, 73, 74).

Articular cartilage

Articular cartilage is a highly specialized tissue overlying the subchondral bone to form the articular surface. Together with the synovial fluid, articular cartilage provides near frictionless movement of joints, enabling efficient locomotion (23, 70, 75). The thickness of articular cartilage varies by age and degree of weight bearing in different locations of the joint; however, on average it is 1- 4 mm thick in horses and people. Articular cartilage is devoid of

blood vessels or lymphatics and is therefore dependent on diffusion from the synovial fluid for

nutrition and waste removal (23, 70, 76). Minute openings at the superficial layer of cartilage,

approximately 6 nm in diameter, allow exchange of small solutes (ions and molecules such as

glucose) between the cartilage and the synovial fluid. Nutrients are produced in the layers of the

synovium, diffuse into the synovial fluid and eventually reach chondrocytes embedded in the

cartilage (77).

Cartilage is primarily composed of water, which accounts for approximately 70% of the

wet weight of adults and up to 80% in growing individuals. The majority of cartilage dry weight

is composed of collagen (~50%), followed by proteoglycan (~35%), glycoprotein (~10%),

minerals (~3%), surface active phospholipids (~1%) and miscellaneous substances (~1%) (4, 52,

75, 77). Chondrocytes represent 1-12% of the cartilage volume while the remaining volume

corresponds to extracellular matrix (ECM) (52, 75, 77).

The cells and matrix of cartilage are organized in a unique manner that is responsible for

its mechanical properties (70, 75). Based on chondrocyte morphology and orientation of

collagen fibers, mature mammalian articular cartilage can be described as containing four

sequential zones (**Fig. 1-2**) (70, 77):

1. The superficial (tangential) zone has an acellular outer most layer, the *lamina
   splendens*, with fibril diameters differing from the cellular portion (~31 nm). Below
   there is a layer with the highest chondrocyte density among cartilage zones. These
   layers are loosely connected, and thus can displace from each other without damaging
   the collagen network, protecting the deeper zones from shear stresses. Chondrocytes
   in the superficial zone are flattened and their cell axis is oriented parallel to the joint

surface similar to the collagen fibers, which in this zone, are more densely packed resembling the skin.

2. The middle (transitional) zone is populated by larger chondrocytes that are more ovoid to round in shape, with thicker collagen fibrils (40-100 nm in diameter) oriented in a random pattern.

3. The deep (radiate) zone contains the largest chondrocytes, which are organized in lacunae. Cells and collagen fibrils orient perpendicular to the joint surface. The collagen fibrils here have the largest diameter, and cross the calcified zone prior to attaching within the subchondral bone.

4. The calcified zone consists of mineralized cells and matrix. The tidemark refers to the junction of the non-calcified and calcified cartilage.

Cartilage collagens

Several types of collagens are described in the articular cartilage and are classified in fibril-forming (types I, II, III, V, and XI) and non-fibril-forming collagens (23). Articular cartilage is composed of 90-95% type II collagen by dry weight, creating the framework for all other ECM molecules. Type II collagen is a fibril-forming collagen that forms a trimer of three identical collagen molecules consisting of approximately 1,000 amino acids each. Trimers are assembled intracellularly as propeptides and become cleaved once transported extracellularly (4). Given its structural organization and mechanical properties, type II collagen counteracts shear and tension at the articular surface (4). Although cartilage collagen is produced by the chondrocytes, the ECM surrounding the chondrocyte has little to no fibrillar collagen. Instead, the pericellular ECM harbors microfibrils of type VI collagen, which interact with cell surface
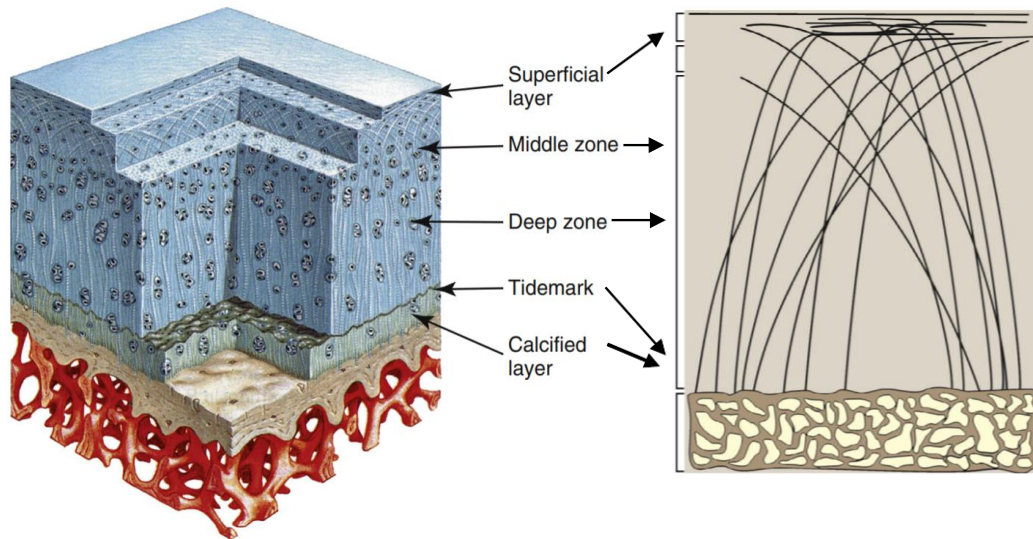
Figure 1-2: Schematic illustration of the zonal stratification of the articular cartilage over on the subchondral plate depicting distribution and orientation of chondrocytes and collagen fibers. Adapted from P.R. van Weeren, 2016, "Joint Disease in the Horse" (4). Reprinted with permission of Elsevier, 2019.

molecules, hyaluronan and small proteoglycans involved in cell signaling (4, 23, 70, 75). Type II collagen fibrils locate outside the peri-chondrocyte matrix at areas called territorial and interterritorial matrix. At those areas, the collagen fibrils are assembled in a quarter stagger fashion, in which each molecule offsets the start of the next by 25% of its length (**Fig. 1-3**) (4). Types IX and XI collagen are also part of the cartilage assembly. Both are intimately associated with collagen type II and are crucial to the crosslinking, and mechanical stability of the fibrillar network. Type IX collagen provides a covalent interface between each type II collagen fibril, forming a template for the lateral growth of collagen II fibrils. Alternatively, collagen type XI ensembles with the type II collagen triple helix providing a core protein that harbors type II molecules as they are produced, especially in the juvenile tissue. Type IX collagen comprises

3% of mature collagen. Other collagen types are believed to interact with fibrillar collagen and ECM components increasing mechanical stability and the life-time of the assembly (4, 23, 70, 75).

Proteoglycans

Proteoglycans are another key integrant of the articular cartilage ECM accounting for ~35% of cartilage dry weight. They are a combination of protein and glycosaminoglycan that act as structure of the matrix and a selective permeability barrier. Aggrecan corresponds to 85% of the proteoglycans in the ECM, and is composed of a core protein which serves as the backbone
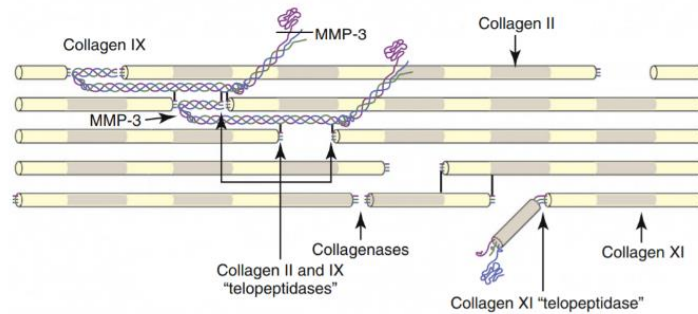
Figure 1-3: Representation of a collagen polymer assembly including different collagen types, and depicting cleavage sites, necessary for either degradation or polymer growth. From P.R. van Weeren, 2016, "Joint Disease in the Horse" (4). Reprinted with permission of Elsevier, 2019.

for the attachment of glycosaminoglycans (GAG). Three main GAG molecules are present in aggrecan: keratan sulfate, chondroitin-4-sulfate and chondroitin-6-sulfate (Fig. 1-5). GAG bind to the core protein through covalent bonds, and approximately 100 chondroitin sulfate units bind

at the carboxy terminal of the molecule, while close to 100 keratan sulfate units bind towards the

N-terminal region of the aggrecan monomer (23). Because of their negative charges, GAGs

repel each other and attract water, creating positive pressure that confers high viscosity to the

molecule (4). As it occupies the interfibrillar space of the cartilage ECM, it confers osmotic

properties to the cartilage, critical to resist compressive loads. In addition to providing

mechanical resistance to the ECM, proteoglycans also play a major role as important modulators

of cell signaling (23, 75). The aggrecan core protein has three main domains (G1, G2, and G3),

which define the sites of cleavage for several enzymes, both during physiological and

pathological conditions (**Fig. 1-4**) (4, 23). The G1 domain directly interacts with hyaluronan, as

part of an assembly that arrange approximately 100 aggrecan monomers alongside a hyaluronan

molecule. The G2 domain allocates to the amino end of the keratan sulfate–rich region, and

defines a cleavage site for aggrecan breaking enzymes. On the other hand, the G3 domain does

not appear in all aggrecan molecules, and therefore is believed to have a less crucial role (4, 23).

Both the side chains and the core protein of the aggrecan monomer decrease in length over time,

leading to age associated alterations in the structure and mechanical efficiency of the cartilage

ECM (4).

Approximately 5% of proteoglycans in the cartilage ECM are small proteoglycans. They

are mostly represented by biglycan, decorin, and fibromodulin (4). Despite limited knowledge of

their participation in fibrillogenesis, cartilage turnover and immune signaling, their roles require

deeper characterization (78).

Chondrocytes

Not only chondrocyte morphology varies according to their location within cartilage zones, but also their metabolism. Therefore, the turnover of cartilage ECM components follows a similar pattern. The cartilage ECM microenvironment fully surrounds the chondrocyte preventing it from migration. Still, chondrocytes have cytoplasmic processes that extend into the cartilage ECM, sensing mechanical loading and the farthermost biochemical microenvironment, which allow them sense chemical, mechanical and electrical stimuli. The survival of chondrocytes depends on balanced chemical and mechanical stimulation (70). As a matter of
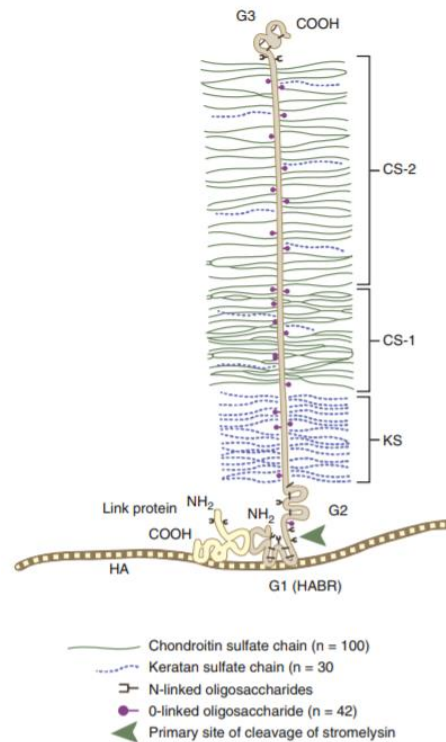
Figure 1-4: Schematic of an aggrecan molecule. A core protein with GAG side chains attached, bound to hyaluronan. Globular domains 1 (hyaluronan binding region [HABR]), 2 and 3 are indicated. From P.R. van Weeren, 2016, "Joint Disease in the Horse" (4). Reprinted with permission of Elsevier, 2019.

fact, cyclic loading of non-injurious intensity is required for the normal turnover of

proteoglycans and other matrix components, and therefore necessary to maintain mechanical

strength and cartilage integrity (75). The mechanical forces of normal dynamic loading cause

deformation of the ECM, stretches the chondrocyte membrane and increases fluid flow due to

changes in hydrostatic pressure. Once the load is retrieved, the ECM recovers its steady-state

organization leading to cyclic osmotic changes, both extracellular and intracellular. Failure in

maintaining the osmotic balance, especially regarding concentrations of Na+, K+ and H+,

dramatically affects cell metabolism, membrane transport and macromolecule synthesis,

ultimately affecting ECM turnover. Diffusion of solutes resultant from cyclic loading also alters

the frequency at which signaling molecules reach the chondrocytes and induce metabolic

changes (75). The limited mobility and replication capacity of chondrocytes, unfortunately

contribute to the restricted capacity of cartilage to heal in response to injury (70, 79).

Joint lubrication and functional biomechanics

Lubrication of synovial joints happens by two main mechanisms: fluid-film and boundary

lubrication. Fluid-film lubrication provides low friction for cartilage surfaces by creating a fluid

layer between the two articular surfaces. Different hypothetical mechanisms have been proposed

to explain this phenomenon, however the elastohydrodynamic model is the best accepted (**Fig. 1-
5**). It is based on the attraction of water by cartilage aggrecan molecules through the porous

cartilage surface. When load is applied, water is squeezed out the cartilage creating a fluid film

that provides a thin separation between the articular surfaces. In the absence of water, negatively

charged proteoglycans in the cartilage ECM are forcefully pulled together, creating repulsive

pressure. Therefore, when loading forces are retrieved this fluid film is resorbed back into the
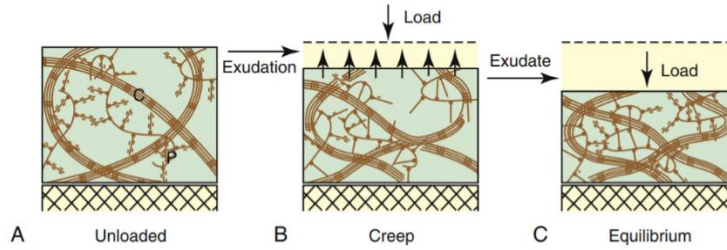
Figure 1-5: Schematics of the sponge-like behavior of articular cartilage. During static state, negatively charged sulfate groups of proteoglycans (p) of the collagen network (c) attract water to the ECM (A). When load is applied (B), water is squeezed out of the ECM (C). Finally, when the load is retrieved, the reverse mechanism happens. From P.R. van Weeren, 2016, "Joint Disease in the Horse" (4). Reprinted with permission of Elsevier, 2019.

cartilage matrix. In a steady-state, an equilibrium of pressures is provided by the aggrecan-water interaction in the articular cartilage. The dynamics of fluid in and out of the articular cartilage is essential to joint lubrication and chondrocyte nutrition, where the synovial fluid is a vehicle to between the cartilage and the joint space (4, 23).

Boundary lubrication prevents abrasions and adhesion between soft tissues and bones but cannot provide frictionless movement between articular cartilage surfaces. Hyaluronan and lubricin are considered the main synovial boundary lubricants (80).

Joint mechanics depends on the biomechanical properties of the cartilage matrix when exposed to motion and loads created across the articular surface. The ability of articular cartilage to undergo elastic deformation is defined as viscoelasticity, as a result of the combination of its biomechanical properties and fluid viscosity (23).

Due to anatomic features, joints do not experience even loading over their surface. The magnitude and types of loading is different across the joint surface, and includes low-level

constant loading, intermittent loading, and high-impact loading areas. Loading differences also vary with the range of motion, where low-motion joints experience more compressive loading, while high-motion joints undergo higher shear loading. This topographic diversity leads to biochemical differences and related biomechanical properties across the cartilage areas (76). High intermittent loading is associated to higher deposition of strongly cross-linked collagen, while consistent loading of lower intensity is associated with higher proteoglycan content. Therefore, to efficiently meet these variable loading conditions, articular cartilage have different biomechanical properties at different sites (4).

Tissue homeostasis

All of the components of the joint establish an elaborated cross-talk, and the functional integrity of such components is vital for the preservation of joint homeostasis, function, and adaptation to the challenges from locomotion and injury. Disruptive function of any of these components is translated in joint disease. Proper balance between anabolism and catabolism of all of the articular tissues is a cornerstone for optimal joint function.

Dysregulation of this homeostatic state can directly affect the response of articular tissues to the challenges regularly posed to the joint. While the joint is a functional organ, the metabolism and turnover rate of each of its components vary widely. Although the articular cartilage undergoes constant remodeling, the turnover rate of cartilage components differs among themselves, affecting the capacity of proper tissue repair following injury (23). Although early studies proposed that the time for turnover of cartilage proteoglycans was from 1 to 20 years (81) or even 350 years for collagen type II (82), it is currently known that these molecules turnover much faster (83). In fact, during disease collagen synthesis is increased (84, 85), nonetheless, so

is its cleavage (86, 87). Therefore, lost ECM components are not efficiently restored to the collagen network, negatively affecting the mechanical properties of the cartilage. As a result, injured cartilage becomes incapable to resist mechanical stress, undergoing further damage and a progressive cycle of relative overloading and joint deterioration. Eventually, structural damage leads to inflammation and perception of pain, the most relevant effect of chronic OA that invalidates horses and people (4).

Chondrocytes are considered to be the sole source of all cartilage ECM components. Chondrocytes control the assembly and remodeling of the collagen network in response to biomechanical stimuli or injury. Collagen is then cleaved as an effect of activation of matrix metallopeptidases (MMP)-1, -8 and -13 and collagenases. MMP-13 is considered the most powerful enzyme to cleave collagen type II (88). MMP-3 cleaves proteoglycans, especially aggrecan between the G1 and G2 domains. Other proteoglycan cleaving MMP in the joint are the gelatinases MMP-2 and -9. These enzymes are part of the ADAMTS (A Disintegrin And MMP with Thrombospondin motif) family, with ADAMTS-4 and -5 contributing to aggrecan degradation in articular cartilage (89, 90). Noteworthy, the activity of these enzymes was defined in face of the limitations of *in vitro* cultures of cartilage challenged with unnatural concentrations of cytokines, including interleukin (IL)-1 and tumor necrosis factor (TNF)-α, since they are considered main drivers of OA (4, 90-92).

The activity of MMP in joints can be regulated through transcription, by the extent to which the pro-forms become activated (e.g. influenced by cytokines), or by the effect of tissue inhibitors of MMP (TIMP) on active MMP, leading to their inactivation. During healthy condition, the articular cartilage can have a mildly increased TIMP: MMP ratio that becomes switched in case of disease. Also, the TIMP-MMP bond is reversible, and free MMP can rapidly

recover activity. Since, the activity of MMP is increased in pathologic conditions, they have become therapeutic targets; however, as they are also important regulators of homeostasis in healthy joints, targeting their therapeutic inhibition stands to provides detrimental effects to the healthy ECM (93, 94).

Mechanical stimuli from joint loading also plays an important role in tissue homeostasis. Cartilage loading induces cellular responses through cell and nucleus deformation. Chondrocytes respond to mechanical stimulation through increasing ECM synthesis, production or activation of cytokines and growth factors (4, 95). As an example, static compression produced by immobilization causes cartilage damage with loss of proteoglycans, increases ROS production and MMP-3 and ADAMTS-5 activity. On the other hand, cyclic loading prevents excessive protease activation, while increasing matrix synthesis. Similarly, *in vitro* dynamic loading of physiological degree is capable of inhibiting ECM degradation following IL-1 stimulation (75, 95, 96). Importantly, at sustained supra-physiologic loading (trauma), the primary response of chondrocytes is to upregulate cartilage anabolism by increasing collagen and aggrecan synthesis (23). However, traumatic injury increases production/activation of cytokines, chemokines and cartilage-degrading proteinases, and the stress-induced production of reactive oxygen species (ROS) causes upregulation of TNF-α, ADAMTS-5, and MMP-13, and chondrocyte death (4, 91, 95). Despite of an initial anabolic response, cartilage catabolism ultimately prevails, likely as a consequence of an overwhelmed anabolic response (23).

Growth factors known to exert an anabolic effect on articular cartilage include insulin-like growth factor-1 (IGF-1) and transforming growth factor-β (TGF-β) (97). TGF-β can act by signaling in different pathways. Via the canonical type I receptor ALK5 (Smad2/3 route), TGF-β stimulates chondrocytes to synthetize proteoglycan collagen type II, and downregulates gene

expression of MMP. TGF-β signaling via the ALK1 (Smad1/5/8 route) pathway culminates in deleterious responses that alters chondrocyte metabolism and stimulate osteophyte formation. Specific pathway signaling seems to be dose and age dependent (98). Similar to TGF-β, IGF-1 promotes cartilage homeostasis by stimulating matrix production and inhibiting its degradation (97, 99), in such a way that IGF-1 deficiency produces OA-like lesions in experimental models (100). In fact, IGF-1-supplemented chondrocytes have been successfully used to treat subchondral bone cysts in horses (101).

Current Concepts in the Pathophysiology of Osteoarthritis

Osteoarthritis is no longer considered a disease of cartilage, but as a whole joint disorder (5, 91). There is increasing evidence that all joint components contribute to the progression of joint disease, leading to mild, chronic non-specific synovial inflammation, gradual cartilage deterioration, osteophyte formation and sclerosis of the subchondral bone (5, 91). OA was traditionally defined as a non-inflammatory disorder where tissue degeneration was believed to happen from gradual wear and tear (17). Instead, it is currently interpreted as an aberrant remodeling of synovial tissues driven by an inflammatory response (5, 91).

Inflammation in OA can be driven by autoimmune mechanisms or those unrelated to auto-immunity. This review will not focus on autoimmune-mediated OA. Aside from arthritides developing from auto-immunity, three hypothetical mechanisms have been considered for OA pathogenesis. The first includes an abnormal cartilage, displaying defective mechanical functions that fails under normal loading. An inherited type II collagen defect exists in people, yet it has not been identified in the horse (17); however, cartilage chronically exposed to inflammation such as in sepsis or other arthritic conditions, can hold defective mechanical

properties. A second mechanism involves changes in the subchondral bone. Even though the subchondral bone is more resistant to loading than cartilage, it undergoes mild deformation and is an important shock attenuator. Normal mechanical stresses often result in subchondral bone micro fractures. When too frequent or intense, remodeling of the subchondral bone result in higher deposition of compact bone to the subchondral plate, increasing its density (sclerosis), decreasing its capacity to absorb impact, thus exposing the cartilage to supraphysiologic stresses and mechanical damage. In fact, cartilage degeneration often happens over areas of sclerotic bone while over areas of normal bone density it remains intact (17).

The most accepted hypothesis of the pathogenesis of OA involves mechanical forces causing damage to healthy cartilage (17, 77, 102-104). Damage of cartilage matrix or cells by excessive or repetitive loading result in altered chondrocyte metabolism and production of enzymes that cause degradation of the ECM components. In equine and human athletes, repetitive trauma is a well-defined risk factor to OA (5, 6). In equine athletes, repeated micro trauma is likely the most common pathogenic factor for the development of OA, as noted by the correlation of joint damage localizing at specific sites more exposed the mechanical stress in certain athletic disciplines (6, 17, 22, 105). The daily trauma and damage experienced by equine and human athletes essentially overcomes homeostatic mechanisms, eliciting chronic joint inflammation (15). The aforementioned observations build the concept of "traumatic arthritis", a collection of clinical and pathologic conditions developing from single or repetitive trauma, potentially leading to OA (e.g., synovitis, capsulitis, ligament sprain, intraarticular fractures or meniscal tears).

The concept of traumatic arthritis is divided into three entities (6):

- Type I: Traumatic capsulitis and synovitis with no alterations to the cartilage or injury of major adjacent structures. Type 1 arthritis comprises acute synovitis and joint sprains.

- Type II: Trauma causing injury of the cartilage or rupture of a supportive structure. This comprises severe sprains, intraarticular fractures, and meniscal tears.

- Type III: Posttraumatic OA, is the inevitable result of severe trauma to the bone or soft tissues of the joint, and often a consequence of ineffective treatment of the predisposing conditions.

Joint disease can initiate from isolated or from intertwined abnormalities in any of the joint components, yet the articular cartilage has been the focus of OA studies because of the evident damage found in pathology specimens and imaging studies (**Fig. 1-6**) (106). Cartilage degeneration is, however, the ultimate effect of the OA process (92, 102). There is growing evidence (clinical and scientific) of the high prevalence of synovitis at all stages of OA (**Fig. 1-7**). An increasing number of studies have demonstrated the relation of synovitis to pain, poor function and that it is often an independent driver of structural changes and progression of radiographic abnormalities (3, 12, 38-40, 107).

Role of the synovium

OA was traditionally classified as a non-inflammatory arthritis, considering the absence of relevant increases in leukocyte counts or other biomarkers of inflammation in synovial fluid, especially when compared to RA (RA) (104). Yet, common clinical signs of OA, such as joint
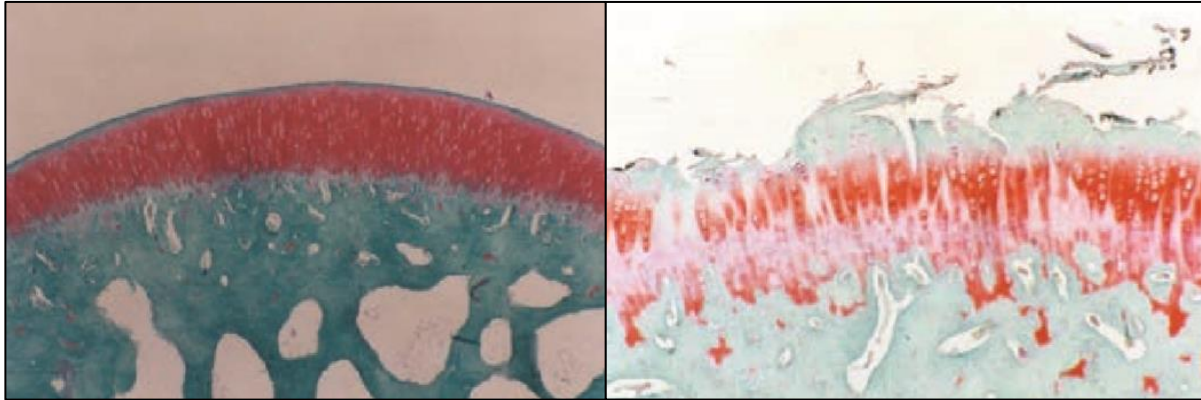
Figure 1-6: Cartilage sections stained with safranin O and fast green. Normal (left): smooth surface with marked proteoglycan uptake of safranin, and a defined tidemark. Osteoarthritis (right): osteoarthritic cartilage showing chondrocyte proliferation, decreased red stain of proteoglycans, and a duplicated tidemark with blood vessels invasion. © 2014 François Rannou. Originaly published in Pathophysiology of Osteoarthritis, Atlas of Osteoarthritis, Nigel Arden, Francisco J. Blanco, Olivier Bruyère, Cyrus Cooper, Ali Guermazi, Daichi Hayashi, David Hunter, M. Kassim Javaid, Francois Rannou, Jean-Yves Reginster, Frank W. RoemerA (Eds), Springer Healthcare (5).

effusion, swelling and reduced range of motion are signs of joint inflammation (106). Studies from the early 1980's, reported histological evidence of synovitis (inflammation in the synovium) of osteoarthritic joints in the majority of patients (108). Synovial inflammation occurs during the early stages of OA and is very often subclinical. In fact, many patients undergoing arthroscopy with no clinical signs of inflammation aside of pain, have localized proliferative and inflammatory synovium (7). Non-invasive techniques such as magnetic resonance imaging (MRI) have improved the ability to identify localized areas of synovitis that previously went undiagnosed either clinically or by ultrasound (7, 109). The intensity of

synovitis varies according to disease activity, duration and related changes in other joint tissues (Figure 1-8) (7, 41, 104). In more advanced stages of OA, synovitis is associated to disease severity and correlates with both the Kellgren-Lawrence scoring system, as well as by narrowing of the joint space (106). Therefore, synovitis is undeniable factor in the pathophysiology of both equine and human OA (3, 110, 111).

Characteristic histological changes of inflamed synovium include synovial hypertrophy and hyperplasia characterized by enlargement of the synovium and increasing numbers of total and hyper activated lining cells, often associated with increased vascularity and scattered foci of lymphocyte infiltration in the subintima (Figure 1-9) (7, 33, 34, 112). In end-stage OA, synovial cells are replaced by fibrous tissue, which affects all of the basic functions of the synovium (42).

In contrast to rheumatoid arthritis (RA), synovitis in OA is primarily restricted to areas adjacent to damaged cartilage and bone (34, 106). In these areas, synovial macrophages respond to damage, at first by inciting inflammation. If the inflammatory response is not efficient in counteracting the damage, and therefore does not self-resolve, it catalyzes progressive degenerative changes that culminate in an altered synovial microenvironment (113).

Inflamed synovium synthesizes a wide range of cytokines, chemokines (such as IL-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, nitric oxide (NO), prostaglandin E_2 (PGE_2)), proteases and neuropeptides (7). Several of these mediators are detected in increased concentrations in synovial fluid and tissues of OA joints, and are considered to have catabolic effects and accelerate destruction of the nearby cartilage (7, 114, 115). These cytokines also upregulate transcriptional networks, including nuclear factor kappa B (NFkB) and mitogen-activated protein (MAP) kinase, which induce synovial cells to increased production of collagenase, proteolytic enzymes and other lipid mediators causing vascular hyperplasia in the

Figure 1-7: OA histology: Normal synovium with thin lining and loose collagenous sublining. OA synovium demonstrates villous and lining hyperplasia (arrows) and increased vascularity. OA cartilage with ECM loss and areas of chondrocyte loss and clustering, tidemark duplication and thickening of the calcified zone, and vascular invasion of the subchondral bone. © 2012 Richard F. Loeser, Steven R. Goldring, Scanzello Carla R., Mary B. Goldring. Originaly published in Osteoarthritis: a disease of the joint as an organ, Arthritis and Rheumatism, Dr. Richard Bucala (Ed), Springer Healthcare, DOI: 10.1002/art.344 (11).

osteoarthritic synovium. Most of these catabolic molecules are produced by the diseased cartilage itself. However, the cross-communication of altered cartilage and the inflamed synovium amplifies this response and creates autocrine and paracrine vicious circles of degradation. Such environment compromises chondrocyte function leading to hypertrophic differentiation and early senescence (110, 116). In addition, synovitis leads to synovial effusion eliminating the normal negative pressure within the joint, which affects synovial fluid turnover, producing pain and micro-instability adding more deleterious effects of inflammation (6).

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Inflammation and neovascularization often develop in combination. Inflammation facilitates angiogenesis maximizing the transport of inflammatory mediators to the sites of inflammation (106). As clinical disease is associated with synovial inflammation leading to joint degradation, therapies targeting the central drivers of synovitis have the capacity to improve OA symptoms and prevent disease progression (110).

Synovial macrophages in health and disease

The immune response and cytokine production in the OA-affected synovium is centrally activated by synovial macrophages (**Fig. 1-8**) (12, 43, 44). The role of macrophages as drivers of synovitis was first evidenced when systemic macrophage depletion dramatically blocked the development of OA in a mouse model (117, 118). Further, the effect of articular macrophage depletion in controlling joint inflammation was corroborated in human patients with RA (119). The anti-inflammatory effects associated to macrophage depletion include decreased expression of MMP1, MMP3, MMP9, MMP13, IL-6, IL-8, and bone morphogenetic protein (BMP)-2 and -4, identifying some of the mediators involved in the process (12, 43, 113, 119-122). Moreover, a recent *in vivo* study showed that, macrophage activation is directly related to disease activity, severity and pain in human patients with OA (41), suggesting that synovial macrophages could be a key therapeutic target.

A detailed characterization of the relative counts of immune cells within the OA synovium identified macrophages as the most abundant leukocyte (65%). While T (22%), B (5%) and plasma cells (>1%), as well as mastocytes can also be detected in the OA synovium, their contribution is hitherto poorly defined (**Fig. 1-9**) (59). Synovial fibroblasts and chondrocytes are crucial for amplification of the inflammatory response in OA, but play a minor role in initiating the process (3, 59, 123). Whether neutrophils appear to have a role in RA (124), they are nearly absent in the synovial fluid and membrane of OA-affected joints (38, 59). Monocytes and macrophages are also the main cell type in the synovial fluid of both healthy and OA-affected joints.
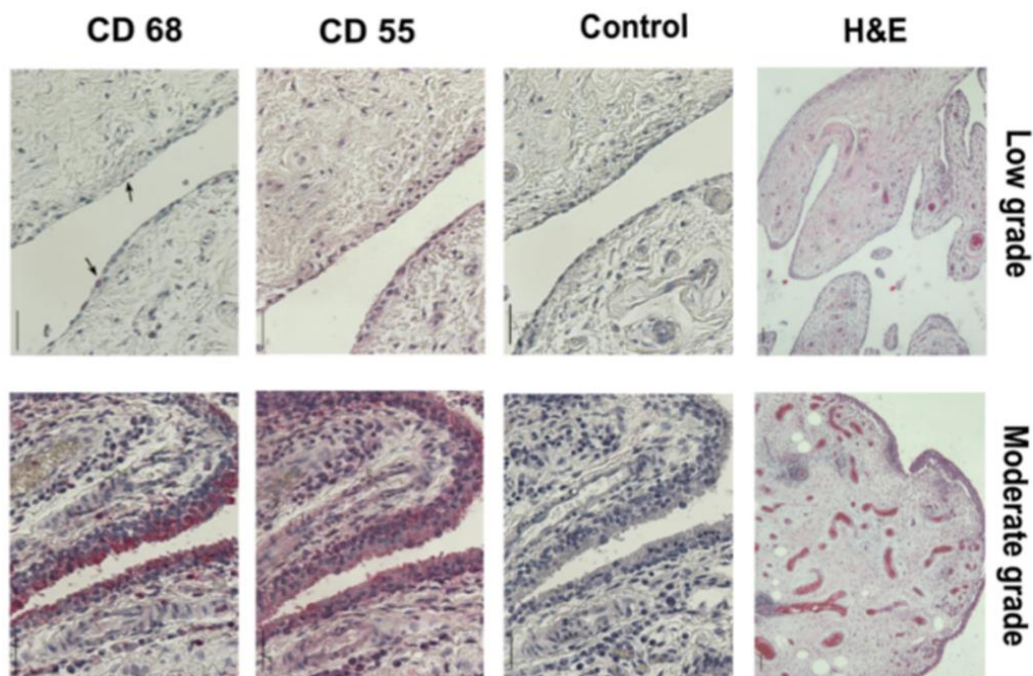
Figure 1-8: Histological features of H&E stained osteoarthritic synovial membranes with low-grade (top) and moderate-grade synovitis (bottom), showing differences in intimal cellularity and subintimal vascularization (40×magnification), as well as increased expression for macrophages (CD68) and fibroblasts (CD55) markers. From Manferdini et al, 2016 (12). http://creativecommons.org/publicdomain/zero/1.0/.

The overall cellular infiltration in the synovium of OA-affected joints tends to be much lower compared to RA joints; however, the macrophage percentage is comparable or even higher in in the synovium from OA-affected joints (38, 122). A predominance of macrophage infiltration in the synovium is especially common during the early stages of OA (42). Macrophages in the osteoarthritic synovium communicate with neighboring synovial fibroblasts and T cells in the perivascular infiltration, through a known group of cytokines that participates in the activation and survival of different types of immune cells. Among these cytokines, IL-4
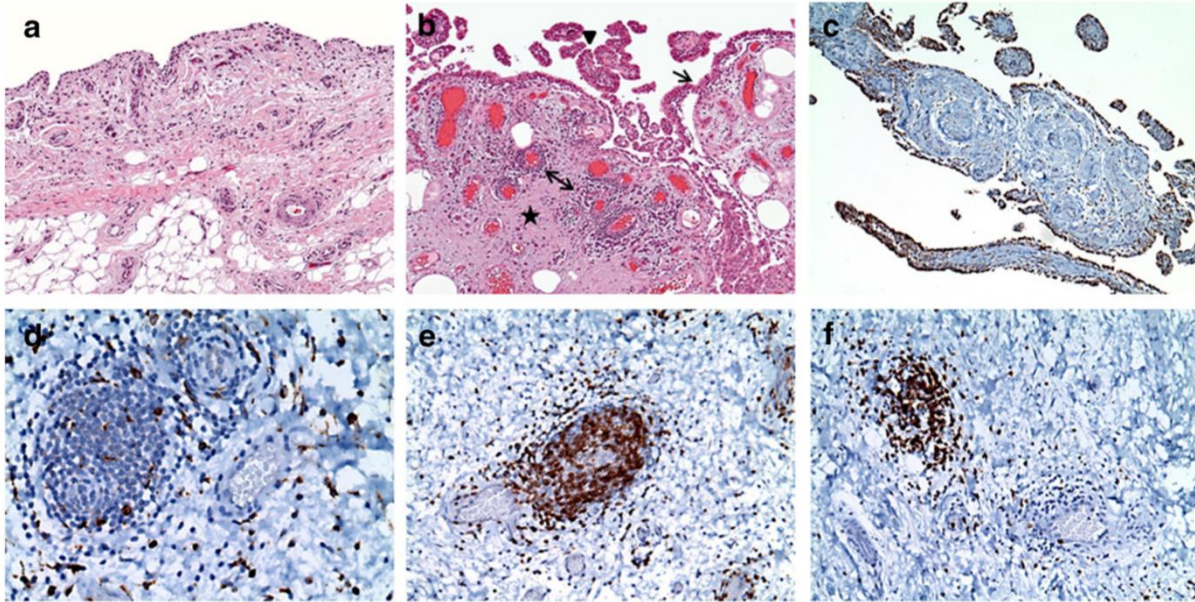
Figure 1-9: Synovium sections from patients with late stage OA subjected to arthroplasty. H&E (**a** and **b,** 20 ×) or IHC stains for the macrophage marker CD 68 (**c** and **d,** ,10X and 40X), for the T cells marker CD3 (**e,** 40 ×), and for the B cells marker CD20 (**f,** 40X). © 2012 Steven R. Goldring, Carla R. Scanzello. Originaly published in The role of synovitis in osteoarthritis pathogenesis, Bone, Dr. Sundeep Khosla (Ed), Elsevier, DOI: 10.1016/j.bone.2012.02.012 (7).

dramatically influence macrophage activation, and IL-15 and IL-17 have been implicated in the T cell activation during the pathogenesis and progression of OA (11, 125).

Synovial fibroblasts play an important role in the perpetuation of the inflammatory response during the development of joint disease. Fibroblasts from the osteoarthritic synovium respond to *in vitro* stimulation with TNF-α, IL-1β and interferon, by releasing IL-6, IL-8 and high concentrations of macrophage chemoattractant protein (MCP)-1. In a cross-talk between synovial macrophages and chondrocytes, these cytokines and chemokines can markedly enhance the inflammatory reaction in OA (126). As most cells within the joint, synovial fibroblasts express Toll-like receptors (TLRs)-2 and 4 have, which are known to participate in the

pathogenesis of OA (127). One of TLR-2 and TLR-4 ligands is the CD14 molecule, highly expressed by activated macrophages. CD14 can exist bound to the cell and also in a soluble form during active inflammation. Binding of soluble CD14 from OA synovial fluid to TLR receptors in synovial fibroblasts has been shown to trigger the production of many cytokines involved in the initiation and progression of OA (127, 128). These cytokines also induce synovial fibroblast to produce MMP-1 and MMP-3, highlighting their role in amplifying inflammation and cartilage degeneration (129).

Signaling pathways such as NF-κB, STAT and Pi3K are persistently activated on macrophages in the arthritic synovium. These signaling pathways are major sources of inducible NO and cytokines including IL-1β and TNF-α (42). Osteoarthritic macrophages also produce enzymes leading to cartilage matrix degradation like MMP-2, MMP-3 and MMP-9 (130). While MMP-3 directly causes destruction of cartilage, MMP-2 induces chondrocytes to express other MMP. Eventually, sustained inflammation and the production of related cytokines and proteolytic enzymes, cause joint degeneration (122, 131, 132).

Although synovial macrophages are associated to deleterious effects during synovitis and OA, they play a crucial role in joint homeostasis and tissue repair, through phagocytic clearance and secretion of cytokines, chemokines and growth factors (37, 44, 124). Macrophages are the main source of IL-10 (133, 134), an essential cytokine for cartilage homeostasis and tissue repair (135-139), whose absence prevents efficient recovery from articular damage (135-137). Importantly, macrophage depletion negatively affects chondrocyte metabolism and chondrocytic differentiation of progenitor cells. Synovial macrophages modulate the transcriptional machinery of chondrocytes and synovial progenitor cells in a paracrine fashion, which is

absolutely required for the expression of central genetic markers of chondrogenesis (SOX9) and synthesis of collagen type II (COL2) (44).

Macrophages therefore perform two seemingly opposite functions in the joint – they are both the key drivers of joint health and, alternatively, of joint disease (107, 140). Despite significant research efforts to understand how synovial macrophages communicate with the articular environment (12, 43, 44, 64, 122, 131), the exact mechanisms responsible for inducing these opposed functions remain unmet. It has been proposed that these opposing functions relate to opposite patterns of macrophage activation (phenotype polarization) (12, 50, 124, 131, 141-145), leading to substantial research regarding the participation of these opposing phenotypes in health, chronic inflammation and disease in many different tissues (146, 147).

Macrophage activation and phenotype polarization

According to environment stimuli, macrophages differentiate into a spectrum of defined cell phenotypes, with the polar extremes represented by cells displaying a classical pro-inflammatory response (M1), or an alternative suppressive/healing response (M2). These activations states were defined based on *in vitro* observations of their respective ability to kill (M1) or repair (M2), in the contexts of infection and wound healing respectively. This nomenclature comes from a comparison to the opposing T cell phenotypes (Th1 and Th2 polarization) (141-143). An increasing number of markers have been proposed to identify these macrophage phenotypes (12, 131, 141, 148, 149). However, there is inconsistency and contradiction regarding their interpretation in the currently available literature. Since most of these markers were identified in the artificial context of in vitro cultures, their accuracy in translating to macrophage phenotypes in vivo has been questioned (50, 150).

The roles of different macrophage phenotypes during joint health, chronic inflammation, and disease have been minimally investigated. Specific information regarding macrophage phenotypes in joint disease is limited and restricted to *in vitro* or experimental data or late/end stage OA (12, 122, 131, 151) and the only report describing healthy joints is limited to the low numbers of synovial fluid macrophages shedding from the synovium following hyperactivation (152). Up to date, it has been reported that *ex vivo* chondrogenesis of synovial progenitor cells is impeded by classically activated (M1) macrophages from the osteoarthritic synovium (131). Nonetheless, alternatively activated (M2) macrophages are required for efficient chondrogenesis (44). Inflammation in arthritic joints is dampened by macrophage-derived MMP-12 (M2), improving clinical and histological signs of joint disease (153, 154). However, joint injuries overwhelming the homeostatic functions (M2) of resident macrophages elicit an inflammatory phenotype (M1), upregulating inflammation and increasing the recruitment of macrophages and immune cells in face of the increased demands for tissue repair (43, 141, 155-157).

In vivo, macrophage phenotypes are not a black and white concept (50, 141, 142, 144, 145). The idea of macrophages as either inflammatory (M1) or suppressive/healing (M2) cells originated from monocyte-derived macrophages treated *in vitro* with defined and overwhelming cytokine stimuli (142, 145, 148). Therefore, clear identification of macrophage phenotypes *in vivo* is significantly more challenging than proposed by *in vitro* models (50, 131, 150, 151). *In vivo*, macrophages activate exhibiting marked plasticity and often a hybrid state between polar phenotypes (50, 146, 147, 150, 151). Current knowledge suggests that *in vivo,* macrophages are by default homeostatic cells that, drive inflammation following injury with the purpose of counteracting tissue aggressors and recruit cells to initiate tissue repair. Macrophages play such a fundamental role in resolving inflammation and promoting tissue healing that their depletion or

exhaustion results in severely compromised wound healing and regeneration, or leads to chronic inflammation (50, 151, 158). Once tissue repair has been efficiently accomplished, macrophages coordinate resolution of inflammation, ultimately achieving homeostasis (47, 50, 157, 159). Therefore, the presence of chronic inflammation suggests that the inflammatory response has not achieved its purpose and could not be efficiently resolved, and overwhelmed local macrophages remain inciting inflammation. In this line of thoughts, is reasonable to consider that increasing the numbers of macrophages in inflamed and OA-affected joints, would optimize the macrophage response in counteracting damage, favoring resolution of synovitis.

Cartilage: a target and a protagonist in OA

Damage to the articular cartilage is the hallmark of OA and is found in varying extents. These changes seem to firstly occur at the superficial layer, at areas more highly exposed to mechanical forces of loading and shear (160). Several events that happen to the cartilage during early OA result from a dysfunctional chondrocyte metabolism. Chondrocytes, as well as macrophages and fibroblasts from the OA synovium produce supraphysiologic concentrations of inflammatory cytokines (IL-1β and TNF-α), which lead to increased production of other mediators of inflammation (IL-8, IL-6, PGE_2 and NO) and the activation of proteolytic enzymes (including MMP) (106).

Although the effect of cytokines is most of the time associated to deleterious effects, in healthy cartilage a balanced influence of cytokines, growth factors and mechanostimulation, is critical for chondrocytes to maintain proper tissue homeostasis, which is represented by a low-rate turnover of cartilage matrix proteins. Disruption of this equilibrium, induce aberrant chondrocyte activation for adaptive remodeling of the surrounding cartilage ECM (11). This

response varies with the intensity of the stimuli. Extreme stimulation induces an injury response that involves degradation of matrix components with the purpose to replace them with an ECM network of higher mechanical resistance. However, efficient cartilage remodeling requires recapitulation of gene expression patterns from early development (e.g. SOX9, COL2a1, GDF-5), which are nonetheless dramatically downregulated or even silenced following birth and adulthood, limiting the extent to which ECM components can be produced and replaced into the adult cartilage (161, 162). Such dysregulated response leads to inappropriate chondrocyte proliferation, cluster formation and hypertrophy-like maturation, with increased synthesis of cytokines and matrix components, but even higher activation of matrix-degrading enzymes (4, 11, 23). The resulting cartilage has altered mechanical and biochemical properties that increase the expression of several molecules involved in cartilage deterioration. The ultimate result is cartilage loss, partial replacement by inferior quality matrix, and calcification of the deep layers of diseased adult cartilage (11, 95, 162).

Mechanical stress induces altered chondrocyte metabolism and increased NO production, which similar to cytokines, dysregulate collagen and proteoglycan synthesis contributing to breakdown of the peri-chondrocyte ECM (106, 163). The mechanoreceptors from chondrocytes are also sensitive to altered ECM components inducing production of cytokines and proteases of the MMP family (e.g. ADAMTS-5 and MMP-3) (23, 164). As the articular cartilage is degraded, fragments of fibronectin, small leucine-rich proteoglycans (SLRP), COMP, and collagen act in a feedback-loop, stimulating further cartilage degeneration (78, 165-168). Exposure of type II collagen to the discoidin domain receptor 2 (DDR2) in the surface of the chondrocyte activates MMP-13, increasing collagen breakdown (169). Fibronectin and collagen fragments also stimulate cytokines and MMP production (165, 166, 168). The effects of

members of the SLRP family (e.g. fibromodulin and decorin) can vary from inducing complement activation or its inhibition (170, 171). Conversely, COMP is a potent complement activator and both of COMP and C3b, as well as their immune-complexes have been identified in synovial fluid of OA joints (11, 113, 123). As a result of the combined activity of these molecules and other cytokines and enzymes, the collagen network often reaches a state of degradation that is unlikely to be efficiently stopped (11).

Another trans-membrane proteoglycan receptor involved in mechanotransduction and cartilage homeostasis is Syndecan-4. During mechanical stress and inflammation this receptor is cleaved affecting cell adhesion (172), and also serves as a positive effector of ADAMTS-5 activation through regulating the synthesis of MMP-3. In the interterritorial regions of the cartilage where these receptors are less frequent, the cartilage is protected from enzymatic degradation by a combination of its physical location and decreased activation of ADAMTS-5, preserving the proteoglycan coat and the mechanical resistance of the matrix (11, 173). In fact, syndecan-4 neutralization impedes progression of OA in murine models (11).

Osteoarthritic chondrocytes, particularly those from clonal clusters, express several cytokine receptors, and genes reported to involved in the modulation or amplification of the inflammatory response (e.g. COX-2, mPGES-1, sPLA2, INOS2). Chondrocyte activation resultant from inflammatory and excessive mechanical stimuli, arises primarily through pathways such as the NF-κB, and Mitogen-Induced Protein Kinase (MAPK) (174). On the other hand, synovial inflammation leads chondrocytes and synoviocytes to secrete alarmins, also known as damage-associated molecular patterns (DAMPs). These molecules are ligands for TLRs or Receptor for Advanced Glycation Endproducts (RAGE) and participate in the amplification of the inflammatory response (175). Chondrocyte expression of TLRs is

upregulated by inflammation. TLR-2 and TLR-4 activation in OA chondrocytes lead to increased expression of genes involved in inflammation-derived catabolism, such as MMP-3, MMP-13 and INOS2, as a downstream effect of NF-κB signaling (175).

The high mobility group box 1 protein (HMGB1) is a dual function alarmin that locates to the cell nucleus, and play key roles in chondrocyte homeostasis. After sensing damage HMGB1 translocates to the cytosol or extracellular space, and signal to amplify an inflammatory cascade. HMGB1 contributes to OA development and progression by effecting on articular chondrocytes and synoviocytes mediating the amplification of inflammation (175-179). Other alarmins such as S100A4, A8, A9, and A11, also signal through RAGE and TLRs amplifying the catabolic effects of inflammation, leading to increased cytokine-derived production of ROS (175, 180). Inflammatory cytokines, NO and ROS also alter the mitochondrial function of chondrocytes through oxidative stress leading to decreased autophagy, senescence and apoptosis (4, 11, 23) (181-183).

Aging is a well-established risk factor for OA due to the related overt matrix remodeling. This process is associated to the accumulation of RAGE ligands, whose signaling increase cytokine activity, and downstream affect cartilage viscoelasticity contributing even more to chondrocytes senescence. Loss of superficial zone chondrocytes can also be a consequence of age-related decreases in the HMGB2, which opposing to HMGB1 have homeostatic effects on cartilage (184). Collectively, these events support the concept that OA, in some circumstances, can indicate premature aging of the joint (106).

Calcification of the articular tissues is often associated to deposition of calcium crystals (pyrophosphate and/or hydroxyapatite) in the joint, and is a common finding in advanced OA (185). These crystals can act as TLRs ligands in chondrocytes and synovial macrophages

upregulating inflammation. In the specific case of hydroxyapatite crystals, they may upregulate

inflammation through activation of the NLRP3 inflammasome, proposing it as a target for novel

approaches for preventing progression of OA (11, 186). Altogether, these events show that

chondrocytes are active participants in controlling the cytokine production and joint integrity,

instead of passive victims of the disruptive force of cytokines and proteolytic enzymes (174,

187).

Repair of damaged articular cartilage

The main limitation joints face in recovering from cartilage injuries is the poor capacity

of the non-vascularized cartilage defects to heal, reassembling the original architecture of its

matrix. Defects in cartilage may span from focal fibrillation (collagen fiber condensation and

fraying) to full thickness eburnation (ulceration). Defects that do not extend past the tidemark

more commonly reach a stagnant non-healing state. However, when crossing past the tidemark

and reaching close to the subchondral bone, there is an improved healing response (6, 77, 188,

189). Cells from hematopoietic and mesenchymal origin from the vascularized subchondral

bone and adjacent bone marrow, fill the defect and undergo differentiation and maturation to

form fibrocartilage (190-192). Although this process recapitulates that from early development,

the newly formed tissue does not have architectural and biochemical differences according to

zonal variation (193, 194), not coping with the different demands of weight-bearing and non-

weight-bearing areas. In addition, repair of articular cartilage is largely affected by the age of the

patient (6).

Three mechanisms are considered the basis for cartilage repair (6, 195):

1. Intrinsic repair (from within the cartilage) - relies on the limited capability of chondrocytes to divide and the dysregulated collagen and proteoglycans synthesis.

2. Extrinsic repair – relies on mesenchymal cells and solutes from the subchondral bone and underlying bone marrow, leading to granulation tissue formation, which is further replaced by fibrous connective tissue that undergoes metaplasia including cartilage elements, and thus is called fibrocartilage.

3. Matrix flow – the perimeter of the cartilage injury contributes to repair by creating lips of cartilage that progress towards the center of the injury.

Partial-thickness defects of cartilage in young and juvenile individuals can regenerate to produce hyaline cartilage that is close to normal, yet this phenomenon is not observed in adult mammals. Superficial defects not necessarily progress in extent or compromise joint function. In fact, it has been demonstrated in horses that the loss of up to 30% of cartilage surface in a single spot does not seem to impair athletic activity. However, losses greater than 50% of the cartilage surface or substantial damage to the subchondral bone significantly worsen the odds of having a fully functional joint. One reason for this attempted repair to fail is that binding of repair cells to the injured surface is ineffective, and chondrocytes in the surrounding area of the injury (150 µm) are also mechanically damaged, which leads to the formation of a zone of "cartilage necrosis" preventing repair (6).

Similar to partial thickness lesions, the location and size of full-thickness cartilage significantly influence the quality of tissue repair, with larger lesions being less likely to heal. The repair tissue formed after full-thickness cartilage injury is primarily composed of type I

collagen and thus named fibrocartilage. As the GAG content in the repair tissue is lower and a tide mark does not form, the biomechanical properties of this tissue are inferior, easily breaking down if exposed to shear stresses (6, 196, 197). A study comparing large (15 mm^2) and small (5 mm^2) full-thickness cartilage defects in weight-bearing and non-weight-bearing areas, revealed that small defects would fill with granulation tissue in 1 month, by both matrix flow and extrinsic repair mechanisms. At 4 months the repair tissue showed slightly higher organization, with defects becoming hardly detectable by 5 months. On the other hand, even though the initial repair of large lesions was good, at 5 months the repair tissue failed to integrate the adjacent tissue developing peri- and intralesional clefts which extended to subchondral (195, 196). Collectively, these studies show that repair of adult articular cartilage depends on the response from surrounding tissues and the varying challenges associated to its incapacity to produce a tissue with properties of native articular cartilage (6, 77, 188, 189, 197). For those reasons, the search for innovative approaches aimed at improving osteochondral and cartilage repair is a constant target in the field of orthopedic research.

Subchondral bone

Increasing evidence shows that the subchondral bone can be a key factor in the development of osteoarthritis across species (198, 199). Changes in bone density leading to early subchondral bone sclerosis can reduce the shock-absorbing ability of the joint, increasing the risk of mechanical failure of the cartilage cross-links, especially under repetitive loading (6, 17, 200). While both the increased density of subchondral bone and osteoporosis are considered predisposing factors for OA development, the complex cross talk between the articular cartilage and the subchondral bone are still a developing research field (201).

When horses are subjected to athletic exercise, subchondral bone micro damage often develop in combination with primary osteocyte death (**Fig. 1-10**) (202, 203). Primary loss of this mechanical support not only causes damage to the subchondral bone, but often progresses to catastrophic fractures. The cytokine output from injured subchondral bone also alters the articular cartilage metabolism, involving increased activity of MMP similar to that observed in the articular side of the cartilage (6, 199-201, 204).

Mechanically induced OA in racehorses can lead to osteochondral abnormalities ranging from commonly seen cartilage fibrillation and erosion, to cartilage indentation and subchondral bone cavitation resembling trauma induced osteochondrosis. Other common findings include thickening and sclerosis of the subchondral bone, associated to increasing osteocyte necrosis and osteoclastic remodeling, microfracture of the trabecular bone leading to increased vascularity, hemorrhage, fibrin, and fibroplasia often associated with obliteration of vascular channels with
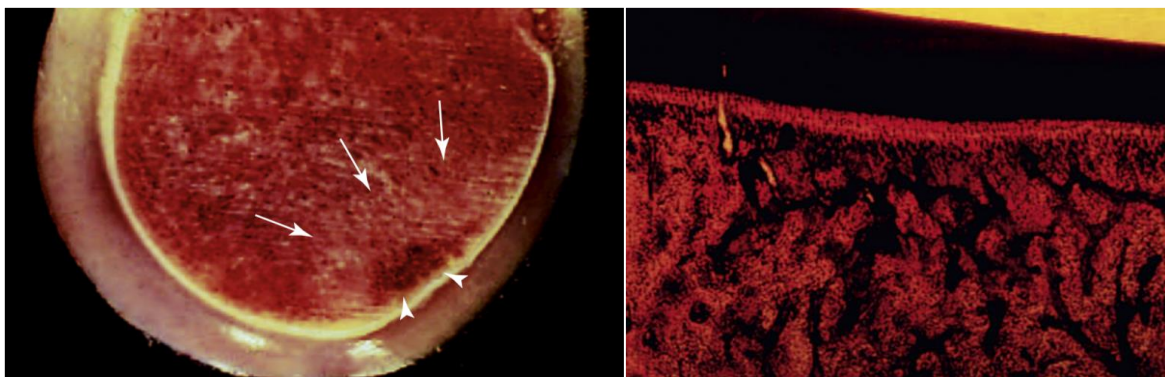
Figure 1-10: Gross pathology from a specimen of a horse euthanized due to catastrophic injury in the opposing limb. Note the area of necrosis in subchondral bone with a surrounding sclerotic area despite of intact cartilage (left). Histologic section (basic fuchsin stain) of articular cartilage and subchondral bone exhibiting subchondral bone microcracks. From McIlwraith et al, 2016, "Joint Disease in the Horse" (6). Reprinted with permission of Elsevier, 2019.

plugs of degraded matrix. Fragmentation of the subchondral bone suggests matrix brittleness. Curiously, many of these findings can exist under an overall healthy looking cartilage, at times showing random indentation and mild degeneration of its superficial layers. Focal cracks in the calcified layer are associated to cartilage in-folding (6, 202). Of note, metacarpal condyles from horses experimentally trained on a treadmill were reported to exhibit milder changes than those exercised on the race track, and changes in the metacarpophalangeal joint are more marked than those found in the carpus (200, 203). Altogether these findings reinforce the role of mechanical impact in the pathogenesis of subchondral bone damage, as joints in the distal limb such as the metacarpophalangeal joint receive a higher load of impact than joints in the proximal limb.

Cytokines

Cytokines are a large group of small regulatory proteins and glycoproteins secreted by a wide array of cells. Historically, they were associated with fever, infection, inflammation and the related catabolic pathways. Therefore, detection of increased levels of cytokines in tissues were historically considered pro-inflammatory and associated to tissue degeneration, and thus, cytokines quickly became a villain in biomedical research (23, 205, 206). In joints and many other connective tissues, cytokines control the metabolism of all joint tissues in healthy state through a complex balance of activities. Following injury, cytokines expression and activity change to counteract aggressors and to drive tissue repair, or may remain altered in diseased tissues that not reach homeostasis (17, 206, 207). There is a growing number of cytokines identified to be involved in articular metabolism, and they possess pro- and anti-inflammatory functions, and can induce both anabolic and catabolic effects (114, 174, 208-212).

In OA, cytokines such IL-1 (α and β) gained substantial attention due to their increased expression in OA cartilage and synovium (39, 115, 206, 213). In osteoarthritic cartilage, chondrocytes upregulate the expression of IL-1 and TNF-α receptors. Binding of these ligands to their receptors alters chondrocyte metabolism, driving increased degradation of ECM components as a result of increased production of hydrolytic enzymes such as MMP-3 (214, 215). IL-1 and TNF-α are generally expressed at very low concentrations in synovial fluid of normal and OA samples (216-218), and differently from synovial fluid samples from RA joints, they are very often not even detectable in samples from OA joints (212, 219). Nonetheless, minimal changes in its concentration (as much as 3-4 fold increase) are enough to dramatically increase the activity of their downstream effectors (214).

A wealth of research targeting IL-1, especially IL-1α, have demonstrated its importance as a pro-inflammatory cytokine in OA. Early studies of *in vitro* culture of cartilage, provided data supporting a role for a synovium derived factor inducing cartilage matrix degeneration; however, the chemical nature and identity of the factor(s) was not characterized (220, 221). Further, "IL-1 like" activity, define by the effective induction of T cells to produce lymphokines, was demonstrated in synovial fluid of OA-affected joints (222, 223) in a similar frequency in which IL-1 is currently detected (216). In continuity, such findings were then supplemented by characterization of equine and other species' IL-1, and detection of increased concentrations of IL-1 in synovial fluid of osteoarthritic joints (224).

While IL-1 is detected in much higher concentrations in OA than in healthy cartilage (206, 214), it cannot be detected in the synovial fluid of at least 50% of samples from OA joints (216, 225-227), and there is no consistency in differences of IL-1 concentrations between healthy and OA joints (228). In the synovium, as in cartilage, IL-1 is also detected in normal joints, but

is more markedly expressed during joint disease. Noteworthy, its native antagonist (IL-1 receptor antagonist (IL-1ra)) is expressed at much higher magnitude, both in the synovial fluid and membrane (39, 225-227, 229). Therefore, observations of the imbalance between IL-1 and IL-1ra reported for RA do not apply to OA tissues (225, 230).

Increases in IL-1 are associated to cartilage matrix degradation and development of repair tissue that is inapt to resist mechanical stress. Increased IL-1 induces altered synthesis of type II collagen and proteoglycans (231-234), as well as production of enzymes that cleave cartilage components, both in a time and dose dependent manner (233-237). IL-1 also potentiate catabolism by inhibiting the synthesis of TIMP (238). Moreover, IL-1 induce production of prostaglandin E2 and NO, which when expressed supra-physiologically can promote increased cartilage degrading activity (111, 235, 237, 239) and may also contribute to osteophyte formation and proliferation in OA through stimulation of osteoblastogenesis (240-243). Of note, the IL-1 concentrations required to induce expression of matrix degrading enzymes is substantially higher than that required for inhibiting/decreasing matrix synthesis (235). Collectively, these isolated observations build some evidence of the destructive role that increased IL-1 can have in OA.

The IL-1 receptor antagonist protein shows a protective *in vitro* effect of blocking many of the typical catabolic events of IL-1, supporting the previously listed roles of IL-1 in OA. While interesting results of experimental IL-1ra therapies were seen in equine joints (244) and in OA-like lesions in canine models of arthritis (241, 245), its use in the clinical scenario failed to provide satisfactory clinical results in people (246, 247) and there are no similar objective assessments for horses. Controversially, recent studies considering an OA model using IL-1α and β knock-out mice, showed that despite of the absence of IL-1, OA development happened similarly to that seen in wild type mice or was even worse (243, 248).

Interleukin-1 has been claimed as the most important cytokine in OA. While studies leading to that conception indeed established the important relevance of IL-1 in cartilage degeneration, those studies where mostly or solely focused on IL-1 instead of assessing a broad range of cytokines (111, 220-224, 231-234). In addition, while both isoforms (α and β) have been identified in OA, the effects of IL-1α in joint disease are much more powerful (214, 216). Nonetheless, most experimental models evaluating the effects IL-1 on chondrocytes and cartilage degeneration have used recombinant human or equine IL-1β, using *in vitro* models considering concentrations of IL-1 that are far beyond limits detected in natural disease (232, 233, 235-237). Since OA is a whole joint disease and not only a cartilage condition, studies focused on the effects of IL-1 on synovial fibroblasts and macrophages, as well as on the subchondral bone, using lower doses of IL-1(α and β) would be necessary to better understand the role of IL-1 in joint disease. While it is clear that IL-1 participates in disease processes of OA, studies supporting its central role are sparse. In fact, recent proteomic analysis of synovial fluid and transcriptomic analysis of cartilage from normal and OA-affected joints did not point IL-1 as a driver of joint disease (175, 178). The role of IL-1 still needs to be assessed in combination with the multiple cytokines, chemokines and lipoproteins involved in the disease process of OA.

TNF-α is also a cytokine classically identified as pro-inflammatory that has been implicated in the pathogenesis of osteoarthritic lesions and detected in increased concentrations in the event of joint inflammation and OA (213, 249-251). Similar to IL-1, under *in vitro* conditions, TNF-α incite the production of cartilage-degrading enzymes and impairs the synthesis of proteoglycan and collagen by chondrocytes (234). Although the effects of TNF-α appear to be less powerful than IL-1 (252), they are described to be potentiated when combined (17, 253). Interestingly, TNF- α has been a much more useful therapeutic target as a disease-

modifying agent in treatment of the exacerbated inflammatory reaction in RA, than IL-1 (254, 255).

Although TNF-α has traditionally been associated with several inflammatory joint conditions, with defined roles in accelerating cartilage destruction through activating matrix metalloproteinases (MMP) and aggrecanases (ADAMTS) (256). However, TNF-α also appears to play critical roles in both inciting and resolving inflammation (257, 258). TNF-α is involved in localizing harmful agents and amplifying local and systemic immune responses and, as observed in our study, coordinates myeloid cell proliferation to attend increased demands during recovery of inflammation-derived damage and tissue repair. These processes also involve other effects of TNF-α such as regulation of apoptosis and efferocytosis (257, 258). In mouse models, TNF-α alone is not arthritogenic or destructive and exerts its arthritogenic potential through IL-1 induction (259). As such, induction of arthritis in TNF-α deficient mice does not produce reduction in clinical, histological, and morphological joint abnormalities, suggesting that other cytokines, such as IL-1, may have a higher role in disease processes (260). In agreement, a study evaluating the *ex vivo* production of TNF-α and IL-1β following LPS stimulation of macrophages failed to identify TNF-α production as a predictor of OA, in contrast to IL-1β (261).

Beside IL-1 and TNF-α, several cytokines classically know as pro-inflammatory including IL-6, IL-8, IL-15, IL17 and PGE_2 have been identified in clinical and experimental contexts of OA and all seem to play relevant roles in the inflammatory and degenerative loop in joint disease and repair of articular tissues (43, 114, 209-211, 218, 250, 262-264). The degradative effects of increased concentrations of inflammatory cytokines, such as IL-1 and

TNF-α, are counteracted by endogenous regulatory cytokines (e.g., IL-4, IL-10, IL-1ra and IL-13) in a homeostatic negative feedback loop (135-137, 209, 210, 225, 251, 265-267).

Current technology has allowed the identification of the above mentioned and other cytokines and chemokines, such as GM-CSF, PPAR-γ (107, 114), interferon-γ (IFN-γ) oncostatin-M (OSM) (228), substance-P (218), CCL5, the axis of CCL19-CCR7, MCP-1, MIP1β (7), whose roles in joint disease and inflammation still remain to be more precisely studied. Also, cytokines known as growth factors (GF) (e.g., transforming GF (TGF), insulin-like GF (IGF), endothelial GF (EGF) and basic fibroblast GF (FGF) have stimulatory or inhibitory effects on synthesis of matrix components. Targeting these pro- and anti-inflammatory cytokines to modulate process involved in joint disease has also been an important area of research, however limited advances have been achieved (17, 101, 114, 187, 268-271).

Prostaglandin E_2 (PGE_2) production is reported to be increased in inflamed and OA-affected joints (272-274), and is associated to reduction in the proteoglycan content of the cartilage ECM when released from chondrocytes following stimulation with IL-1 and TNFα *in vitro* (275). Therefore, similarly to IL-1 and TNF-α, PGE_2 quickly became a villain in the OA context. While it could be accurately considered as a key mediator of vascular changes and pain in the inflammatory reaction in OA (276), it is also associated to multiple physiological functions (277), is vital for chondrocyte survival (278), and is the key for benefits of stem cell therapy (279).

The enzymes cycloogenase-2 (COX-2) and prostaglandin E synthase are required for the production of PGE_2, and both of them can be activated by IL-1β. Curiously, blocking PGE_2 creates a feedback loop potentiating the catabolic effect of IL-1β (193, 262). Quantification of PGE_2 concentrations is currently considered routine method to objectively assess synovitis in

experimental conditions (6, 272, 274); however, the isolated effect of PGE_2 in joint disease may need revision. To date, negative effects reported for PGE_2 in joints include vasodilation, increased perception of pain, proteoglycan degradation and inhibition of its synthesis, and intra-articular calcification (6, 276). Nonetheless, PGE_2 generated during the early inflammatory response can also induce resolution of inflammation by upregulating the transcription of enzymes required for the synthesis of other classes of bioactive lipids that are potent mediators of tissue repair and inflammation resolution (47, 49, 280). Quantifying other oxylipids, such as the specialized pro-resolving molecules (resolvins, maresins and protectins), may provide more information regarding stage of inflammation, and even provide insights on the likelihood of its resolution (49, 281).

SDF-1 has multifaceted roles in the biology of synovial tissues, including homeostatic and pro-inflammatory functions. It is not only associated with chemotaxis of immune cells, but also through direct SDF-1/CXCR4 signaling on different cells such as chondrocytes, synovial fibroblasts, and macrophages. SDF-1 is expressed proportionately to inflammation or disease activity, with higher concentrations reported in inflamed joints (282-284). Increased MCP-1 concentrations in synovial fluid and expression in the synovium from OA joints are associated with monocyte/macrophage recruitment to the damaged/inflamed synovium, the presence of gross signs of inflammation, and expression of other inflammatory cytokines (i.e., IL-1β and IL-6), as well as cartilage degradation (282, 285-287).

IL-10 exerts anti-inflammatory and chondroprotective effects, favoring chondrocyte recovery from damage (136). However, its concentrations in synovial fluid and tissues increase proportionate to injury during inflammation, especially in highly inflamed joints with RA (288, 289). During chronic low-grade inflammation, as in OA, IL-10 concentrations in synovial fluid

are comparable to healthy joints (289, 290). Interestingly, a study challenging monocytes from OA and healthy human patients *in vitro,* reported that patients whose cells showed no significant IL-10 increase following challenge with LPS were 3 times more likely to develop OA compared to those responding with a significant increase, reinforcing the role of IL-10 in driving resolution of inflammation and promoting joint homeostasis (261).

Cytokines classically known as pro-inflammatory can induce tissue anabolism and catabolism, and drive or amplify disease processes. These effects can vary according to magnitude of cytokine expression. Also, cytokines signal through a variety of receptors leading to opposing downstream effects, activating different cell pathways such as NF-kB, WNT, and Notch, as well as complement cascades (7, 251, 291-293). Paradoxically, cytokines classically know as anti-inflammatory and chondroprotective such as IL-10 (135, 136), are often detected in osteoarthritic tissues, as a negative feedback to counteract the tissue inflammation and destruction (261, 265, 294). It is important to consider that while many of these molecules identified in the diseased joint, they are also present at very similar concentrations in healthy joints. Insults to joint tissues induce changes in the magnitude of cytokines expression, in order to promote remodeling and adjustment to the biomechanical challenges. Overwhelming changes in cytokine expression lead to altered metabolism of joint tissues. While the effect of many of these cytokines have been evaluated *in vitro*, and their effect was tested using doses that are by far supraphysiologic, the integrated role of them *in vivo* is poorly characterized, and thus do not reflect the in vivo conditions of the joint during OA development and progression (92). Therefore, further studies using in vivo models are required to better define the role of cytokines involved in joint disease, repair and homeostasis.

Current therapies for OA

In face of no available cure for OA, therapeutic goals are to quickly recover joint function, prevent reactivation of synovitis, and minimize structural damage to the cartilage, thus decreasing pain and joint deterioration. Management of the osteoarthritic patient, human or equine, should be elaborated based on the clinical findings of each patient, in order to specifically meet their individual needs (54, 105, 295-297). Since the joint should be seen as an organ, treatments should consider all of its components, however, rapid resolution of synovitis is critical to prevent byproducts of inflammation to induce degradation of cartilage matrix (298, 299).

Comprehensive management of the osteoarthritic patient should always include a combination of treatment options considering the patients' weight, fitness, behavior and joint stability. Treatment plans defined solely on the radiographic basis build a poor and outdated approach (296). General recommendations defined by international consensuses, are based on the sequential hierarchy of nonpharmacological modalities, followed by judicious selection of drug options, and finally surgery when the previous treatments resulted unsuccessful (54, 300, 301). Exceptions should be made for patients with congenital or developmental conditions such as osteochondritis dissecans (OCD), especially in the horse, where early surgical treatment should most often be the right choice (302, 303). Differently from people, partial or complete joint replacements are not a realistic option for horses with late stage OA. However, arthrodesis of some joints can often provide clinical relief (304).

Nonpharmacological treatments

In contrast to horses, the majority of people with OA are either overweight or obese. Diet management and exercise leading to approximately 10% weight loss yield around 50% improvement in OA symptoms (305). Weight management is always advocated, but not frequently implemented. Another pivotal, yet often ignored aspect of the conservative management of OA is exercise (295, 297, 299). Fitness increasing muscle tone decreases loading impact to affected joints and increase the intra- and peri-articular production of anti-inflammatory cytokines (290, 306). However, since OA places an enormous physical burden, under-water exercise minimizes the impact created by load-bearing by 50%, and can be an initial approach for people and horses to develop muscle tone and further engage in traditional exercises (295, 301, 306, 307).

Pharmacological treatments

Current drug-based options to treat OA mostly reduce symptoms, but are limited in providing long-term improvement (299). Medications that provide pain relief, with no effect at cartilage matrix are termed symptom-modifying OA drugs (SMOADs) (298). Such drugs include non-steroidal anti-inflammatory drugs (NSAID), analgesics with no effect on inflammation, opioids, capsaicin, corticosteroids, and a couple more less representative alternatives including antiphlogistics. NSAIDs and corticosteroids have cyclooxygenase (COX) inhibitor activity with local and systemic adverse side effects, raising a number of long-term safety concerns (298, 299).

Drugs capable of beneficially altering the articular cartilage or the synovial environment, in a so-called "chondroprotective" fashion are called disease-modifying OA drugs (DMOAD)

(298). Those can include inhibitors of MMP, ADAMTS-4 and 5, cathepsin K and iNOS. Also, in this class comes bisphosphonates, calcitonin, strontium, doxycycline, estrogens, sprifermin (rhFGF18), parathyroid hormone, as well as antibodies targeting IL1, TNF-α and IL-6. A new peptide called TPX-100 that stimulate bone and cartilage production in a tissue specific manner, is under investigation (308).

Symptom Modifying OA Drugs

1. Analgesics

The ability of acetaminophen (Paracetamol®) to produce pain relief from OA is limited. In face of not unfrequently reported gastro-intestinal (GI) toxicity, cardiovascular complications, or even mortality, the use of acetaminophen for treating human OA has been reconsidered (309, 310). In horses, reported use of acetaminophen is very limited and off-label (311-313), since the pharmacokinetics and safety of acetaminophen in horses was just recently established (314). When combined with tramadol, it has been effective in the control of acute foot pain in horses (313). While varying results have been reported for its use in equine musculoskeletal pain (311, 312), ongoing research in that field should soon provide updates.

2. Nonsteroidal anti-inflammatory drugs (NSAID)

NSAID are considered a cornerstone in the treatment OA and can provide significant pain relief and control of inflammation in horses and people. Recently, the widespread and uncontrolled use of oral (or injectable) NSAID was revisited due to the presentation of significant GI, renal and cardiovascular complications (298, 315, 316). However, NSAID are non-homogeneous, and there are noticeable differences between them regarding complications

and risks for GI and cardiovascular events. Therefore, NSAID can provide an effective and safe treatment for OA only if properly prescribed and monitored.

NSAID can produce beneficial as well as adverse effects through the same mechanism of action—inhibiting the cyclooxygenase (COX)-dependent conversion of arachidonic acid into prostaglandins and thromboxanes. All NSAID inhibit COX activity to some degree. COX-1 activity is important for normal GI and renal physiology but plays a lesser role in inflammation. COX-2 relates to inflammatory events, in particular those driven by synovial macrophages and fibroblasts during joint inflammation, holding a minor role in normal physiology (298). Although COX-2 selective drugs are available, total COX-2 inhibition is also undesirable for either the joint or the individual (317, 318). Inhibiting COX-2 with nonsteroidal antiinflammatory drugs (NSAID) can relieve pain, but has no effect in cartilage recovery. In fact, complete blockage of COX-2 has detrimental effects for the cartilage metabolism (317).

Among the most-used COX-2 selective drugs in people are: aspirin, diclofenac, ibuprofen, indomethacin, ketoprofen, fluorbiprofen, naproxen, nimesulide, piroxicam, meloxicam, celecoxib, etoricoxib, celecoxib and lumiracoxib (299). Lately, ibuprofen, naproxen, piroxicam and celecoxib have been the most used (319). When it comes to the associated risk of GI complications, drugs such as ketorolac, piroxicam, naproxen, ketoprofen, indomethacin and meloxicam present significantly higher odds of complications, decreasing with the use of ibuprofen, rofecoxib, aceclofenac, and celecoxib respectively (315). Also, a recent review showed that celecoxib may present a lower risk of cardiovascular events, posing it as a safer drug option (316).

Topical NSAID are within international guidelines recommended for the treatment of early OA in people, and suggested to be used previously to moving into oral NSAID due to

superior safety profile, to allowing 40% reduction in the need for systemic NSAID and thus related side effects, as well as due to rescuing pain control with simple analgesics (299, 317). Topical NSAID are heterogeneous regarding formulation. Etofenamate and diclofenac have evidence for accumulation in synovial tissues, improving joint pain and function (320). Anti-inflammatory effects were demonstrated using a topical diclofenac liposomal cream in the treatment of subcutaneous and joint inflammation in horses, showing preservation of the GAG content in cartilage and preserving physiological levels of PGE_2 in the synovial fluid (318, 321, 322).

Although it is common knowledge that the selective inhibition of COX-2 is an ideal target in anti-inflammatory therapy, phenylbutazone, a non-COX-selective drug is the most commonly used NSAID in horses (298). Banned from human use in 1985 due to causing a range of side effects including death, it remains in the equine industry where such complications happen much less frequently (319), and is considered to provide the highest analgesic effect among NSAID commonly used in horses (323). Variable results have been seen in horses with both experimentally induced and naturally occurring OA (298). Although combined administration of phenylbutazone and flunixin meglumine provides higher analgesia compared with phenylbutazone alone (323), secondary side effects (including acute necrotizing colitis) are a serious concern (324).

Flunixin meglumine is also commonly used in horses, and comparisons to phenylbutazone suggest no difference in the efficacy to control pain (325). Recent studies comparing both drugs to ketoprofen, observed equivalent analgesic effects among drugs, however, phenylbutazone followed by flunixin, were more often associated to renal damage, while GI complications were only observed in the phenylbutazone or flunixin treated horses

(326, 327). Both studies concluded that ketoprofen stands as an effective analgesic with lesser likelihood of complications.

Inhibiting prostaglandins has shown to relief symptoms of OA, however, long-term the use of some NSAID have deleterious effects of on resolution of inflammation (47, 49, 280) and cartilage metabolism (317). Actually, phenylbutazone has been shown to experimentally induce decreased proteoglycan synthesis to a similar degree of recombinant human interleukin– 1β (328). However, since associations between phenylbutazone use and articular cartilage degeneration have not been reported in the clinical scenario, its cautious use is deemed justified (298).

Firocoxib, a COX-2 inhibitor is available for prolonged use to treat OA-associated pain and inflammation in horses (329), but its efficacy in musculoskeletal pain control is often underappreciated. The safety and efficacy for the chronic use of meloxicam in horses has been defined and provides satisfactory outcomes in musculoskeletal pain relief in horses (277), and its use is common practice in Europe and South America (274, 277, 330).

Intra-articular medications

1. Corticosteroids

Intra-articular corticosteroids are widely used in the treatment of OA in people and horses. Their use is shown to reduce cartilage degeneration associated with inflammation but it also induces deleterious effects on cartilage and periarticular bone, depending on the drug and dosage of choice (331). Corticosteroids' effects are dose dependent and binding to its receptor induces nuclear transportation, binding to specific DNA sequences (glucocorticoid responsive elements - GRE), leading to expression of corticosteroid regulated genes (positive GRE), or by

repressing expression of some genes through negative GRE (nGRE). They can induce direct transcription (cis) or protein coupling that modulates indirect (trans) activation of other transcription factors including AP-1, STAT-5 and NF-κB (332).

Among corticosteroids most used for intra-articular injection in people are: hydrocortisone (acetate, 10–25 mg), triamcinolone (acetonide (TA 10–40 mg, hexacetonide 10–20 mg), methylprednisolone (acetate (MPA) 20–80 mg), and betamethasone sodium (phosphate & acetate, 40 mg) (299). Global data analysis has suggested that clinical improvement related to joint injections with corticosteroid is somehow short-termed, lasting in average 1–4 weeks (54, 333, 334). Not surprisingly, more recent data have shown that corticosteroid joint injection at earlier stages of joint degeneration are more likely to produce long lasting results, with ≈25% of patients at different stages of disease presenting pain relief for over 6 months (335). Another recent study suggests that repeated triamcinolone administrations at three months intervals do not provide long term clinical benefit, and is associated to increased MRI cartilage loss at two years. Nonetheless, the triamcinolone dosage used in that study, was at least 4 times higher than what is validated and safely used in horses (298, 331). Findings from a recent review support the use of corticosteroids for short-term management knee and hip OA (300, 301). However, the overall data suggests that the short-term benefit, potential adverse effects, and limited evidence of mid- to long-term benefit, make the use of corticosteroid in human OA questionable (299). Therefore, it is suggested that corticosteroids should be considered as an adjunct treatment to relief moderate to severe pain (299, 333, 336, 337). Unfortunately, most review studies do not clearly define and compare the result of different types of corticosteroids and doses used.

The use of intra-articular corticosteroids to treat equine OA has been widely reviewed, and benefits and negative side effects have been assessed and clarified (338, 339). Extensive

clinical and experimental experience on equine OA provides information from over 25 years of evidence-based use of intra-articular corticosteroids in equine OA (15). The experimental use of MPA (340), TA (341), and betamethasone (342) were evaluated in horses using an osteochondral fragment–exercise model of OA, where horses were followed for 6-8 weeks. The effect of corticosteroids in horses were associated to presentation and quantification of lameness, as well as assessment of clinical, radiographic and synovial fluid parameters. Following euthanasia, gross pathology and histological assessment of the joints and synovial tissues were performed. Results from each of these studies were analyzed by comparisons of joints treated with corticosteroids with no osteochondral fragment, as well as with joints with osteochondral fragment but treated with polyionic fluid (339).

Since the first study in 1994, numerous similar studies have been carried and recently collectively analyzed, with overall coincident findings (338, 343). There is compelling evidence that MPA have detrimental effects on cartilage healing and turnover, with limited clinical benefit. TA reduces lameness and has positive effects on synovial health but can cause GAG loss that may not be counteracted by the addition of HA or nutraceuticals. The risk of drug associated infection is minimal (1 in 1338 cases). Although complications such as laminitis post corticosteroid joint injection are reported in horses, risk factors have been poorly and only anecdotally documented. Deciding if, when, what to use and where to medicate an OA joint, requires scientific knowledge, but perhaps more importantly, appreciate the unknowns relating to the intraarticular corticosteroid medication (338, 343, 344).

2. Polyacrilamide hydrogel (PAAG)

PAAG is a non-degradable non-toxic, biocompatible synthetic product, composed of polyacrylamide and silver ions. PAAG has water exchanging abilities and is used in the augmentation of soft tissues. Preliminary results treating 43 horses with low grade OA through intra-articular injection of PAAG, suggested long-lasting beneficial effects (up to 24 months), with over 80% of horses improving lameness, synovial effusion and returning to previous activities (345). A prospective, controlled, longitudinal study investigated if intra-articular injection of PAAG would integrate into the synovium of normal rabbit joints and equine osteoarthritic joints, and the durability in time of this integration. Normal rabbit joints injected with PAAG were followed for 1 year. Also, post mortem examination of 18 equine OA joints previously treated with PAAG for as far as to 2 years were performed. Findings revealed that PAAG integrated to the synovium at 10 - 14 days and synovial cells invaded the gel and proliferated forming a new synovial layer crossed by very thin collagen strands, which included newly formed vessels and was delimitated by a synovial lining facing the joint cavity. These findings were persistent for up to 2 years post-injection (346). It is speculated that this tissue layer creates an immune barrier to the cross-communication between the reactive synovium and the hyper responsive cartilage during the vicious circle of inflammation in OA. Despite no published human clinical trial, PAAG is currently advertised in Europe as an intra-articular device for the treatment of OA (NoltrexTM)[1]. Complications in one human patient following injection was reported (347).

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[1] http://www.noltrex.info/preparation/preparation

Disease Modifying OA Drug

1. Hyaluronan

The use of intra-articular injections of hyaluronan (HA), has also been extensively researched (54, 300, 348-351). Although a recent meta-analysis found that treatment with HA is associated with limited improvement in pain and an increased risk of complications (300, 349), a variety of studies have objectively identified opposing outcomes. A comparison between patients receiving intra-articular HA or MPA, and followed for 26 weeks, found that from 0 to 4 weeks, intraarticular corticosteroids were more effective than HA in controlling pain. At 4 weeks, both HA and MPA had similar results, however, after 8 weeks HA provided superior results (334). Another multicenter, randomized, double-blinded controlled study evaluated the effect of four cycles of five intra-articular injections of HA or placebo, where patients were followed for 6 months after the 1st and 2nd cycles, and after 1 year following the 3rd and 4th cycles. At the 40th month more patients treated with HA significantly improved over time as compared with placebo, suggesting that HA not only improve symptoms of OA but also exert an important carry-over effect for at least 1 year. No safety problems were recorded (352). Based on those findings, HA has now been proposed to be a DMOAD. It has also been advocated that HA should be the first intra-articular therapy to be used in patients not responding to NSAID, and that its use over steroids may delay need to joint replacement for an average of 2 years (350). Moreover, considering HA's DMOAD effects, it should likely be considered as a first option therapy.

Similar to people, the clinical use of HA in equine OA has been extensively evaluated and discussed (295). In horses, HA produces limited short-term analgesia, but its anti-inflammatory effects have received more attention, and were associated to decreased free

radicals and prostaglandin synthesis inhibition following IL-1 stimulation (353, 354). Claims that HA preparations of high molecular weight provide superior chondroprotection and clinical results than low molecular weight HA are controversial (298). In a randomized, double-blind, and placebo-controlled clinical study, horse (n=77) with moderate to severe lameness associated to OA were treated with HA, polysulfated GAGs (PSGAG), or placebo for 3 weeks. Both drugs showed to be superior to placebo for reduction of lameness scores (355). More recently, the use of intra-articular poly-sulfated GAG as well as intra-articular HA was evaluated in the osteochondral fragment-exercise OA model, resulting in significantly lower cartilage fibrillation in HA treated joints, despite of less marked decreases in synovial effusion or histological changes in the synovium, as compared to PSGAG treatment (356). Such findings support that, similarly to people, HA may be a DMOAD in horses as well.

The combined use of HA with intraarticular corticosteroids is a common practice, due to a concept that HA might mitigate deleterious effects of corticosteroids. Although performed on empirical basis for many years, there is ancillary evidence that using HA in combination with TA can be beneficial, at least in equine athletes (339). Current data from a multi-center study showed that their combined use induced fewer acute complications, and despite of inferior short-term results when compared to TA injection alone, TA+HA injection produced equivalent results to TA alone at 3 months post treatment (357). In addition, a 1-year, randomized, single-blind study evaluated 24 people treated with HA joint injections once weekly for 3 weeks and then at 6 months (6 injections total). Sixteen patients were treated the same but with the addition of TA prior to the first and fourth HA injection. Patients were evaluated with the Western Ontario and McMaster University Osteoarthritis Index (WOMAC) and the visual analog pain scale (VAS). Following 12 months, progression of OA was assessed using MRI. Although all patients showed

improvement in pain and function, those receiving HA+TA had superior pain relief than HA alone and there was no progression of OA in either group for early OA cases (358). Similar findings of a similar study were recently reported by Campos et al, 2017 (351). Combined, the chondroprotective effects of HA and the anti-inflammatory effects of TA build a logical therapeutic approach.

2. Polysulfated glycosaminoglycan (PSGAG)

The main GAG in PSGAG is chondroitin sulfate (CS). The therapeutic use of PSGAGs is aimed at preventing or retarding irreversible cartilage degeneration (298). As such, an early *in vitro* study demonstrated that among several drugs tested (phenylbutazone, flunixin meglumine, betamethasone, and HA) PSGAG was the only drug that could inhibit MMP activity (359). PSGAG were also shown to increase synthesis of collagen and GAG in both chondrocyte and cartilage explant cultures from normal and osteoarthritic equine samples (360). Several studies have provided data that support the use of intra-articular PSGAG in horses. These findings coming from clinical studies using aggressive OA models such as Freund's adjuvant-induced OA and a monoiodoacetate carpal synovitis model (361-363), certainly strengthen the power of this evidence, showing significant reduction of chondrocyte death, articular cartilage erosion, fibrillation, and markedly improved GAG staining (361).

3. Pentosan polysulfate (PPS)

PPS is a heparinoid compound derived from beechwood hemicellulose instead of animal sources. In experimental ovine studies, joint injections of PPS every week for 4 weeks improved joint function and lead to lower radiological and Mankin histological scores compared to control (364). Further, the same group of researchers, as well as recent Japanese study evaluated the

effects of intra-articular PPS injection for the treatment of knee OA in people, showing significantly improved clinical signs and ability to perform daily activities (365, 366). However, the latest also detected altered coagulation and triglyceride profiles in PPS treated patients, yet within safe ranges. Recently, a study investigating the effects of PPS in the osteochondral fragment–exercise model (Pentosan Equine Injection), found that the Pentosan treatment significantly decreased cartilage fibrillation, and showed a marked trend for overall improvement of cartilage histological aspects (367).

4. Stanozolol

A recent study verified the clinical and pathological effects of weekly intra-articular treatment with stanozolol using a sheep OA model. The gross anatomy and histological features of articular cartilage was well-preserved in stanozolol-treated joints, while inflammation, cartilage degeneration, osteophyte formation and subchondral bone reaction were observed in placebo treated joints (368). The same authors further verified the clinical effects of intra-articular stanozolol in 60 cases of acute and chronic equine OA, in a double-blinded clinical trial. Improvements were observed in over 80% of cases and lameness was abolished in 15 of 21 acute cases, and 11 of 19 chronic cases, with no complications observed long term (369). Moreover, the effects of stanozolol on gene expression and chondrocyte viability in normal equine chondrocytes evaluated under an inflammatory *in vitro* system determined that cell viability was not affected by stanozolol, which induced a reduction in gene expression of MMP-13, MMP-1, IL-6, COX-2 and COL2A1in both normal and IL-1β treated chondrocytes (370).

5. Oral Joint Supplements

Oral joint supplements or nutraceuticals are dietary supplements aimed at treating or preventing joint disease. Despite logical questioning on their role in preventing OA given the lack of quality of supporting evidence (371-375), there is growing evidence of some benefit (274). The majority of supplements available in the market comprise varying amounts of CS and/or glucosamine in combination with other ingredients. There are currently many commercial products including variable concentrations of glucosamine, CS, methylsulfonylmethane (MSM), fish oils, vitamin C, manganese, and other constituents. However, the hidden formulas make objective comparisons impossible.

Combination of chondroitin sulfate and glucosamine have been suggested to reduce equine cartilage degradation *in vitro* with potential anti-inflammatory effects. Cartilage incubated with lipopolysaccharide (LPS) and glucosamine, CS, or both revealed that glucosamine decreased NO and PGE_2 production, but CS alone had no effect (376). Also, glucosamine combined with CS has been shown to decrease MMP-9 activity, with no effect on MMP-2, and a trend for decreasing concentrations of MMP-13 (377, 378). Glucosamine and CS combined and alone also showed a protective effect on IL-1–conditioned articular cartilage explants (379), suggesting that a combination of CS and glucosamine might decrease inflammation (380) and may be beneficial in preventing GAG degradation (381). Although the bioavailability following oral administration of CS and glucosamine in horses is reported to be low (382, 383), an *in vivo* study showed that supplementation with glucosamine lead to significant increase in synovial fluid glucosamine following inflammatory challenge (384), and clinical improvement in osteoarthritic horses after treatment with glucosamine, CS and undenatured collagen type II (UC-II) compared to placebo (385).

Supplementation with UC-II has been demonstrated to extend the pain-free period after strenuous exercise and to alleviate joint pain arising from such activities (386). It was also shown to decrease cartilage degradation in a mouse model of OA (387) and to provide significant clinical improvement in OA patients (388). Other formulations of oral collagen hydrolysate have been shown to stimulate chondrocytes to synthetize type II collagen *in vitro* (389), to have a high affinity to cartilage *in vivo* (390), and to have beneficial effects on joint pain associated with OA in people (391-393). Methylsulphonylmethane (MSM), a natural anti-inflammatory agent (394, 395) was shown to decrease joint pain and swelling in OA patients (396, 397) and to significantly improve exercise-related inflammatory changes and oxidative stress in equine athletes (398).

A recent comparison of the preventive effects of collagen hydrolysate, meloxicam and a new compound (glucosamine sulphate 2KCL, shark chondroitin sulphate sodium, MSM, boswellic acid dry extract 65%, Ananasus comosus extract 2500 GDU, L-glutamine, feverfew dry extract PE 4:1, hyaluronic acid) showed that after experimental induction of synovitis, the placebo group had significantly higher synovial fluid total protein (TP), total nucleated cell count (TNCC) and PGE_2 than the meloxicam group. Both supplements induced significantly lower synovial fluid TP, TNCC and PGE_2 production in comparison to placebo (274).

Despite the benefits of injectable HA in treating OA, oral supplementation has not shown benefits in the osteochondral fragment–exercise model. On the other hand, supplementation with avocado and soybean unsaponifiable (ASU) extract showed significant decreases in synovitis, cartilage disease scores and a trend for decrease in lameness (298). Omega-3 polyunsaturated fatty acids (PUFAs) reduce inflammatory mediators in equine synoviocytes, with anti-inflammatory potential for the reduction of equine synovial inflammation (281, 399)

Biologicals

1. MMP inhibitors

MMP inhibitors include peptide-based (e.g hydroxamic acids), non–peptide-based (e.g.
modified tetracyclines for example doxycycline), and natural inhibitors (including omega-3 fatty
acids, i.e., fish oil). *In vitro* studies assessed the effects of MMP inhibitor named BAY 12-9566
on canine and equine cartilage explants and identified a marked dose-dependent reduction in the
proteolytic effects of IL-1α on type II collagen and proteoglycans (400), but failed to provide
benefits in people (401) and a canine OA model (298). Investigations on the efficacy and safety
of orally administered PG-116800, a pan-MMP-inhibitor in human patients with knee OA,
revealed no benefits, and the highest dose tested was discontinued due to musculoskeletal
adverse reactions (402). Another set of inhibitors that are more selective MMP-13 (including
CP-544439, AZD-8955, and WAY-170523) has been investigated, however, clinical data from
these clinical trials are not yet unavailable (308).

2. Cytokine inhibitors

Although IL-1 has been claimed as the main driver of OA, TNF has received more
attention in RA leading to the development of anti-TNF neutralizing antibodies, as well as
soluble TNF receptor, with interesting therapeutic results (403, 404). Inhibition of production or
activity of IL-1, has been investigated for various treatments including: application of IL-1
receptor antagonist proteins, soluble IL-1 receptors, blocking the formation of active IL-1β,
blocking the IL-1 cellular signaling pathways, or gene therapy, have been investigated in
preclinical and clinical human studies. However, the results were not satisfactory (405), and
differently from TNF, soluble receptors to inhibit IL-1 are not currently available for therapeutic

use (299). On the other hand, therapeutic IL-1ra application in horses was initially done by direct transfection of the IL-1ra gene to equine to synoviocytes, and was reported to achieve complete inhibition of carpal OA (406). Even though both disease- and symptom-modifying effects were identified to a striking magnitude, repetition of this technique requires a better gene transfer vector than the adenovirus.

The limitations associated to gene therapy forced the investigation of alternative approaches for delivering IL-1ra to joints (298). A multi-compound product centered in IL-1ra, IRAP®, was developed for horses in Europe in which peripheral blood is harvested into a syringe containing glass beads coated in chromium sulfate, then incubated for 24 hours prior to centrifugation. It is currently known that the beads and syringe induce monocytes to up-regulate production of several signaling proteins, more than just IL-1ra. Substantial elevation of IL-1ra with minor changes in IL-1 or TNF expression was documented (407). In a double-blinded, placebo-controlled study using the osteochondral fragment– exercise model, IRAP® showed both symptom and disease-modifying effects in horses (408). Further, blood derived compounds processed in a similar fashion and called ACS (autologous conditioned serum) gained popularity, with most reports concerning its clinical efficacy being anecdotal with little or contradictory scientific evidence in dogs and horses and people (409-414). Controversially, increased plasmatic concentrations of IL1ra are also correlated in a causal fashion to the progression and severity of symptomatic knee OA, independently of other risk factors (415), questioning if IL-1ra is the (main) active component in ACS providing clinical benefits.

3. Mesenchymal stem cells (MSC)

MSC have been applied in the attempted management of several joint conditions with and initial goal of restoring damaged/lost tissues, due to their ability to differentiate into cells of different lineages. In that perspective, injected MSC would ideally host within the damaged tissue, and ultimately differentiate into chondrocytes, restoring cartilage damage. It is currently known that MSC engrafting is very low, and more likely to happen to synovial soft tissues than cartilage (416-419). Up to date, beneficial effects of MSC therapy are known to result from a paracrine response, modulating the activity of other cells within the joint, especially macrophages (155, 279, 420). Following stimulation by cytokines, chemokines and growth factors classically involved in inflammation, MSC function as anti-inflammatory cells increasing the production of anti-inflammatory and immunomodulatory factors such as TSG-6, IL-6, IL-10 and PGE_2, proportionally to inflammation (138, 155, 420-423). However, it has been shown that overt inflammation overwhelms this regulatory response and decreases chondrogenic capacity of synovial progenitor cells (44, 131, 424). This stimuli-dependent response account for at least part of the variation between studies evaluating MSC for joint therapy, using models with different severity of joint inflammation.

Equine studies demonstrate that MSC exposed to healthy joints can induce mild, transient inflammation, reflected by synovitis and increased synovial fluid TP, TNCC and inflammatory cytokines (425-427). However, these findings can be an artifact caused by the use of allogeneic cells, or culture conditions where even serum starved cells display some degree of xenogeneic protein/antigen (fetal bovine serum), that is recognized by the host's immune system eliciting an immune reaction.

There is general agreement from clinical and research data pointing bone marrow as the optimal source of MSC for therapeutic purposes, regarding to both their plasticity and immunomodulatory effects (428-431). There are also important differences between culture-expanded MSC and those cell compounds available through non–culture-expanded sources, in which MSC are a minute part (0.001–0.01%) and mononuclear cells are the main component, represented mostly by myeloid and hematopoietic progenitors if original from bone marrow (194, 432-437). Intra-articular injection of scaffold-free and scaffold-in MSC has been investigated in cartilage repair studies (438, 439), both in naturally occurring and experimentally induced equine OA (418, 428), and *in vitro* studies suggest an anti-inflammatory effect associated to MSC-derived PGE_2, with poor cartilage saving effects (279, 423, 427, 428). On the other hand, articular soft tissues such as the anterior cruciate ligament and the meniscus, were shown to benefit from MSC therapy with increased repair and regeneration (417, 431, 440).

There have been several reports advocating in support of the clinical use of allogeneic MSC (423, 426, 427, 441). However, the use of autologous versus allogeneic cells do differ, in which allogeneic cells can induce immunogenicity and adverse reactions (425, 442-445). While the magnitude of the adverse reactions reported to present may not be relevant, the expected beneficial results may as well not be achieved due to a graft-host reaction and such treatment option may not be justifiable.

The use of autologous MSC aimed at improving osteochondral defect healing have also been shown to provide better results if used in scaffolds or constructs then injection of cells in suspension, and both have favored modest improvement in healing, especially when a scaffold is used in combination (419, 438, 439, 446-448). While a variety of studies support the notion that MSC therapy may have a positive effect on OA patients, there is limited high quality evidence

and long-term follow-up, especially in face of the extent to it has been clinically used in Veterinary Medicine (416). Such limitations include lack of objective outcome measures, and variation in cell source, joints treated and lack of control groups. Two recent reviews on the use of intra-articular MSC for joint therapy in people, also called the attention for the limited number of high-level evidence studies, with high risk of bias, and identified a lack of consistency regarding MSC preparations, and thus a lack of reproducibility of reported outcomes, preventing objective recommendation of MSC therapy for knee OA for human patients (449, 450).

4.   Platelet rich plasma (PRP)

PRP is a plasma compound derived from peripheral blood centrifugation, red cells removal (at least most of it) and concentration of platelets, with or without leukocytes. A growing amount of studies, systematic reviews and meta-analyses evaluating PRP as treatment for knee OA have been published with favorable results, where intra-articular PRP injections produced superior clinical results over either placebo or HA. Reported benefits were observed starting at 2 months post injection all the way to 12-month follow-up, with patients with early OA achieving superior outcomes in comparison to those affected by advanced OA. All studies found significantly improved WOMAC Index and International Knee Documentation Committee scores (451-457). The effect of leukocyte concentration on PRP's clinical outcomes have been investigated with diverging findings (451, 458). PRP also became a common therapy for equine OA and synovitis. Despite of a multitude of studies in horses, there is limited information regarding the mechanisms by which PRP provide improvements on joint pain and function (68, 439, 459, 460).

Platelets are a rich in growth factors, chemokines and cytokines, which altogether are released during the initial stages of inflammation associated to reparative processes of tissue healing. PRP enhances physiological processes of clotting and wound repair, and is believed to recapitulate events intrinsic to tissue repair in which platelets play a key role. PRP also stimulates angiogenesis, migration and proliferation of fibroblasts at the site of injury, synthesis of collagen and macrophage chemotaxis, which are absolutely required for tissue repair. Several growth factors are involved in tissue repair including, IGF-I and IGF-II, TGF-β1, FGF, vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and platelet derived epidermal growth factor (PDEGF), and are released from the degranulation of alpha granules in the platelet cytoplasm following activation (461). Increases in cytokines such as IL-1, IL-1ra, IL-4, IL-6 and TNF-α, as well as PDGF and TGF-β1 have been documented in equine joints treated with PRP (68, 460, 462).

There is some clinical evidence of the beneficial effects of PRP in horses with naturally occurring OA (463-465). PRP is commonly used therapeutically either as a sole agent, or combined with other regenerative therapies where it seems to provide a scaffold and a concentrate of growth factors supporting cellular repair of musculoskeletal injuries. Listed advantages to the use of PRP include its minimally-invasive collection and processing, rapid preparation and overall its autologous nature (460, 463, 466).

Bone marrow mononuclear cell therapy

The use of bone marrow components in the treatment of musculoskeletal conditions dates from over a century ago, when Goujon et al, published one of the first papers highlighting the

properties of bone marrow in inducing heterotopic bone formation (467). Dozens of studies were published in the following 150 years showing similar findings as an effect of whole bone marrow grafting in many different tissues. The osteogenic capacity was attributed to all of the bone marrow components, or a variety of different cells including endosteal osteoblasts, but special attention given to mesenchymal and reticular cells within the bone marrow (468, 469). The remarkable early findings of those studies evolved with some modifications, to establish what was the center piece of today's gold standard for the treatment of non-union fractures: bone marrow grafting (469-471).

Non-union fractures were not the only medical application of bone marrow transposition that resulted in successful outcomes. Injection of bone marrow aspirates into injured suspensory ligament in horses became a common practice, due to improved healing and return to exercise (472). Microfracture and subchondral bone drilling, which are today's most common an validated techniques to improve osteochondral healing, account in providing means for bone marrow cells to reach the osteochondral defect (190, 436, 473-476). In that regard, Shapiro et al, studying the cells responsible for filling the osteochondral defect and guiding the healing process following microfracture, defined them as progenitor cells from mesenchymal origin (190). At that same time, the concept of mesenchymal stem cells (MSC), with multi or pluripotency to differentiate in distinct different tissues, nourished an avalanche of research characterizing the fibroblastoid, plastic adherent cell selected from bone marrow multi-passage culture (477). Most following studies focused on characterizing their plasticity, as defined by tri-lineage differentiation, and many pre-clinical trials aimed at improving cartilage and tendon repair (438, 442, 478-481). These studies led to the observation that the most beneficial effect of such cells was a paracrine modulation of tissue inflammation and repair (155), and therefore some recent

studies have been exploring that subject (421, 423). In addition, it became well known that MSC poorly engraft to osteochondral defects, and there is no evidence of their *in vivo* differentiation into chondrocytes and very little improvement of osteochondral healing (416, 419, 446, 482). Therefore, the search to define the cell(s) type(s) within bone marrow that is (are) responsible for guiding improved tissue repair and decreasing inflammation continued.

Since the red cell portion from the bone marrow was unlikely to have a relevant role in guiding tissue repair, and actually play an important role in inciting destructive inflammation within joints (483), further studies included an effort to remove red cells from the research scope, focusing on myeloid granulocytes and mononuclear cells. Also, the use of whole bone marrow aspirate in tendons or ligaments, despite of providing marked improvement in healing and return to exercise, was sporadically associated to intra-tendinous bone formation, requiring also an effort to remove bone spicules from the aspirate and preventing heterotopic bone formation (481). One simple way of achieving both goals was to centrifuge the bone marrow aspirate, where heavy bone spicules and iron loaded erythrocytes become packed in the bottom of the cell pellet, and the thin buffy layer on top of the red pellet, containing white and progenitor cells is recovered for research and clinical use. Such product was called bone marrow aspirate concentrate (BMAC), which is rich in myeloid leukocytes and other hematopoietic and mesenchymal progenitors. Its application in osteochondral repair has been studied with very successful outcomes, even when the subchondral bone is not drilled or picked, reinforcing that the cells responsible for osteochondral repair were within the BMAC (194, 484).

Current knowledge in leukocyte biology has shown that granulocytes and monocytes not only drive and participate in inflammation but also have a key role in driving tissue repair and homeostasis (inflammation resolution) (47, 141, 158, 485). Immediately after tissue injury,

neutrophils are the main granulocyte involved in sterile inflammation, and their phagocytic clearance is relevant for tissue repair. However, their persistent identification in tissues is associated to chronic inflammation and delayed repair, leading clinicians and scientists to avoid their inclusion in biological joint therapies (485). Therefore, after removing bone spicules, erythrocytes and granulocytes from the investigative scope, the mononuclear fraction from bone marrow cells is what is left, which has been shown to retain the regulatory and tissue repair properties from the bone marrow (486).

Bone marrow mononuclear cells (BMNC) have been substantially investigated over the last 20 years, in the fields of tissue repair and treatment of chronic inflammation. They were shown to reduce the inflammatory phase of healing and to improve tissue quality in both tendons and ligaments (479-481, 487, 488), as well as in osteochondral defects (452, 489-491). BMNC therapy in the management of chronic airway inflammation was well studied in murine models and equine clinical cases, with clinical improvements comparable to corticosteroid treatment (134, 432). BMNC has also provided improvement in the functional recovery from acute liver failure and related survival in mouse models (492-494), and most impressively, have made possible the successful transplantation of pancreatic islet in both murine and primate models (495). Finally, the management of patients with cerebral palsy with intra-thecal injections of BMNC substantially improves gross motor function, muscle spasticity and tone (496-498). With many beneficial effects, autologous BMNC therapy have become an attractive therapeutic option for chronically inflamed tissues with limited regenerative capacity, and is the focus of a growing amount of research.

Due to its autologous nature BMNC therapy does not present risk of host-graft disease or any adverse reaction derived from cell culture, and therefore is FDA approved, exempt from

legal restrictions applying to MSC therapy. Moreover, BMNC are rapidly isolated from the bone marrow aspirate through gradient centrifugation with minimal manipulation, and readily available for immediate administration using minimal equipment (489, 499, 500). The resulting cell isolate, is composed mostly by mononuclear cells (<90%). Although the majority of cells in this fraction are macrophage and monocyte committed progenitors (<50%), there are also hematopoietic stem cells (~25%), lymphoid cells (~10%), fibroblastic reticular cells (~10%), and a minute portion of MSC (432, 433, 435). Macrophage progenitors are the cells primarily responsible for downregulating inflammation in BMNC. Other hematopoietic and mesenchymal stem cells contribute, but to a lesser degree (432, 494). BMNC have been used successfully in animals and people to improve healing in inflamed tissues refractory to other treatments, however, the mechanisms by which a response is achieved are not yet fully elucidated and may vary by tissue.

Interestingly, following sterile damage, the injury site becomes invaded by myeloid cells including neutrophils from the blood stream, which are the predominant cells for the initial 48-72 hours, during the acute inflammatory phase (158, 501, 502). Further, they are rapidly replaced by mononuclear cells, displaying specific monocyte/macrophage markers, which are responsible for tissue remodeling and secretion of cytokines and growth factors throughout the process of tissue repair (141, 502, 503). Coincidently, *in vitro* and *in vivo* studies have shown that following a couple days of inflammatory stimuli, the leukocyte fraction from bone marrow can become composed by over 90% macrophages (433). These macrophages respond through a biased regulatory response in face of inflammation and tissue damage, producing a myriad of mediators aimed at counteracting the deleterious effects of inflammation, and support tissue repair (157). Moreover, there is current evidence that these same cells invading the repairing

injury display marked plasticity, initially expressing macrophage markers, they further undergo differentiation in to fibroblast looking cells displaying a different set of markers typically seen in fibroblasts. These cells holding this transmutation ability are currently called fibrocytes and have a central, intrinsic role in tissue repair (192, 504, 505).

There are limited reports regarding the use of BMNC in the treatment of chronic synovitis and joint disease. However, BMNC was shown to reduce clinical signs of synovitis and moderate OA, decreasing degenerative changes in the joint (506, 507). As previously mentioned, synovial macrophages are the main drivers of joint health and homeostasis. Once the mechanisms leading to these physiological and homeostatic functions of macrophages become overwhelmed, they become the main responsible for inciting joint inflammation. Therefore, the ability to capitalize the macrophage derived effects from BMNC to reestablish joint homeostasis, by maximizing the macrophage-driven anti-inflammatory response directly seem to provide a more targeted OA therapy compared to currently available options. By comparison, conventional therapy with corticosteroids have the potential to impair cartilage metabolism and inhibits endogenous production cytokines essential for joint homeostasis and repair such as PGE_2 of IL-10 produced by resident macrophages, which are essential for optimal function of chondrocytes and other joint cells (134, 135, 139, 279). Re-establishing the homeostatic effects of regulatory macrophages through BMNC joint injection, may recover the functional population of the main guardians of joint health, and stands to play an important role in decreasing synovial inflammation in OA by optimizing homeostatic mechanisms and favoring synovial tissue repair. BMNC therapy has the potential to benefit thousands of human and veterinary patients suffering of OA, and builds relevant part of this study.

Surgical treatment

The surgical approach to OA can mainly go two different ways: conservative treatments, in which the injured cartilage is preserved, and radical treatments, in which the cartilage is replaced by prosthetic implant, a procedure often called arthroplasty. These therapies are the alternative approach to patients unresponsive to other therapies. Arthrodesis is yet another possible surgical intervention for treating OA which eliminates the joint's mobile function, and in people it is performed mostly in small joints like ankles and wrists (299, 508).

Conservative surgical treatments are based on the principle that OA symptoms result from the imbalance between the load applied to a joint and the extent of articular surface available to efficiently cushion that load. Thus, surgical procedures such as a tibial valgus osteotomy are aimed at decreasing the load to which the most affected area of joint is exposed. Conservative treatments are usually offered to young patients in order to delay, if not avoid, the need for a joint prosthesis. Joint arthroplasties from nowadays have excellent post-surgical recovery and functional outcomes, for both partial and total joint replacement, nonetheless, complications with joint arthroplasties can occur and have very little salvage solutions. Hence, such procedures are only considered for patients that failed to respond to adequate medical management of OA and experience justifiable morbidity (508).

Arthroscopic lavage or debridement (or both) and meniscal resection, are standardized procedures performed in patients with mechanical or disabling symptoms, and are aimed at removing triggers of inflammation from the joint and treating concomitant meniscal tear. Despite of questionable benefits on pain management, such procedures remain among the most frequently performed by orthopedic surgeons (299, 509). Whether partial meniscectomy (PM) provides benefits to patients with symptomatic meniscal tear and concomitant OA is unclear,

there is evidence that patients undergoing PM exhibit earlier and more severe OA than age matched patients who have not had PM (509).

Arthroscopic surgery also remains the gold standard for diagnosis and assessment of pathological joints in horses. However, depending on the extent and distribution of cartilage injury, it is possible that little can be done arthroscopically to provide benefit, aside of a clear diagnosis. The use of arthroscopy in horses has been validated in the management of several conditions such as osteochondral fragmentation, slab fractures, osteochondritis dissecans (OCDs) and subchondral cystic lesions. Injection of corticosteroids into the lining membrane of subchondral cysts has been facilitated by means of arthroscopy. As in people, equine arthroscopy is used in the diagnosis and treatment of meniscal tears and cruciate ligament injuries (298).

1. Surgical approaches to improve articular cartilage healing

Arthroscopic techniques that enhance both the quantity and quality of cartilage repair tissue, have been attempted (298, 475). Overall, attempts at improving the repair of articular defects can be divided into stimulation of endogenous repair and articular grafting.

2. Stimulation of endogenous repair

Most techniques stimulating endogenous repair rely on the principle of providing access for myeloid cells within the subchondral bone marrow to reach the articular defect and drive the development of repair tissue. Then, fibroblastoid cells of mesenchymal origin and other myeloid leukocytes in the bone marrow coordinate tissue repair leading to the formation of a granulation tissue, which further differentiate into fibrocartilage. Yet, this newly formed tissue comprises high concentrations of type II collagen, it has very low GAG content (190, 197, 298, 510).

Among techniques that rely in this principle are: debridement, spongialization, abrasion arthroplasty as well as subchondral bone drilling or microfracture. However, diverging results were seen using those methods in different clinical settings and in vivo models of OA. An exception applies for subchondral bone drilling and microfracture, which have been the most successful technique (298, 436).

While the surgical principle of debriding to the level of subchondral bone was just described above, it is important to notice that the calcified cartilage needs to be removed in order to increase the amount of repair tissue, its adequate attachment to the subjacent bone and calcified cartilage reformation (511). Subchondral drilling had similar rationale in providing access through the cancellous bone plate, while still preserving most of the subchondral bone plate. Subchondral microfracture is extensively used in people (436, 473, 476). Whether it is debatable if it should be considered the up to date gold standard for cartilage repair or not (473), it is shown to provide equivalent repair to the commercially available autologous chondrocyte implantation process (474), is a simple and atraumatic way to provide progenitor cells and growth factors, and the subchondral bone architecture provides a foundation to enhance attachment of the repair tissue. It has been shown that microfracture combined with debridement significantly increase the amount of repair tissue when compared to debridement alone, increasing type II collagen deposition (512).

Partial-thickness chondrectomy was early described as a potential technique that consists in shaving cartilage down to a healthy layer, in the hopes this would decrease further tissue exfoliation; however, no benefit seemed to appear from it (513). A similar procedure using radiofrequency probes have also been attempted in vitro (514, 515) and recent results from a randomized clinical trial suggested positive long-term outcomes, with no significant difference

from debridement (516, 517). Similar findings where experimentally seen in horses (518). Spongialization is the removal of sclerotic subchondral bone from the base of a full-thickness defect, so that the vascularized spongy subjacent bone allows marrow contents to reach the defect. Abrasion arthroplasty, consists in superficial debridement of sclerotic degenerative lesions, with or without combined debridement of other diseased tissues such as the synovium. The concept is an outdated, highly controversial approach, because it is necessary to expose a substantial area of the articular surface cancellous bone in order to reach blood supply and progenitor cells (298, 519, 520).

3. Implantation of autologous chondrocytes

Early research aimed at grafting chondrocytes or chondrogenic cells within a matrix were relatively unsuccessful (521-523). Nowadays a commercial technique is available for autologous chondrocyte implantation into human knees, and is particularly applied into focal erosive defects and OCD as a two-stage procedure (524). After harvesting a sample of cartilage, chondrocytes are isolated and cultured for 3 weeks. A graft of autologous periosteum is sutured to the edges of the defect to create a shield, to which the cultured chondrocytes are injected beneath it. The addition of growth factors to the cell suspension, especially IGF and TGF has been reported (101). Although considered a very successful technique, this is a very laborious procedure that can sometimes fail due to detachment of the graft. Comparisons of this technique to microfracture on a long-term follow up failed to show superiority for people and horses (474, 525).

4. Joint replacement

Surgical joint replacement was firstly introduced in the 1960s, and due to growing success of the technique it is estimated that over 3 million total joint replacements will be performed annually in the US by 2030. Approximately 20% of patients are dissatisfied with the surgical outcomes as they experience pain, their level of activity is inferior to pre-surgery, or inexact fitting of the prosthesis results in poor kinematics of the joint. Current technology allows development of implants attending the patients' individual anatomical needs, providing better joint geometry and soft tissue preservation (526). Most recently, partial joint replacement has become a common procedure for patients suffering for unicompartmental knee OA. A recent meta-analysis did not find any significant difference between total or partial joint replacement for the management of knee OA, however, the quality of evidences available was low, especially regarding follow up data (508, 527). Secondly, arthrodesis of the ankle and wrist joints are reliable, reproducible, and effective alternatives to total joint replacement and restore a reasonable level of function with good relief of pain (509).

Experimental models to study OA

Several *in vivo* and *in vitro* models for the study of OA are available and have been widely used (32, 92, 262, 528). *In vivo* models can be subdivided according to the primary mechanism by which the degenerative process is triggered (mechanical of chemical), and *in vitro* models according to the complexity of tissue or cell components (monoculture, multiple cells, tissue explants). Subtle variations to these models translate into a myriad of different effects which often prevent accurate comparison between models' results.

While *in vivo* models include the complex inter-relationship between all anatomical components of the joint, and thus provide an irreplaceable way to obtain the most accurate representation of naturally-occurring OA, they are generally costly and require substantial numbers of animals in order to provide means of interpreting the effects of individual variability (32, 92). Therefore, the reduced costs and ease of manipulation from *in vitro* systems, added the 3R philosophy of refining, reducing and replacing the use of animals in science, makes *in vitro* modelling of OA desirable (92, 529).

In vivo models of OA offer the opportunity to study events from the developmental stage of disease, providing means to elaborate therapeutic interventions targeting disease processes prior to fulminant tissue degeneration, and thus more likely to produce long term benefits (31, 92). There is no consensus on the most appropriate model for the representation of specific features of OA, as each model has its own particularities, which better fit the different phenotypes of OA to be studied, such as PTOA or OA caused by chronic repetitive trauma. However, for logical reasons, models such as the collagen induced arthritis and the monoiodoacetate model, create immune responses and a degree of inflammation that clearly do not correspond to that seen in naturally occurring disease, and so, despite of their potential usefulness to study conditions such as RA, they have limited value in translating features of OA. Multiple rodent models have been developed and uniquely provide means of modeling the effect of gene knock-outs and are widely used in this field. However, the genomic response during inflammation in those species, especially murine models, poorly translate to people, and therefore the utility of the findings is limited or questionable (530, 531).

The femorotibial patellar (knee) is the joint regularly used in mice, rabbits, sheep, goats, and dogs to model OA (31, 32, 440, 489, 532-537). In the case of horses, metacarpophalangeal

and middle carpal joints are the most studied. The equine metacarpophalangeal joint has great similarities to the human knee (15, 34, 538, 539), and in face of many ethical issues for the use of non-human primate models, the horse became one of the most representative models for the study of human OA (15, 539).

In vitro models can be useful, and allow modeling the effects of specific substances in a short period of time, especially in testing cell-based genetic manipulation and the validation of research concepts and hypothesis prior to moving to in vivo pre-clinical research. However, the relevance of such findings to clinical disease always needs careful interpretation, since even the best designed in vitro models lack the complex physiology of a live joint. While some differences from in vivo and in vitro models can be an effect of treatment delivery and metabolism and duration and variation of osteoarthritic processes, in vitro models should be ultimately aimed at closely reflecting the natural in vivo disease. This has special concern to stage of disease, where nearly all in vitro models are designed to replicate events of terminal OA, with little or no consideration to early disease events, where chondroprotection is probable to provide greater clinical benefits. In addition, most in vitro models challenge cells and tissue explants with cytokines doses that are far beyond supra-physiological, and therefore the cellular responses do not reflect the one corresponding to that of cells during natural OA (92). Such models have focused mostly on studying cartilage and chondrocytes, and only more recently the addition of synovial explant or synoviocytes, and the subchondral bone has been included (239).

Spontaneous OA in horses has a similar pathogenesis to that observed in people. Decades of experience in managing clinical and experimental OA in horses created strong cumulative knowledge regarding development and treatment of clinical OA in this species (15). This generates opportunities to obtain samples from naturally-occurring, early-stage disease,

following a surgical intervention or from necropsy of deceased equine athletes. The combination of these factors makes horses a valuable source of clinical material for study disease mechanisms and response to therapy (15, 92). Experimental *in vivo* models of equine OA and synovitis include mainly three established models. The carpal osteochondral fragment-exercise model developed at the Colorado State University, and thus also called the CSU model, the lipopolysaccharide (LPS) or IL-1 induced synovitis models. These models have been widely used, and therefore the resulting clinical changes, levels of inflammation and cartilage degradation have been well documented (15, 111, 540-543). Most recently other models such as the groove model in the equine metacarpophalangeal joint, and the blunt trauma in tarsal joints have been reported (544, 545), however, further characterization of these models is necessary.

In the CSU model an osteochondral fragment is created in the middle-carpal joint, and horses are then exercised for periods of 6-9 weeks. This model produces low levels of inflammation in a similar degree to what is observed in naturally occurring OA. It allows experimental follow-up through the disease processes for relatively long term, in similar fashion that race horses experience disease following naturally occurring osteochondral fragmentation. For this reason, it became one the most valuable models to study OA development, and test the validity of emerging therapies. Nonetheless, it is an expensive model that can be time and horse consuming (15).

On the other hand, acute joint inflammation can be induced by intra-articular injection of a low dose of bacterial lipopolysaccharide (LPS), which causes a transient synovitis without long lasting deleterious effects (111, 112, 423, 540, 542). The LPS-induced synovitis model has been widely used to investigate mechanisms of early joint pathology and evaluate joint therapies aimed at synovitis and osteoarthritis (112, 213, 357, 540, 541, 546-552). Although this is a

model of acute inflammation based on clinical and synovial fluid cytological data, it has been reported to induce significant synovial fluid increases in general MMP activity (MMP1, MMP8, MMP13) and biomarkers of type II collagen and proteoglycan degradation (C2C and 3-B-3) and synthesis (CPII and CS-846) that last for at least 1 week (274, 427, 541). Disadvantages of the LPS model could be the use of a bacterial by-product, and expression of mediators involved in septic arthritis not present during sterile inflammation such as in OA. Another synovitis model has also been used, and is created by injection of IL-1β. This model creates a similar response to that observed in the LPS model, with similar clinical and pathological findings with the advantage of producing sterile inflammation (111, 543, 553, 554). Of notice, all of these models require that equivalent joints from the same individual to be compared in order to obtain accurate observations, since the response differ according joint types (543).

Investigation and treatment of animals with naturally occurring OA is also a source of treatment–response information, however variability in the individual response to injury, therapy, and affected joints prevent accurate interpretation of the research outcomes, frequently providing inconclusive data. In the absence of a perfect model, the multifactorial nature of OA should be considered when designing a model to reproduce disease events accurately, even when testing a single parameter (15, 92). For the reasons mentioned above, the horse stands to be the model to most closely translate human OA, and therefore was elected as the model for these studies.

Summary

OA is an important degenerative disease of the articular tissues, causing significant morbidity to innumerous people and horses. The burden of OA is not only physical but also financial, representing increased costs of health care to affected patients and the equine industry.

The joint is a multicomponent organ, which depends on the integrity of each of its structures to perform efficiently. Therefore, if any of these structures undergo injury leading to dysfunction, the physiology of the entire joint is affected initiating a cascade of inflammatory processes that alter the metabolism of joint tissues, which if not efficiently and timely interrupted leads to eventual degeneration of the whole joint.

The articular cartilage is one the tissues most grossly affected, and therefore cartilage damage has been the hallmark of OA and a diagnostic criterion. However, there is increasing understanding of the role of all joint tissues, including the subchondral bone and the synovium on triggering the inflammatory process of OA, with especial attention to synovial macrophages as the main drivers of synovial inflammation, often a sole driver of OA. Intriguingly, synovial macrophages centrally drive joint health and joint disease, and the common denominator between these two opposing effects is the varying efficiency of synovial macrophages to promote joint homeostasis and recover it following injury.

To date, most therapeutic options available to manage the negative effects of OA are limited in providing long term durable results, and commonly used therapies, such as corticosteroids negatively interfere with many cell functions intrinsic to homeostasis and repair of synovial tissues. Increasing the number of macrophages in several chronically inflamed tissues through BMNC therapy provides recovery of homeostatic effects, and stands to provide similar effects in inflamed osteoarthritic joints.

Horses and people share many immune and inflammatory features, and the vast experience with research and treatment of naturally occurring OA in in equine athletes make horses one of the most validated models to study OA. Therefore, in the studies comprising this thesis, horses were selected as the species to investigate the *in vivo* phenotypes of synovial macrophages in normal and osteoarthritic joints, as well as to evaluate the effects of BMNC therapy in regulating joint inflammation using *in vitro* and *in vivo* models of equine synovitis.

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CHAPTER 2: SYNOVIAL MACROPHAGE POLARIZATION IN HEALTHY AND OSTEOARTHRITIC EQUINE JOINTS

ABSTRACT

Synovitis, a major component of osteoarthritis, is driven primarily by macrophages. Macrophages induce an inflammatory response (M1 phenotype), but also drive tissue repair and resolve inflammation (M2 phenotype). Macrophage phenotypes in the synovium from osteoarthritic and healthy joints have not been characterized. The objective of this study was to compare the patterns of macrophage phenotypes (M1:M2) in healthy and osteoarthritic equine joints. We hypothesized that synovium from osteoarthritic joints would have increased M1:M2 ratios compared to normal joints. Immunohistochemistry for M1 (CD86), M2 (CD206, IL-10), and pan macrophage (CD14) markers was performed on synovial biopsies from healthy (n=29) and osteoarthritic equine joints (n=26). Cytokines and growth factors in synovial fluid were quantified. All markers were co-expressed in all joints with minimal differences between groups. Intensity of expression varied with degree of synovial inflammation. CD14, CD86, CD206, and IL-10 were highly expressed in grossly inflamed osteoarthritic joints, with CD86

most highly expressed. Synovial fluid MCP-1 was higher in osteoarthritic joints while SDF-1 and IL-10 were lower.   Increased CD86/CD206/Il-10 expression was associated with synovial hyperplasia, consistent with macrophage recruitment and activation in response to higher demands for repair.   Macrophages are vital in modulating joint homeostasis and are not as clearly defined in vivo as they are in vitro. Our findings suggest that homeostatic mechanisms from synovial macrophages are impaired in OA, preventing inflammation resolution, resulting in chronic inflammation. Therapeutic approaches aimed at recovering mechanisms of macrophage-driven synovial homeostasis may be more effective in treating osteoarthritis than inflammation inhibition.

Keywords: osteoarthritis, macrophage activation, synovitis, inflammation resolution, joint homeostasis.

INTRODUCTION

Osteoarthritis (OA) is a leading cause of morbidity and presents significant treatment challenges (1, 2). The pathophysiology of OA is incompletely understood. Increasing evidence suggests that the innate immune system plays a role in disease development (3, 4). Early macrophage depletion studies established that macrophages drive synovial inflammation in experimental arthritis (5, 6) and in clinical cases of rheumatoid arthritis (7). The specific role of synovial macrophages as the main drivers of synovial inflammation was recently confirmed when inflammatory markers were dramatically decreased in the absence of synovial macrophages (8, 9) and considering that activation of macrophages in the osteoarthritic synovium is directly related to disease activity, severity, and pain (10, 11). Under homeostasis, 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 (4, 12). When these homeostatic functions become overwhelmed, synovial macrophages recruit other immune cells (e.g., lymphocytes and neutrophils) to upregulate inflammation and respond to the increased demands for repair and homeostasis (9, 13, 14).

In vitro, macrophages activate into a spectrum of phenotypes, with the extremes represented by cells displaying classical inflammatory (M1) or suppressive/healing (M2) responses (15). *In vivo*, macrophages activate in response to environmental stimuli, exhibiting marked plasticity, and play a fundamental role in resolving inflammation and promoting tissue healing. Their depletion or exhaustion results in severely compromised wound healing or chronic inflammation (16-18). The roles of different macrophage phenotypes during health,

chronic inflammation, and disease are described (19, 20). *Ex vivo* chondrogenesis of synovial progenitor cells is impeded by classically activated (M1) macrophages from the osteoarthritic synovium (21). Alternatively activated (M2) macrophages are required for efficient chondrogenesis (12). Inflammation in arthritic joints *in vivo* is dampened by M2 macrophages, improving clinical and histological signs of joint disease (14, 22). Collectively, these findings support the premise that enhancing the M2 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* or experimental data or end stage OA. Reports describing healthy joints are limited to the low numbers of synovial fluid macrophages shedding from the synovium following hyperactivation (6, 9, 12, 21, 23). Defining patterns of macrophage activation in normal and osteoarthritic synovium is paramount for understanding their *in vivo* roles and for designing strategies targeting macrophage-driven joint homeostasis. The aim of this study was to define the patterns of macrophage phenotypes in biopsies of healthy and osteoarthritic equine synovium and to correlate macrophage phenotypes with histology and synovial fluid cytokine and growth factor concentrations. We hypothesized that synovial macrophages in osteoarthritic joints would exhibit increased ratios of M1:M2 phenotypes 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 macrophage ratios in synovial 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 or evidence of lameness referable to the harvested joints and with grossly normal articular surfaces at euthanasia. All procedures were approved by the IACUC and written owner consent. Synovial inflammation and concentrations of pro- and anti-inflammatory cytokines, growth factors, and prostaglandin E_2 (PGE_2) were assessed by synovial membrane histology, synovial fluid cytology, and immunoassays. Synovial macrophage polarization *in situ* was defined by immunohistochemistry.

Inclusion criteria

Horses 3-15 years old (skeletally mature, but not aged) were recruited and lameness exams performed, including response to joint manipulation, gait analysis at the trot, joint flexion, and radiography. Diagnostic analgesia was performed at the discretion of the veterinarian in charge of the case, 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) (24). Only moderate OA (OARSI grade 2) joints were included, as representative of those most commonly treated clinically and when synovial cellularity is highest (25). Horses with a history of septic

arthritis, non-steroidal anti-inflammatory therapy or intra-articular diagnostic anesthesia within 2 weeks, or intra-articular corticosteroids within 2 months prior to sample collection were excluded.

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 (26). Control samples were harvested at sites where each joint is traditionally most commonly affected (24). Samples were fixed (AZF Fixative®; Newcomer Supply, WI) at room temperature for 24 hours, rinsed and stored in PBS at 4°C until processing.

Synovial fluid analysis

Synovial fluid cytology was processed for total nucleated cell count (TNCC) by hemocytometer and total protein (TP) by refractometer. Differential cell counts were performed following Romanowski stain (Microscopy Hemacolor®, Merck, Germany). Pro- (IL-1β, IL-6, GM-CSF, TNF-α) and anti-inflammatory cytokines (IL-10, IL-1ra), chemokines (MCP-1, SDF-1), growth factors (IGF-1, FGF-2), and PGE_2 in synovial fluid were quantified. Thawed samples (200 µL) were hyaluronidase-digested (10 µL of 100 IU hyaluronidase/mL acetate buffer; Worthington Biochemical Corporation, Lakewood, NJ) for 30 minutes at 37°C, centrifuged (12,000xg; 10 min; 4°C), and the supernatant recovered. Based on previous experience and

interfering factors in cytokine detection in synovial fluid (27, 28), spike-and-recovery assays were performed for the PGE$_2$ ELISA and 4 representative serially-diluted targets in the multiplex assay (IL-1β, IL-6, IL-10, TNF-α). Based on the results, a dilution of 1:2 was selected for PGE$_2$ quantification and no dilution was deemed necessary for the multiplex assay.

PGE$_2$ was quantified by ELISA (R&D Systems, Minneapolis, MN). Hyaluronidase-digested samples were solid-phase extracted (500 µL synovial fluid in 490 µL 100% ethanol and 10 µL glacial acetic acid incubated at 23°C for 5 minutes), centrifuged (2,500xg; 8 min; RT), and the supernatant collected. Remaining analytes were quantified by bead-based multiplex assay (MILLIPLEX MAP Equine Cytokine/Chemokine Multiplex Assay with manufacturer modification to include IGF-1, SDF-1, and IL-1ra; Luminex 200 plate reader Millipore Sigma, Burlington, MA).

Synovial membrane histology and immunohistochemistry

Fixed synovial membrane biopsies were paraffin-embedded, sectioned (5 µm), and H&E-stained. Synovitis was scored based on the OARSI histopathology guide and included cell infiltration, vascularity, hyperplasia, edema, and fibrosis (24). 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^{TM} 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). Cell staining (absent (0); mild (1); moderate (2); or intense (3)) and staining distribution across

synovial villi (base of the synovial villus only (1); reaching portions of the synovial villus tip (2); and throughout the entire synovial villus (3)) were scored on 3 tissue sections and averaged. Composite scores for each marker were compared between groups for both histology and immunohistochemistry.

Statistical analysis

Data analysis (SAS version 9.4, SAS Institute, Inc, Cary, NC) was performed using General Estimating Equations to assess effects of different joints sampled and effects of disease (normal vs. OA). Least square means adjustment for multiple comparisons was performed using the Tukey-Kramer test with significance set at $P<0.05$. Immunohistochemistry data were scored for comparison, but were not analyzed due to lack of a validated scoring system.

RESULTS

Characterization of study population

Normal and OA samples were harvested from 13 horses, OA samples only from 8, and 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 lameness (1-3/5(29)) 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 2-1**). No overall differences were detected between normal and OA

joints for differential cell counts. No significant differences were observed between normal and OA carpal or MCP joints for any 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. Overall, MCP-1 concentrations were significantly higher in OA than normal joints (p=0.0443) (**Table 2-2**). SDF-1 concentrations were significantly lower in OA compared to normal joints overall (p=0.0243) and within MCP joints only (p=0.0378). Overall, there was no difference in IL-10 concentration 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, while those for cell infiltration (p=0.0818), vascularity (p=0.1398), and fibrosis (p=0.3053) were higher for OA joints, but were not significant (**Table 2-3**) (**Fig. 2-1**).

Table 2-1: Synovial fluid cytology from normal (control) and osteoarthritic (OA) equine metacarpophalangeal and carpal joints. Median (95% confidence interval).

Synovial Fluid Cytology						
		Total Protein g/dL	TNCC Cells/μL	Monocytes %	Lymphocytes %	Neutrophils %
Metacarpo-phalangeal Joints	**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=0.5702*	*P=0.5805*
Carpal Joints	**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=0.2167*	*P=0.2251*
All Joints Combined (Overall)	**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=0.2699*	*P=0.1995*

TNCC= Total nucleated cell count.

Table 2-2: Synovial fluid cytokines, chemokines, and growth factors in normal (control) and osteoarthritic (OA) equine metacarpophalangeal and carpal joints. Median (95% confidence interval).

		FGF-2	IGF-1	IL-1β	IL-6	IL1-ra	MCP-1	SDF-1	IL-10	PGE$_2$	TNF-α
Analytes											
	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
Metacarpo-phalangeal Joints	**Control** N=15	N= 4 44* (23-137)	U	N=4 335* (55-617)	N=5 6* (3-19)	U	N=10 799 (128-1508)	N=14 241 (129-292)	N=15 86 (55-97)	N=14 69 (53-73)	N=5 3.5* (2-10)
	OA N=16	N=3 20* (13-53)	N=1 506*	N=5 44 (28-4014)	N=5 25 (3-65)	N=1 3*	N=13 773 (128-1463)	N=13 137 (89-208)	N=11 68 (40-96)	N=13 71 (53-75)	N=3 5* (3-24)
	P-value	-	-	-	-	-	P=0.0803	**P=0.0378**	**P=0.0462**	P=0.7206	-
Carpal Joints	**Control** N= 14	N=2 78* (16-141)	N=2 1917* (196-3639)	N=7 199 (21-5501)	N=6 13 (3-89)	N=4 6* (1-219)	N=14 786 (230-1867)	N=14 334 (152-467)	N=14 64 (41-98)	N=14 67 (53-73)	N=5 9* (6-35)
	OA N=10	N=3 18* (16-42)	N=4 270* (83-863)	N=6 169 (18-695)	N=5 11 (3-172)	N=1 15*	N=10 933 (260-2526)	N=10 267 (73-498)	N=10 64 (57-108)	N=10 73 (62-83)	N=5 5 (3-41)
	P-value	-	-	-	-	-	P=0.1360	P=0.1943	P=0.7362	P=0.3740	-
All Joints Combined (Overall)	**Control** N= 14	N=2 44 (16-141)	N=2 1917 (196-3639)	N=11 283 (42-1014)	N=11 8 (3-22)	N=4 6* (1-219)	N=31 799 (240-1508)	N=30 276 (188-320)	N=31 80 (55-92)	N=29 68 (58-72)	N=10 6 (3-11)
	OA N=10	N=5 19 (12-53)	N=5 407 (83-863)	N=11 64 (18-4014)	N=12 18 (4-40)	N=2 9* (3-14)	N=24 880 (442-1096)	N=24 150 (109-278)	N=22 66 (57-92)	N=23 71 (64-75)	N=5 5 (3-25)
	P-value	-	-	-	-	-	**P=0.0443**	**P=0.0243**	P=0.2052	P=0.5159	-

U= Undetected; - = p-values could not be determined due to small number of samples in which the analyte was detected; * = the actual confidence level is < 95%.

Table 2-3: Individual and composite histological parameters for H&E-stained normal (control) and osteoarthritic (OA) equine synovial membrane from metacarpophalangeal and carpal joints Median (95% confidence interval).

Synovial Membrane Histology							
		Cell Infiltration	Vascularity	Intimal Hyperplasia	Subintimal Edema	Fibrosis	Composite Scores
Metacarpo-phalangeal Joints	**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
Carpal Joints	**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
All Joints Combined (Overall)	**Control**	2 (1-2)	2 (1-2)	1‡ (0-1)	1 (1-2)	2 (1-3)	8 (6-9)
	OA	2 (1-3)	2.5 (0-4)	1‡ (0-2)	2 (1-3)	2.5 (2-3)	9.5 (7-12)
	P-value	P=0.0810	P=0.1398	**P=0.0076**	**P=0.514**	P=0.3053	**P=0.0122**

The composite of these individual scores was significantly higher in overall 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 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 (**Fig. 2-2**).
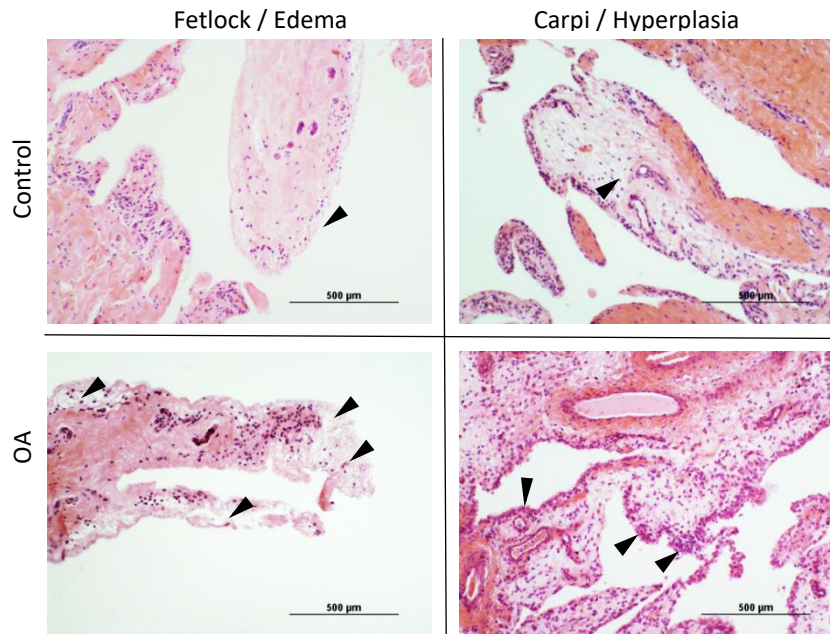
Figure 2-1: Representative images demonstrating (arrowheads) the differences between normal and osteoarthritic (OA) joints for Intimal Hyperplasia and Subintimal Edema.

Synovial membrane immunohistochemistry

Overall, expression of CD14, CD86, and IL-10 was higher in OA compared to normal joints and these differences were most apparent in synovium from MCP joints (**Table 2-4**). No overall difference was observed for CD206 expression; however, in carpal joints expression of CD206 was higher in OA joints. Staining of all markers was most intense around blood vessels, especially over endothelial cells (**Fig. 2-3**). 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.
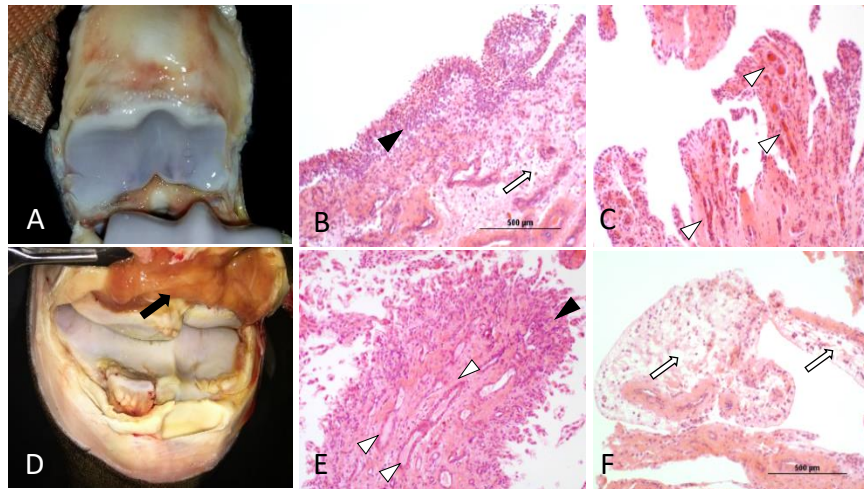
Figure 2-2: Compared to osteoarthritic joints with no or minimal signs of inflammation (A), OA joints exhibiting gross signs of synovitis (D; black arrow), exhibited increased histological changes such as severe cell infiltration and hyperplasia of the synovial intima with shedding of its outermost layer (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).

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 the synovial villi, while in OA joints the tip of villi was also frequently stained (**Fig. 2-4**). 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 was more diffusely distributed in the synovial lining around cell nuclei. In 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

Table 2-4: Composite immunohistochemical scores for CD14, CD86, CD206, and IL-10 in normal (control) and osteoarthritic (OA) equine synovial membrane from metacarpophalangeal and carpal joints. (Median, 95% confidence interval).

Synovial Membrane Immunostaining					
		CD14	CD86	CD206	IL-10
Metacarpo-phalangeal Joints	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)
Carpal Joints	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)
All Joints Combined (Overall)	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)

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 consistent. A similar but less intense 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 (**Fig. 2-4**). 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**.**
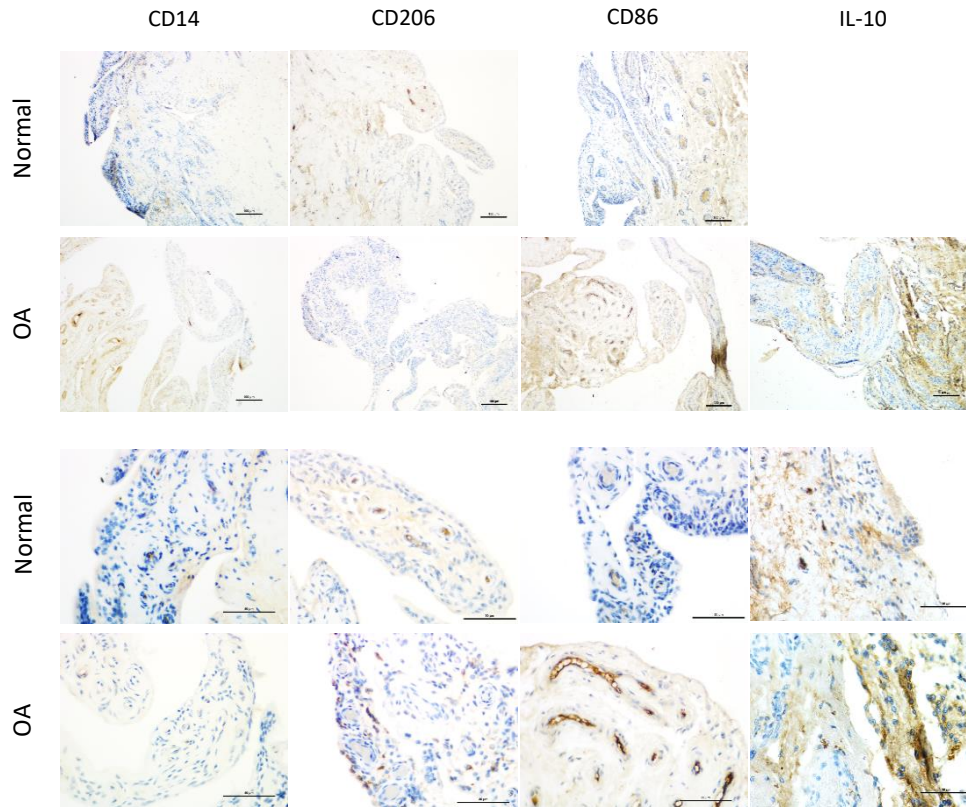
Figure 2-3: Representative immunohistochemistry sections from normal and OA equine synovial membrane at low (top 2 rows; scale bar=100 µm) and high magnification (bottom 2 rows; scale bar=50 µm) from the same histological section and demonstrating the median staining scores for macrophage markers (CD14, CD206 [M2], CD86 [M1], and IL-10 [M2]).
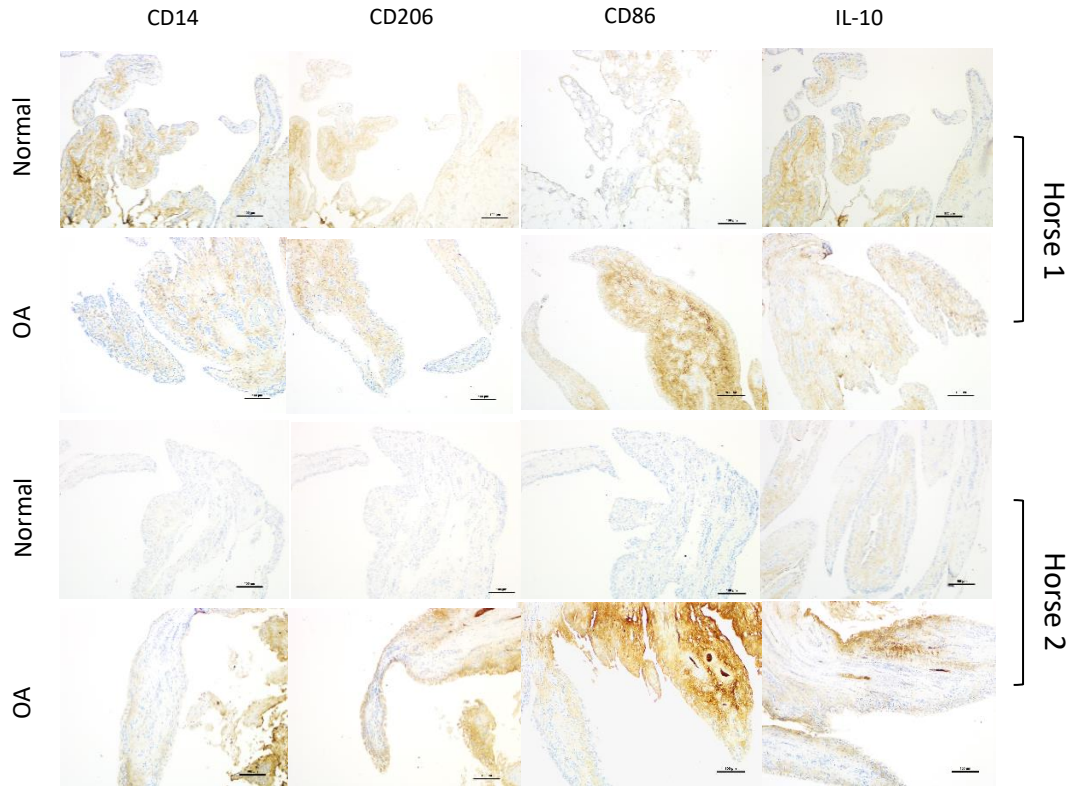
Figure 2-4: Sets of representative immunohistochemistry sections from normal and grossly inflamed OA equine synovial membrane from the same horse (2 different horses; scale bar = 150 µm) demonstrating increased staining intensity and distribution for all selected markers in OA joints, denoting more consistently marked increases for CD86 staining.

DISCUSSION

This is the first study comparing patterns of macrophage phenotypes between synovium from normal joints and those with naturally occurring OA. All markers, widely-used to define M1 and M2 macrophages, were co-expressed in normal and OA joints with minimal differences in expression intensity. Intensity of expression did vary with degree of synovial inflammation. Mildly increased expression of CD14, CD86, CD206, and IL-10 was observed in OA joints with low-grade histologic inflammation. All markers were highly expressed in grossly inflamed OA joints, especially CD86, which was most highly expressed. Histologic findings were consistent with lower synovial fluid concentrations of IL-10 and SDF-1 and higher MCP-1 in OA joints with chronic low-grade inflammation. Combined expression of M1 and M2 macrophage markers (6, 21, 30) and increased expression of macrophage markers proportionate to inflammation (9) are reported in joints with naturally occurring and experimentally-induced OA. Our findings, along with previous reports (22, 31-36), suggest that there is higher macrophage recruitment, proliferation, and activation in synovial tissues from osteoarthritic joints, a physiologic response required to incite inflammation prior to damage repair and subsequent inflammation resolution (11, 16, 17, 32, 33, 35, 37). Decreased IL-10 and SDF-1 in OA synovial fluid also suggests these compensatory mechanisms of inflammation were insufficient, preventing effective re-establishment of joint homeostasis (16, 17, 32-35).

Although CD86 and CD206 expression have historically been considered markers of M1 and M2 macrophages (9, 21, 38, 39), this is an oversimplification of events that occur *in vivo* (11, 16, 40). Attention to the specific function of each cell surface marker is necessary to accurately interpret the meaning of their expression during macrophage activation *in vivo*. For example, CD86 is constitutively expressed at low levels in monocytes and resting macrophages

and increased CD86 expression is part of the events involved in cellular checkpoints required for monocytic lineage commitment, activation, and survival (33). Therefore, as observed in our control joints, CD86 is poorly associated with a classically-described inflammatory phenotype *in vivo*. Likewise, the mannose receptor (CD206) has a pivotal function in host defenses during inflammation, clearance of tissue debris, wound healing and remodeling, and resolution of inflammation (homeostasis). CD206 is constitutively expressed in mature mononuclear phagocytes and the intensity of its expression is associated with demands for anabolic cytokine secretion and clearance of dying cells and damage-associated molecular patterns (35). 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 indicators of disease. Even though expression of CD86 and CD206 was reported to associate to M1 and M2 in synovial fluid macrophages from normal and OA joints (23), such observation are in disagreement with the profiles of macrophages in the synovium found in this and other studies from our lab (unpublished data) and previous reports (6, 21).

Like CD86 and CD206, expression of IL-10 in the synovial membrane in our study was associated with the degree of synovial inflammation, with higher expression occurring in the inflamed synovium. Following injury, macrophage activation is associated with 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 (34). Consequently, the production of these pro-inflammatory cytokines decreases[36]. 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 those grossly inflamed (41, 42). 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, reflecting the classically known vicious cycle of inflammation seen in OA (6, 11, 33, 34, 41-45).

Cell surface markers (CD14, CD86, and CD206) in synovial tissue were localized to cells, as expected and reported in an experimental model of OA (6), while IL-10, a soluble secreted cytokine, was diffusely detected throughout tissues. Markers were absent or lowly expressed at the tips of synovial villi in normal joints, but more highly expressed at villous tips in OA joints. This observation, combined with increased cellular hyperplasia indicates increased macrophage recruitment and a heightened immune response (32, 33). Our composite synovitis scores are similar to those reported in people (26), where the degree of inflammation between individuals was highly variable, but the majority of OA joints exhibited low grade synovitis. The macroscopic and histochemical findings in our study were associated with mild to moderate synovial intimal hyperplasia and subintimal edema, commonly observed in joints with mild to moderate arthritis (46). These findings reinforce the similarities between horses and people and the validity of using horses to model human OA (1).

Increased overall MCP-1 in synovial fluid from OA joints was associated with gross signs of inflammation, consistent with the literature (42, 43). MCP-1 is constitutively expressed by synovial macrophages and increase in inflamed synovium in association with increased expression of classically termed pro- (IL-1β, IL-6) and anti-inflammatory cytokines (IL-4, IL-10), as well as cartilage degradation (47-52). During synovial inflammation, MCP-1 contributes to recruitment, homing, and accumulation of mononuclear cells in the synovial fluid and membrane (synovial lining hyperplasia) (53, 54), which is part of the homeostatic response

against joint damage (37). Without this homeostatic response, MCP-1-deficient mice are unable to home macrophages to sites of injury and are prone to infection and chronic inflammation (51, 54).

Higher SDF-1 concentrations in synovial fluid from normal versus OA joints in our study is inconsistent with previous studies (55-57). SDF-1 has multifaceted roles in synovial tissue biology, including homeostatic and pro-inflammatory functions. It is not only associated with chemotaxis of immune cells, but also through direct SDF-1/CXCR4 signaling in different cells (e.g., chondrocytes, synovial fibroblasts, and macrophages) (55, 57). SDF-1 is expressed proportionately to inflammation or disease activity, with higher concentrations reported in inflamed joints (55-57). Our results showing lower synovial fluid SDF-1 in OA joints is comparable to two other studies from our lab (unpublished data), where inflammation decreased synovial fluid SDF-1. While our data differ from previously published data, the magnitude of differences in SDF-1 concentrations between OA samples in reported studies is very high, ranging from 95 pg/mL (55) to ≥200 ng/mL (56). Methods for quantification of SDF-1 in synovial fluid may be inaccurate and require optimization.

The overall lack of difference in IL-10 in synovial fluid from normal and OA joints is not surprising. IL-10 concentrations in synovial fluid from normal and OA joints are widely reported to overlap (41, 45, 58). Intra- and peri-articular IL-10 oscillates and increases after exercise as a homeostatic response to cope with anabolic demands following physiologic mechanical stress, a variable not accounted for in our study (58). Although IL-10 exerts anti-inflammatory effects, it also increases proportionate to inflammation following injury to counteract the production of pro-inflammatory mediators. This explains why IL-10 concentrations can be increased in OA, but especially in highly inflamed joints with rheumatoid

arthritis (41, 45). During chronic low-grade inflammation, IL-10 concentrations in synovial fluid regress close to baseline (45, 58). Interestingly, 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 (59). 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 joints mechanisms compensating for tissue damage may be impaired or overwhelmed. Injection of experimentally inflamed joints with autologous bone marrow-derived macrophages resulted in markedly decreased gross, laboratorial, and histological markers of inflammation (unpublished data). Recovery of overwhelmed mechanisms occurs in association with higher concentrations of IL-10 and IL-10-expressing macrophages in synovial fluid. Inflamed joints treated with macrophage progenitors were comparable to healthy joints histologically, whereas PBS-treated controls remained severely inflamed. Combined, our ongoing experimental studies reinforce the important role of IL-10-producing macrophages in driving resolution of inflammation and promoting joint homeostasis.

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 (37) 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, allowing free exchange of cellular and molecular components between intra-articular and sub-synovial spaces. This could explain the overall similar IL-10 staining patterns in normal and OA carpi

(37). Importantly, each of these mechanisms can be affected by the stage of the inflammatory

response (acute-chronic / mild-severe) that could not be precisely assessed in our study.

PGE$_2$ is considered an important marker of joint inflammation based on reported

increases following injury (60-62). However, PGE$_2$ also plays anti-inflammatory and anabolic

roles such as inhibition of inflammatory cytokines and neutrophil infiltration to the site of injury,

chondrocyte protection, activation of suppressive macrophages, and actively driving resolution

of inflammation and tissue repair (17, 39, 63, 64). PGE$_2$, generated during the early

inflammatory response can induce resolution of inflammation by upregulating enzymes required

for synthesis of other bioactive lipids that are potent mediators of resolution (17). Therefore,

PGE$_2$ is involved in both inciting and resolving inflammation and concentrations in synovial

fluid vary with the stage of response to injury. As a result, its use as a marker of inflammation in

OA may not be as effective as reported (65, 66) and may explain the lack of differences between

normal and OA joints in our study.

Traditionally, IL-1β and TNF-α have been considered the main drivers of disease

processes in OA (67-70). 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 (71, 72). Limitations in the detection of IL-1 in synovial fluid are

widely reported, even in samples from patients experiencing marked inflammation (27, 28, 66,

73). While early studies suggesting IL-1 as a central driver of OA emphasized the role of IL-1α

(71, 74), the vast majority of later studies have explored the effects of IL-1β in *in vitro* models of

joint disease, using concentrations of IL-1 many folds higher than the limits detected in natural

disease (68, 75). IL-1α was not detectable in our screening assay (minimum detection level

23.87 pg/mL) and was not included in our immunoassay. Recent proteomic analysis of synovial

fluid and genome-wide transcriptomic analysis of cartilage comparing samples from OA and healthy joints did not identify IL-1 or TNF-α as central targets (76, 77). Although limited detection of both molecules could have been an effect of the intensity of disease activity (27), this is unlikely due to their lack of detection in many samples from actively inflamed joints in our study.

The concept of macrophage activation as either inflammatory (M1) or suppressive/ healing (M2) originated from monocyte-derived macrophages treated *in vitro* with defined and overwhelming cytokine stimuli (15, 38, 39). Clear identification of macrophage phenotypes *in vivo* is significantly more challenging than proposed by *in vitro* models (6, 11, 16). Although synovial macrophages can be the main instigators of inflammation in response to damage (7-9), they are also crucial for normal synovial biology (12, 78). Current knowledge suggests 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. Once tissue repair has been efficiently accomplished, macrophages coordinate resolution of inflammation, ultimately achieving homeostasis (16, 17, 64, 79). The presence of chronic inflammation suggests that the inflammatory response has not achieved its purpose efficiently resolving inflammation.

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 (17, 34, 44). 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 (17). 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, such as by delivering autologous macrophage progenitors into the joint, rather than simply blocking inflammation through the use of non-steroidal anti-inflammatory drugs and corticosteroids. The results of our study provide important information useful for the design and interpretation of future studies. 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.

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AUTHORS' CONTRIBUTIONS

BCM, DHR, SRW, and LAD contributed substantially to study conception and design. BCM and DHR collected samples. BCM 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.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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CHAPTER 3: INFLAMED SYNOVIAL FLUID INDUCES A HOMEOSTATIC RESPONSE IN BONE MARROW-DERIVED MACROPHAGES IN VITRO: IMPLICATIONS FOR JOINT THERAPY.

ABSTRACT

Synovial inflammation is a central feature of osteoarthritis (OA), elicited when local regulatory macrophages (M2) become overwhelmed, activating an inflammatory phenotype (M1). Bone marrow mononuclear cells (BMNC) are a source of naïve macrophages capable of reducing inflammation and producing essential molecules beneficial for cartilage metabolism. This study investigated the response of BMNC to normal (SF) and inflamed synovial fluid (ISF). Equine BMNC cultured in autologous SF or ISF (N=8) developed into macrophage cultures with phenotypes similar to cells native to normal SF. BMNC became more confluent in ISF (~100%) than SF (~25%). BMNC cultured in SF or ISF were neither M1 nor M2, but exhibited aspects of both phenotypes and a regulatory immune response, characterized by increasing counts of IL-10-expressing macrophages, decreasing concentrations of IL-1β, and progressively increasing

concentrations of IL-10 and IGF-1. These changes were more marked in ISF. These findings suggest that homeostatic mechanisms were preserved over time and were potentially favored by progressive cell proliferation. Collectively, our data suggest that intra-articular BMNC therapy could increase synovial macrophage counts, potentiating the macrophage- and IL-10-associated mechanisms of joint homeostasis lost during the progression of OA. Moreover, BMNC therapy preserves signaling pathways involved in tissue repair, including PGE_2 and IL-10.

Keywords: osteoarthritis, macrophage, joint, homeostasis, cell therapy.

INTRODUCTION

       Osteoarthritis (OA) is the most common degenerative joint disease in people and horses, causing major physical disabilities and medical expenditures (1, 2). Based on close similarities in clinical significance, disease pathobiology, and nature of the inflammatory response, the horse is a useful model for studying the pathobiological events of and treatments for OA in people (1). Chronic synovitis is a central process in the development of OA and is often the single driver of catabolic disease processes (3-5). Synovial macrophages are the main drivers of cytokine production and the inflammatory response in the OA-affected synovium (6, 7). Early *in vivo* studies have reported that depletion of synovial macrophages decreases synovial inflammation in experimentally-induced arthritis (8), clinical cases of rheumatoid arthritis (9), and more recently *ex vivo* (6, 7). Taken together, these studies demonstrate that controlling the response of synovial macrophages can benefit the treatment of OA and other types of arthritis.

       Under physiological conditions, macrophages are vital regulators of articular integrity, promoting joint health through the secretion of synovial fluid cytokines and growth factors essential for joint homeostasis, as well as the clearance of tissue debris, foreign material, and efferocytosis (10, 11). Synovial macrophages also form a protective immunological barrier in the synovial lining, providing a tight-junction-mediated shield for intra-articular structures (12). When these physiological functions become overwhelmed, synovial macrophages upregulate inflammation, recruiting neutrophils and lymphocytes in response to the increased demands for repair (7, 13). Sustained joint damage impedes resolution of the inflammatory response and related cytokines and catabolic enzymes lead to joint degeneration (6, 14). Therefore, resolution

of inflammation is a needed event to interrupt the catabolic processes in OA, and to re-establish a homeostatic environment favorable for tissue anabolism and turnover (15, 16).

In vitro, macrophages differentiate into a spectrum of phenotypes, with the extremes represented by cells displaying a classical pro-inflammatory response (M1) or a suppressive/healing (M2) response. These different activation states gave rise to the concept of macrophage activation or polarization (17). *In vivo*, macrophages activate in response to environmental stimuli, exhibiting marked plasticity and mixed responses (18, 19). Macrophages play such a fundamental role in resolving inflammation and promoting tissue healing that their depletion or exhaustion results in severely compromised wound healing and chronic inflammation (18, 20-22). Inflammation in arthritic joints is decreased by an M2-type response, which resolves inflammation and improves clinical and histological signs of experimentally-induced joint disease (13, 23). Classically activated (M1) macrophages from the osteoarthritic synovium impede *ex vivo* chondrogenesis of synovial progenitor cells (24). On the other hand, alternatively activated (M2) macrophages are required for efficient chondrogenesis (11). Collectively, these findings suggest that enhancing the M2 response in diseased joints may boost homeostatic mechanisms required for resolving joint inflammation and restoring a healthy and anabolic synovial environment.

The complexity of the inflammatory response culminating in joint degeneration presents a significant challenge in the treatment of OA. There is clear evidence that macrophages participate in both the initiation and resolution of joint inflammation and thus have become the target of significant research efforts to understand their roles in these opposing responses, with the long term goal of uncovering novel therapeutic strategies (6, 7, 9, 11, 24). There is a critical need for therapies that, instead of simply blocking inflammation, favor an inflammatory reaction

that more efficiently clears the pro-inflammatory triggers, to favor a path towards resolution (16, 20). Modern biologicals, such as mesenchymal stem cells (MSC), platelet rich plasma, and autologous conditioned serum, provide modest benefits (25-30). MSC act through modulation of macrophage activation and response (31). Notably, corticosteroids, routinely used in the treatment of OA, block the inflammatory reaction and are known to have detrimental effects on cartilage metabolism more damaging than simply chondrocyte quiescence. Corticosteroids antagonize common cellular pathways innately required for efficient tissue homeostasis and repair (e.g., IL-10, prostaglandin, and NF-kB) (32, 33). Developing approaches that carefully resolve synovial inflammation, preserving cellular and tissue mechanisms intrinsically involved in physiology, would mark key progress in the treatment of OA.

Bone marrow mononuclear cells (BMNC), a rich source of macrophage (>50% macrophages) and hematopoietic progenitors and stem cells (~30%), are readily isolated from bone marrow aspirates (34). Macrophages in bone marrow, by default, promote a pro-resolving response that counteracts the deleterious effects of inflammation and therefore are considered biased M2 progenitors (34, 35). Macrophages in BMNC resolve inflammation in association with increased production of IL-10 (33, 36), a key cytokine in chondrocyte metabolism and recovery from injury (37, 38). BMNC provide potent anti-inflammatory and pro-regenerative effects in injured tissues, including those with minimal regenerative capacity, such as the pancreas, central nervous system, and osteochondral defects (39-44). BMNC can also induce resolution of inflammation in chronically inflamed tissues refractory to other treatments, such as OA-affected joints and the respiratory system (33, 45). Macrophage progenitors are the primary cells in BMNC responsible for downregulating inflammation, while other hematopoietic and mesenchymal stem cells contribute, but to a lesser extent (34, 46). These homeostatic effects of

macrophages in BMNC suggest that increasing the numbers of these cells responsible for maintenance of joint health may provide therapeutic means for restoring joint homeostasis.

Despite their high therapeutic potential, the mechanisms by which macrophage progenitors may achieve a beneficial response to resolve synovitis are not yet fully elucidated. The aim of this study was to define how BMNC differentially respond to culture in normal and inflamed autologous synovial fluid to produce an anti-inflammatory effect. We hypothesized that although both normal and inflamed synovial fluid would induce BMNC to a homeostatic (M2) phenotype, the magnitude of response would be greater in inflamed synovial fluid.

METHODS

Eight adult Thoroughbred horses (3-9 years of age, median 5 years; 6 castrated males and 2 females) donated to the Veterinary Teaching Hospital for reasons unrelated to OA or severe systemic inflammation were used in this study under IACUC approval (#17-052) and oversight. Bone marrow was aspirated and a model of acute synovitis was used to produce inflamed synovial fluid so as to generate more homogeneous synovial fluid than could be acquired from naturally occurring cases with variable degrees of inflammation. Clinical, hematological, and orthopedic examinations, including radiographs, were performed to ensure general and musculoskeletal health. As detailed below, an *in vivo* model of synovitis was created by injection of LPS into each radiocarpal joint. Intact middle carpal joints were used as the source of normal synovial fluid (SF). After 8 hours, SF and inflamed synovial fluid (ISF) were harvested for use *in vitro*. BMNC were cultured in 100% SF or ISF and cells and conditioned media were assayed at 48 and 96 hours, and 6 and 10 days using flow cytometry and gene

expression assays to characterize the phenotypic response of BMNC. ELISA and a multiplex

bead-based immunoassay were used to quantify cytokine and growth factor production in the

conditioned media. Controls included fresh uncultured BMNC (baseline), and fresh SF and ISF.

Bone marrow aspiration and BMNC isolation

Bone marrow aspirates were performed immediately prior to induction of the synovitis

model to prevent potential changes in the myeloid niche as a consequence of LPS injection.

Horses were sedated and the sternum aseptically prepared and locally anesthetized. Bone

marrow aspirates (25 mL each; selected to minimize peripheral blood contamination (47) were

collected from the 4th and 5th sternebrae using an 8 gauge Komiyashiki needle and a heparinized

60 mL syringe (15,000 IU/aspirate) (47). In a laminar flow hood, bone marrow aspirate was

filtered (blood administration set), gently layered over Ficoll-Paque™ Plus (GE Healthcare Life

Sciences, St Louis, MO), and centrifuged (500g; 30 min; 4°C). The mononuclear cell layer on

top of the Ficoll ring were aspirated, transferred to a sterile 15 mL centrifuge tube, washed twice

in DPBS, centrifuged (300g; 10 min; 4°C), and resuspended ($4x10^4$ cells/µL DPBS).

Induction of synovitis and synovial fluid collection

Lipopolysaccharide (*E coli* strain 055:B55; Sigma-Aldrich, St. Louis, MO) was

reconstituted according manufacturer instructions in silanized vials using low retention pipette

tips (02-717-156; Fisher Scientific, Pittsburg, PA) to prevent LPS adsorption, and stored at -

20°C. Synovitis was induced by LPS injection (0.5 ng in 2 mL DPBS/radiocarpal joint; *E coli*

strain 055:B55; Sigma-Aldrich, St. Louis, MO) in both radiocarpal joints (48). After 8 hours (0

hour time point), when inflammation was expected to be at its peak, SF and ISF were collected

from middle and radiocarpal joints, respectively, using aseptic technique, and transferred to 50 mL polystyrene tubes (~12.5 mL/joint). From each joint, 0.5 mL of synovial fluid was subjected to cytology to confirm health of normal joints and that LPS-induced synovitis was effective. An additional 1 mL aliquot was reserved for flow cytometry to define the profile of macrophage marker expression in the synovial fluid of healthy and acutely inflamed joints. The remaining synovial fluid was then pooled according to its nature (normal or inflamed), mixed gently, and centrifuged (5,000g; 20 min; 4°C) for cell depletion (**Fig. 3-1**). The cell-free supernatant was carefully recovered and used for cell culture as growth media. Aliquots of SF and ISF (2 mL each) were reserved in Protein LoBind microfuge tubes for cytokine and growth factor quantification as baseline controls. For all procedures, and at all times, only low retention pipette tips were used to prevent analyte adsorption.

Cell culture

BMNC were plated in 24 well culture plates (2×10^6/50 µL DPBS/well) and wells brought to 500 µL using SF or ISF. Well contents, cells, and synovial fluid samples, were mixed by gentle repeated pipetting and plates were incubated at 37°C in 5% CO_2 and 90% humidity. Culture medium (SF or ISF) was preserved at 4°C. Every 48 hours, culture media were warmed to 37°C for 10 minutes and 200 µL was added to each well, according to treatment, in order to replenish cell nutrients. All conditions and time points were performed in duplicate and separate plates were used for each harvesting time point. Cells from one well were used for flow cytometry, while cells from the duplicate well were used for gene expression analysis. Conditioned culture medium from both wells was combined for cytokine and growth factor quantification. Micropictographs were taken at each time point to record cell morphology and
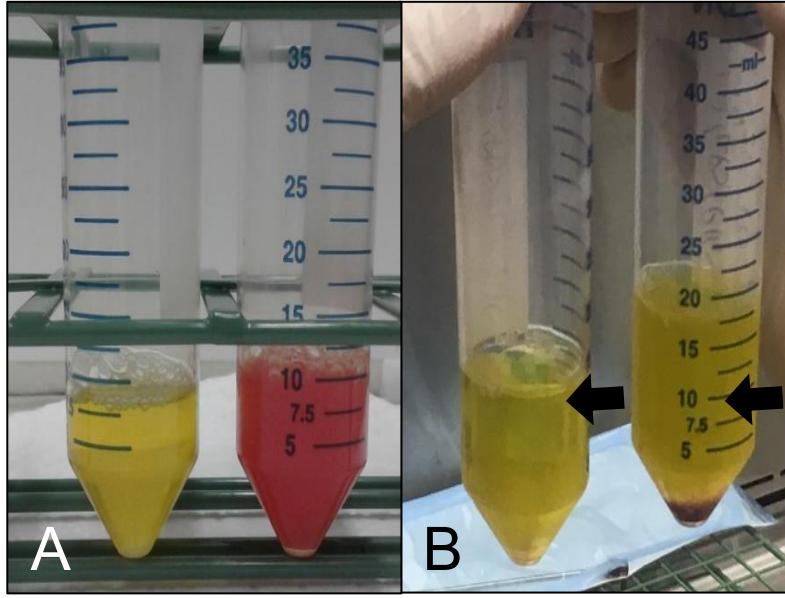
Figure 3-1: Normal (SF) and inflamed (ISF) autologous synovial fluid used as growth media. Synovial fluid was harvested from normal (A, *left*) and experimentally inflamed joints (A, *right*). Native cells from SF or ISF were depleted by centrifugation (B), and the cell-free supernatant (*black arrows*) was used as culture media. The cell pellet was subjected to flow cytometry to evaluate CD14, CD86, CD206, and IL-10 expression.

behavior (Infinity 2-2, 2.0 Megapixel USB 2.0 CCD, Ottawa, ON). Plastic adherence and changes in cell morphology and confluence were described and compared between groups and time points.

Flow cytometry

Following aspiration of conditioned media, 1 mL of ice-cold DPBS/10 mM EDTA was added to each well. Plates containing adherent cultured cells were placed on ice for 10 minutes to release cells, gently pipetted up and down to disperse, transferred to LoBind microfuge tubes,

centrifuged (1,500g; 5 min; 4°C), and washed with DPBS. Cells were fixed in 2%

paraformaldehyde at room temperature for 20 min, washed and stored in DPBS for up to 14 days

at 4°C for batch staining and cytometry. Staining solution included cell permeabilization buffer

(00-8333-56, eBioscience™; Santa Clara, CA) and the following primary antibodies: CD14 for

mature monocyte/macrophages (0.1:100; Wagner Lab, Cornell University, equine specific);

CD206 for M2 macrophages (0.02:100; mouse anti-human, COIM2741, 3.29B1.10 clone;

Beckman Coulter; Brea, CA); CD86 for M1 macrophages (3:100; mouse anti-human, 555666,

IT2.2 clone; BD Biosciences, Franklin Lakes, NJ); and IL-10 (0.25:100; Wagner Lab; Cornell

University). Markers were selected based on the literature (CD14 (49-53), CD 86 (54-57),

CD206 (58-63) and IL-10 (37, 64-66)) and specificity or validated cross reactivity to equine

samples (67-71). Gates were set and auto fluorescence compensated using unstained cells. All

antibodies were validated with positive and negative controls using cells known to express such

markers or not. Antibody dilutions were determined by individual titration assays (70).

Gene expression

Steady-state mRNA levels for 27 targeted gene loci was determined using data from an

ongoing RNA-sequencing project. Gene loci were selected based on established annotation to

represent commonly used markers of inflammatory M1 (CD86, TNF-α, IL-1α, IL-1β, IL-6,

IDO1, STAT1, STAT4, CXCR4 and SDF-1), regulatory M2 (CD206, IL-10, IL-4, IL-4r,

PTGS2, IGF-1, TGF- β1, GPR86, EGR2, CXCL14, STAT3 and STAT6), and hematopoietic

progenitors or naïve unpolarized macrophages (MØ) (CD34, CD14, CD68 and CSF-1), or involved in cell adhesion and membrane reorganization (CD44)(7, 57, 62, 63, 72-74).

RNA isolation

Cells were recovered in ice-cold PBS/10 mM EDTA and centrifuged in RNAse-free Protein LoBind microfuge tubes (Eppendorf®, #13-698-79) (12,000g; 10 min; 4°C). BMNC pellets were harvested into guanidinium chloride-phenol (Trizol®, Life Technologies, 15596018, Carlsbad, CA) and stored at -80°C. Following homogenization by pipetting, RNA was isolated by column purification with on-column DNase digest per manufacturer instructions (DirectZol™ RNA microprep kit, R2061, Zymo Research, Irvine, CA), quantified (Qubit® 3.0 Fluorometer, 33216, ThermoFisher Scientific, Carlsbad, CA), and stored at -80°C. RNA quality was assessed (Bioanalyzer 2100, Agilent Technologies, Santa Clara, CA) at the Roy J. Carver Biotechnology Center (University of Illinois, Urbana, IL).

RNA sequencing and analysis

RNA-seq libraries were prepared by the High-Throughput Sequencing and Genotyping Unit at the Roy J. Carver Biotechnology Center using TruSeq DNA Library Preparation kits (Illumina, Inc., San Diego, CA), followed by sequencing (NovaSeq 6000 S4, Illumina) to generate stranded paired-end reads (2 x 150 nt). Briefly, mRNA was fragmented and converted to first strand cDNA. cDNA fragments underwent end repair and ligation of adapters, and were indexed individually. Libraries of cDNA were sequenced to obtain a minimum of 24 million reads per sample. Sequencing reads were mapped to the equine reference genome (EquCab 3.0) (75) with the software MapSplice3 (76) and expression determined using Cufflinks 2.2.1

(Trapnell Lab, University of Washington, Seattle, WA) (77) as fragments per kilobase of transcript per million mapped reads (FPKM). FPKM were used to determine fold changes in gene expression of cells cultured in SF and ISF in relationship to fresh BMNC, and significant differences (false discovery rate) between conditions at each time point. Genes for which transcripts were identified to have a fold change > 2 and a p-value < 0.05 were categorized as differentially expressed genes between normal and inflamed synovial fluid cultures.

Cytokine and growth factor quantification

Conditioned media were aspirated and deposited in Protein LoBind microfuge tubes and centrifuged (10,000g; 30 min; 4°C). The supernatant was gently recovered for cytokine and growth factor quantification and preserved at -80°C. The few cells in the formed pellet were reserved to add to those recovered for flow cytometry. Baseline controls (SF and ISF) and conditioned media were assayed for growth factors and anti- and pro-inflammatory cytokines and chemokines (FGF-2, GM-CSF, IL-1B, IL-6, MCP-1, IL-10, TNF-A, SDF-1, IGF-1, IL-1ra, and PGE$_2$) using a commercial PGE$_2$ ELISA kit (Parameter Test, KGE004B, RnD Systems, Minneapolis, MN; SpectraMax M5 plate reader; Molecular Devices, San Jose, CA) and an equine multiplex bead-based immunoassay for the remaining analytes (EQCTTMAG-93K; Milliplex MAP Equine chemokine/cytokine; Luminex 200 plate reader; Millipore; St. Louis, MO). Modifications were made by the manufacturer to include IGF-1, SDF-1, and IL-1ra, and to validate inclusion of one additional point at the lower end of the standard curve to maximize detection of low analyte concentrations. Synovial fluid samples were thawed and digested with 10 uL hyaluronidase solution (100 IU testicular hyaluronidase/mL acetate buffer; Worthington # LS005474) in 200 µL of synovial fluid and incubated for 30 minutes at 37°C.

Samples were then centrifuged (12,000g; 10 min; 4°C) to remove any particulate matter, and the supernatant recovered (Protein LoBind tubes). Based on our previous experience, and limitations caused by interfering factors in cytokine detection in synovial fluid (78, 79), spike-and-recovery assays were performed for the PGE_2 ELISA and 4 representative serially-diluted targets in the bead-based assay (IL-1β, IL-6, IL-10, TNF-α). Based on the results, a dilution of 1:2 was selected for PGE_2 quantification and no dilution was deemed necessary for the bead-based assay. Following hyaluronidase digestion, PGE_2 samples were subjected to solid-phase extraction. Synovial fluid (500 µL) was mixed with 500 µL of 100% reagent ethanol and 10 µL glacial acetic acid, the mixture homogenized and incubated at room temperature for 5 min, centrifuged (2,500g; 8 min; room temperature), and the supernatant harvested and assayed. Remaining analytes were quantified using hyaluronidase-digested samples without additional processing.

Statistical Analysis

Sample size (N=8) was determined using estimated 2-fold differences in expected gene expression and SD=0.5 based on gene expression and flow cytometry from preliminary data (α=0.05; β=0.80). Data analysis was performed using General Estimating Equations (GEE) to assess the effects of different culture media, as well as the effects of time. Least square means adjustments for multiple comparisons were performed using the Tukey-Kramer test. All analyses were performed using commercial software (SAS version 9.4, SAS Institute, Inc, Cary, NC). Significance was set at $P<0.05$.

RESULTS

Cell culture behavior

BMNC cultured in both SF and ISF developed into macrophage-rich cell populations. However, cell morphology, distribution, and behavior differed consistently between growth media. At 48 hours, cells cultured in the thick, mucin-rich SF tended to remain in suspension, while BMNC cultured in less viscous ISF settled to the bottom of culture wells (**Fig. 3-2**). Although no plastic adherent cells were observed at 48 hours, changes in shape (from round to piriform cells) and size (large round cells) were noted and were more marked in cells cultured in ISF. At 96 hours, cells in both SF and ISF were settled to the bottom of the wells, but with a minimal percentage of plastic adherence. Non-adherent cells remained heterogeneous in shape and size. A small proportion of cells looked crenate in both SF and ISF. At 6 days, most cells in SF (~ 75%) remained unchanged compared to 96 hours. Plastic adherence was slightly higher, with most adherent cells displaying a flat, irregularly round macrophage shape. At 6 days, BMNC cultured in ISF were 60-70% confluent with typical macrophage morphology, compared to <20% confluence in SF. A substantial number of clumps of non-adherent cells were seen in both groups. These cells were refractile and looked as though they could be dividing cells. Crenated cells began to disappear at 6 days. At 10 days, a minimal number of cells were not plastic adherent in SF, and most crenate cells had been cleared by phagocytosis and could be seen inside larger macrophages (efferocytosis). In ISF, a high rate of cell proliferation was observed, shown by increasing numbers of plastic adherent and non-adherent cells, with clumps of very refractile non-adherent cells scattered over a confluent macrophage culture. At 10 days, BMNC grown in ISF were more confluent (~100%) than BMNC grown in in SF (~25%).

| | 48 hrs | 96 hrs | 6 days | 10 days |
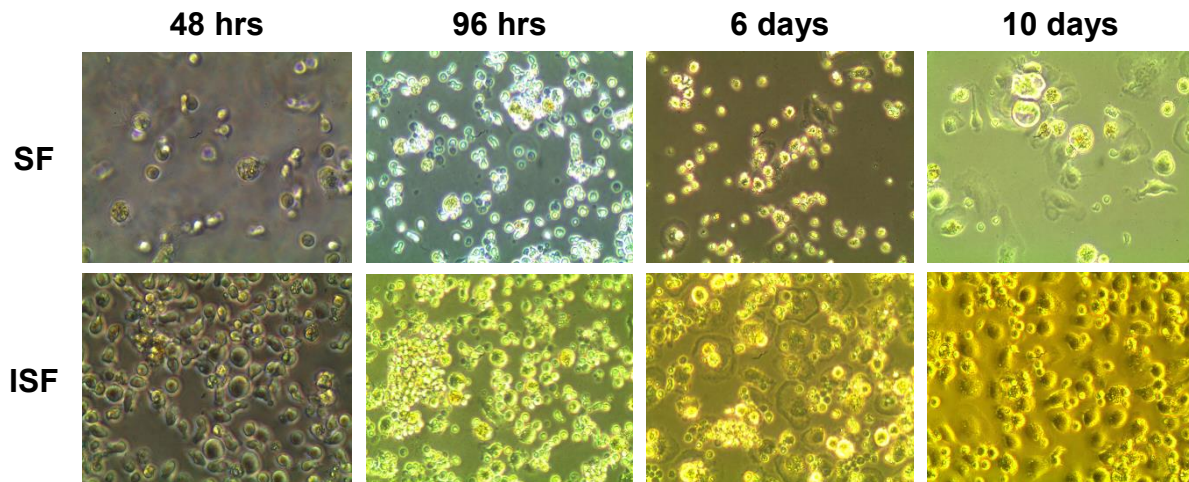
Figure 3-2: Equine BMNC cultured in autologous normal (SF) and inflamed synovial fluid (ISF) for up to 10 days (*100x magnification at 48 hrs; 40 x magnification at 96 hours and 6 and 10 days*). BMNC developed into macrophage-rich cell populations in both culture media. Cell morphology, distribution, and behavior differed consistently with growth media. Plastic adherence was evident after 6 days and was more marked in ISF. Cell proliferation and monolayer confluence at 10 days were higher in ISF (~100%) as compared to SF (~25%).

Flow cytometry

Freshly isolated BMNC contained low numbers of cells expressing CD14 (13%), CD86 (13%), and CD206 (7%), yet 82% of all cells were IL-10$^+$ (**Fig. 3-3A, left**). Native cells from uncultured normal synovial fluid expressed CD14, CD86, CD206, and IL-10 at moderate levels, with similar numbers of macrophages (CD14$^+$; 67%) and IL-10$^+$ cells (65%; **Fig. 3-3A, middle**). However, for cells isolated from inflamed synovial fluid, where neutrophils predominated (89%) following LPS injection, this relationship between CD14$^+$ and IL-10$^+$ cells was not observed (**Fig. 3A, right**). Culture of BMNC in both SF and ISF altered expression of all markers. Following 48 hours of culture, expression of CD14 (mature macrophages) dramatically and

significantly increased in SF (59%; P=0.0013) and ISF (71%; P<0.0001) compared to fresh BMNC. These values remained significantly increased over the course of the 10 days in cultures of both SF and ISF (P<0.009 for all). On average, CD14 expression was 6-12% higher in cells grown in ISF compared to SF; however, this difference was only significant at 96 hours (P=0.0064; **Fig. 3-3B**). After 48 hours in culture, expression of IL-10 increased in both SF (86%; P=1.0000) and ISF (88%; P=0.0141) compared to fresh BMNC and was significantly higher in ISF compared to SF (P=0.0289). Although most cells in SF (71%) and ISF (68%) cultures were still IL-10$^+$ after 10 days, expression of IL-10 significantly decreased over time in culture in both SF and ISF compared to 48 hours (P<0.001 for all). Expression of CD86 increased significantly by 48 hours in cells cultured in both SF and ISF compared to BMNC and remained increased throughout the 10 days of culture ($P \leq 0.0006$ for all). On average, CD86 expression was 5-7% higher in cells cultured in ISF compared to SF; however, this difference was only significant at 96 hours (P<0.0001). CD206 expression increased by 48 hours in cells cultured in both SF and ISF compared to BMNC and remained increased throughout the 10 days of culture. This increase was significant for all time points ($P \leq 0.0001$) except SF at 48 hours (P=0.0714). At 48 hours, CD206 expression was 11% higher in cells grown in ISF compared to SF; however, there were no significant differences in CD206 expression between cells grown in SF and ISF at any time points. Overall, expression of all markers was higher in ISF than in SF. Despite some early differences between SF and ISF, by 10 days in culture, expression values of all markers were similar for BMNC grown in SF and ISF and had developed a comparable expression profile to that of the native cells from normal synovial fluid. No differences were observed in the mean fluorescence intensity expressed for CD86 and CD206 for any experimental conditions.
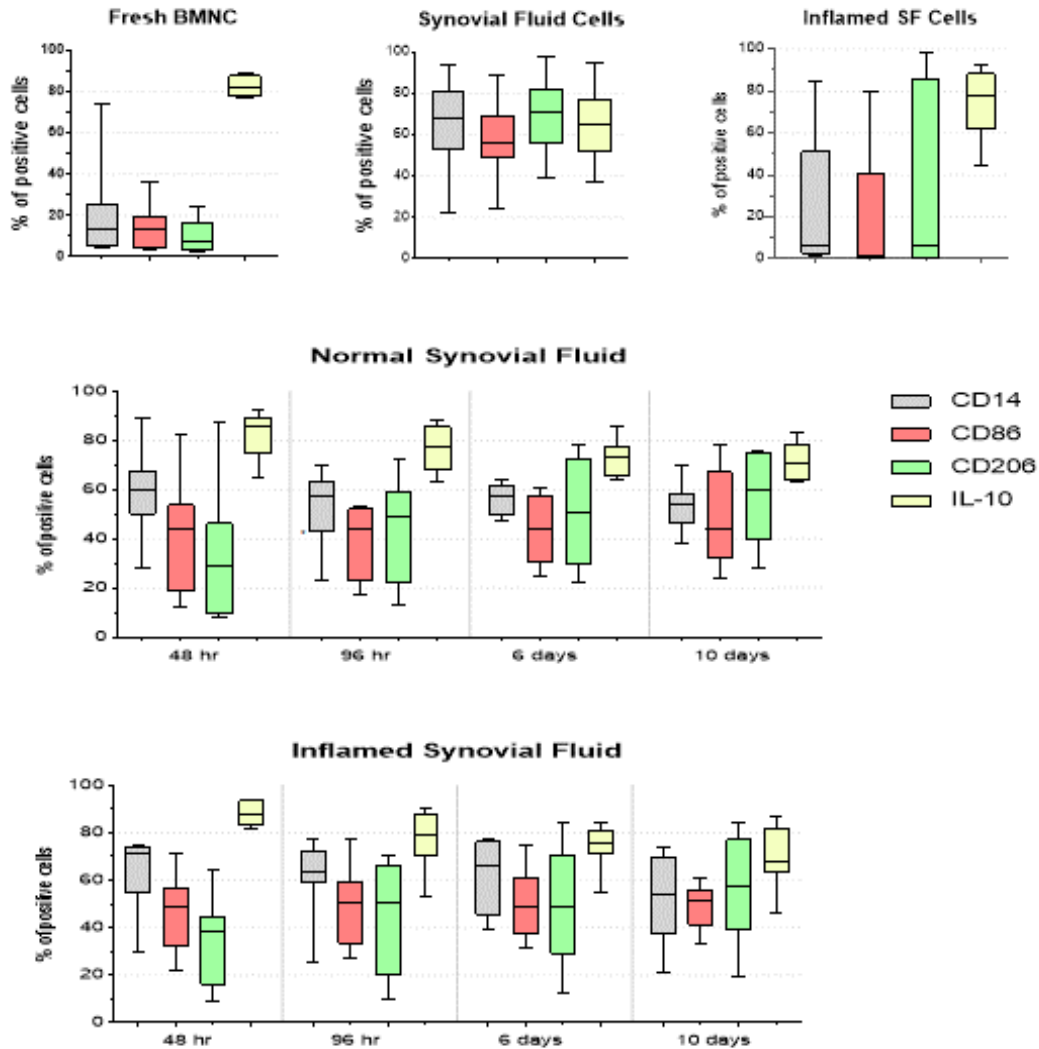
Figure 3-3: *(A)* Flow cytometry from fresh BMNC and native cells from normal (SF) and inflamed synovial fluid (ISF). In fresh BMNC (left), only 13% of cells were positive for the mature macrophage marker CD14$^+$, yet 83% were IL-10$^+$. In native cells from normal synovial fluid (center), there was a correlation between baseline numbers of macrophages (67%) and IL-10$^+$ cells (65%), not observed in cells native from inflamed synovial fluid. *(B)* Flow cytometry from BMNC cultured in autologous SF and ISF. At 48 hours, macrophages (CD14$^+$) in SF and ISF dramatically increased (P<0.001 for both). Also, at 48 hours, expression of CD14 and CD206 was higher (12% and 10%, respectively) in ISF than SF. However, it was at 96 hours when expression of CD14 (P=0.0064) and CD86 (P=0.0001) were significantly higher in ISF. At 10 days, expression of all markers was similar between SF and ISF and both were comparable to that of cells native to normal synovial fluid.

Gene expression

Of the 27 genes analyzed, 12 were identified as upregulated on an overall basis following culture in both SF and ISF as compared to fresh, uncultured BMNC ($P<0.0001$ for all; **Fig. 3-4**). Amongst those, are 7 of 11 genes generally used as M2 markers, 4 of 10 genes considered M1 markers, and 1 gene corresponding to a growth factor essential to chondrocyte homeostasis (IGF-1). Of the 12 upregulated genes, expression of 7 genes significantly differed between BMNC cultured in SF and ISF (**Fig. 3-4**). Levels of mRNA for the early naïve macrophage marker CD68, and M2 markers, including CD206, EGR2, CXCL14, and GPR86, did not significantly differ between cells grown in SF or ISF. Significantly higher read counts were observed for the M2 marker IL-10 ($P=0.0011$) and the growth factor IGF-1 ($P=0.0011$) for cells cultured in SF. Transcript levels significantly higher in ISF cultures included the pan macrophage marker CD14 ($P<0.0001$), the M2 proliferation-related genes IL-4r ($P<0.0001$), and CSF-1 ($P=0.0129$), as well as the M1 markers CD86 ($P=0.0309$) and IL-1α ($P=0.0008$). Other gene loci, including PTGS2, TGF-β1, STAT3, and STAT6, displayed reduced levels of mRNA in SF and ISF cultures. Interestingly, IL-1β was only insignificantly upregulated at 48 hours in both SF and ISF. In summary, as reflected by the heatmap, genes traditionally proposed as markers of homeostatic M2 macrophage markers displayed increased levels of mRNA compared to those traditionally considered M1 markers, in both SF and ISF cultures. No expression for IL-4 was detected.

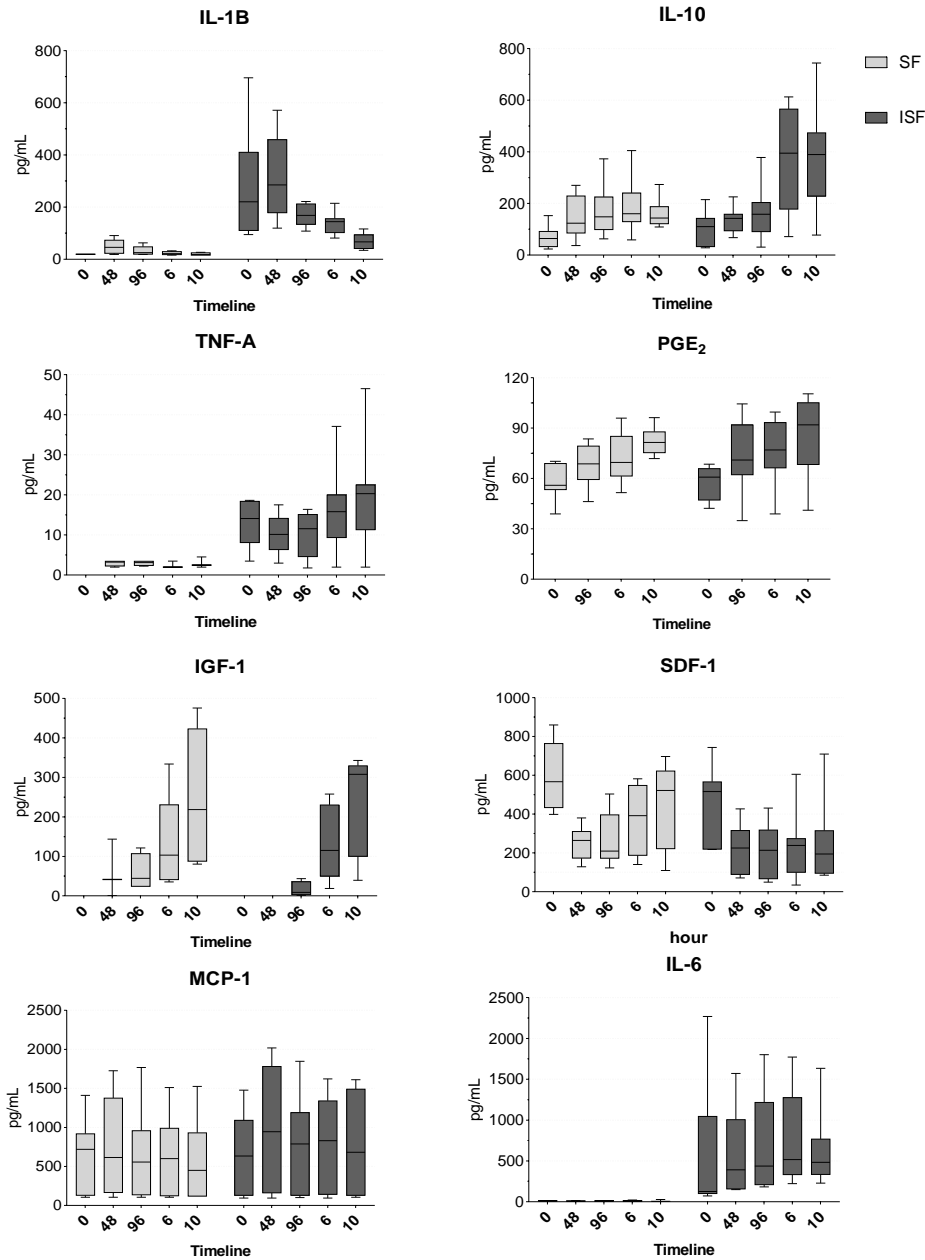**Cytokine and growth factor quantification**

Concentrations were within detectable limits for 9 of the 11 analytes assayed. MCP-1, SDF-1, and IL-10 were detectable in all samples. PGE$_2$ was equally detectable in samples from both SF (29/38) and ISF cultures (29/38). IL-1β and IL-6 were detectable in all ISF and 21 and
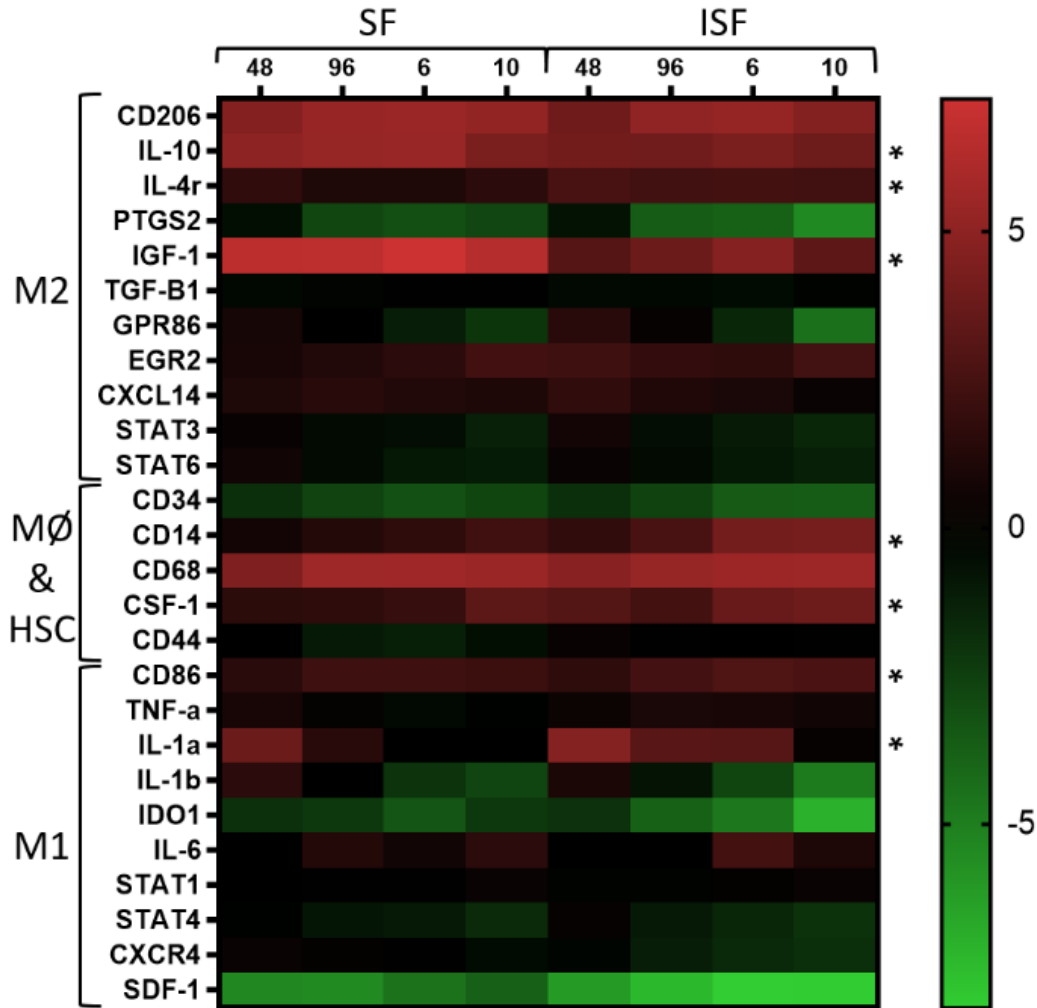
Figure 3-4: Differential expression of genes regularly assessed as markers of macrophage polarization in BMNC cultured in autologous normal (SF) and inflamed synovial fluid (ISF) over 10 days. The heatmap shows Log_2 fold changes of up- and down-regulated transcripts. Gene expression profiles of BMNC cultured in SF or ISF are shown in rows. Color toward red indicates increased gene expression and color toward green indicates decreased expression as compared with uncultured BMNC. Genes traditionally proposed as markers of homeostatic M2 macrophage were more frequently upregulated and in higher magnitude than those traditionally considered M1 markers, in both SF and ISF cultures. Asterisks (*) on the right side of the heatmap depict significant overall p-values ($p \leq 0.05$).

29 of 38 SF samples, respectively. TNF-α was detected in only 15/38 samples from SF cultures, but in 37/38 samples from ISF cultures. IGF-1 was detected in 17/38 SF and 15/38 ISF cultures, and was most commonly detectable at 6 and 10 days. IL-1ra was detected in only 6 samples (3 SF and 3 ISF) and only at 48 and 96 hours, precluding statistical analysis. The remaining analytes were below detectable limits for all samples (FGF-2, 11.5 pg/mL; GM-CSF, 3.7 pg/mL).

At the time of synovial fluid collection from the horses (0 hour), IL-1β ($P=0.1360$) and IL-6 ($P=0.1331$) concentrations were higher in ISF compared to SF, but failed to reach significance. TNF-α concentrations were significantly higher in ISF than SF ($P=0.0002$). At all other time points, concentrations were significantly higher in ISF cultures compared to SF cultures for IL-1β ($P<0.0001$ for all), IL-6 ($P\leq0.003$ for all), and TNF-α ($P\leq0.0005$ for all; **Fig. 3-5**). IL-1β initially increased in both SF ($P=0.0275$) and ISF ($P=0.8818$), then progressively decreased from 48 hours in culture in both SF (6 days [$P=0.0432$] and 10 days [$P=0.0376$]) and ISF ($P\leq0.01$ for all comparisons). In SF, TNF-α increased from 0 to 48 ($P=0.0313$) and 96 hours ($P=0.0309$) and then remained steady, whereas in ISF, TNF-α remained elevated without significant change. In SF, IL-6 remained at low concentrations; however, in ISF, IL-6 increased significantly from 48 hours to 96 hours ($P=0.00190$) and 6 days ($P<0.0001$) and from 96 hours to 6 days ($P=0.0012$). By 10 days, IL-6 had decreased in ISF, though not significantly. IL-10 was significantly higher in ISF compared to SF at 6 ($P=0.0108$) and 10 days ($P=0.0028$) and generally increased over time in culture in both SF ($P<0.05$ for all comparisons) and ISF ($P<0.05$ for all comparisons except 48 hours to 10 days where $P=0.0852$). IGF-1 generally increased over time in culture in both SF ($P<0.04$ from 0 and 48 hours to 10 days) and ISF ($P<0.04$ for all comparisons) and was higher in ISF at 10 days; however, not significantly. SDF-1 decreased

Figure 3-5: Cytokines and growth factor concentrations in normal (SF) and inflamed (ISF) conditioned synovial fluid at 0, 48, and 96 hours, and 6 and 10 days. IL-1β, IL-6, and TNF-α were significantly higher in ISF at all time points following culture. While IL-1β progressively decreased, TNF-α and IL-6 tended to increase overtime. At 6 days, IL-10 was significantly higher in ISF, suggesting a homeostatic effect. PGE$_2$ and IGF-1 did not significantly differ between SF and ISF at any time point. SDF-1 was similar between SF and ISF at baseline and 48 hours, but significantly increased at all remaining time points in SF. Finally, MCP-1 was only significantly higher in ISF at 96 hours.

significantly in SF from baseline to 96 hours ($P<0.01$ for all comparisons) and then increased

significantly from 6 to 10 days ($P<0.04$ for all comparisons). In ISF, SDF-1 decreased

significantly from baseline to 96 hours ($P<0.008$ for both comparisons) and then was constant.

There was no difference in SDF-1 between SF and ISF at baseline ($P=0.0971$) and 48 hours

($P=0.0617$); however, SDF-1 concentrations in SF recovered sufficiently to be significantly

higher than those in ISF at 6 ($P=0.0213$) and 10 days ($P=0.0031$). MCP-1 concentrations were

higher in ISF compared to SF at 48 ($P=0.0373$) and 96 hours ($P=0.0147$), and 6 ($P=0.0175$) and

10 days ($P=0.1197$). PGE_2 concentrations did not significantly differ between SF and ISF at any

time point. However, PGE_2 concentrations in SF at 10 days was increased significantly from

baseline ($P=0.0003$), while in ISF it significantly increased from baseline at all time points

($P<0.005$).

DISCUSSION

Measurable immune responses developed from macrophage-rich populations following

culture of BMNC in both SF and ISF. This is the first study investigating cell culture in 100%

autologous synovial fluid, without introducing artifacts from typical *in vitro* models, such as the

addition of xenogeneic (fetal bovine) or allogeneic (horse) serum, known to artificially induce

macrophage proliferation and a dominant, nonspecific immune response (80, 81). Modelling the

response of BMNC to normal and inflamed synovial fluid, as performed in our study, produces

an *in vitro* environment that most closely mimics that to which BMNC or other cell therapies

would be exposed following joint injection. Our combined findings demonstrate that BMNC

cultured in both SF or ISF ultimately exhibited macrophage profiles similar to that of cells native

to synovial fluid from healthy joints, displaying a homeostatic response that was proportional to inflammation and characterized by increasing counts of IL-10-expressing macrophages, decreasing concentrations of IL-1β, and increasing concentrations of IL-10 and IGF-1. All of these changes were more marked in ISF. Both IL-10 and IGF-1 play vital roles in chondrocyte metabolism and recovery from injury, as well as repair and homeostasis of synovial tissues (37, 38, 64, 82-84). In fact, our overall findings *in vitro* are similar to those observed in our *in vivo* model of equine synovitis, in which joint injection with BMNC dramatically reduced gross and analytical markers of inflammation, in association with increasing counts of IL-10^{+} macrophages and synovial fluid concentrations of IL-10 (85). Combined, these findings explain, at least in part, the benefits BMNC produce in the treatment of OA (45).

The regulatory response of BMNC leading to significantly higher concentrations of IL-10 in ISF is also consistent with findings from horses with chronic airway inflammation treated by tracheal infusion of BMNC. Broncho-alveolar lavage fluid of these horses contained significantly higher macrophage counts and concentrations of IL-10 compared to those treated with dexamethasone, although reduction of airway inflammation was similar between groups (33). Similar anti-inflammatory effects were observed in the treatment of a murine model of airway inflammation, and these effects were abolished when BMNC were depleted of macrophages prior to treatment (34). Combined, these studies reinforce the important role of IL-10-producing macrophages from BMNC in resolving inflammation (20). In agreement, marked increases in ex-vivo IL-10 production by LPS-challenged macrophages are also associated with a protective effect against the development and progression of human OA (86). Although mRNA transcripts for IL-10 were higher in BMNC cultured in SF, the markedly higher proliferation of IL-10-producing cells in ISF explains the higher IL-10 concentrations in ISF at 6 and 10 days.

Together, these findings reinforce the benefits of increasing counts of IL-10-producing macrophages in inflamed joints through BMNC therapy, maximizing local production of IL-10 and related anti-inflammatory effects in joints (45).

The higher concentrations of IL-1β, IL-6, and TNF-α in conditioned ISF are expected given the context of acute synovitis from which the fluid was obtained. The production of these cytokines increases to signal joint damage, and if uncontrolled, are known activators of enzymes leading to cartilage breakdown, such as MMP-3, MMP-13, and ADAMTS5 (87-90). Although IL-10 is an anti-inflammatory cytokine, following injury its secretion also increases proportionate to damage as a negative feedback to limit the production of inflammatory cytokines (86, 91). As such, in our study, both gene expression and protein content for IL-1β peaked at 48 hours and markedly decreased proportionate to increases in IL-10. However, if the inflammatory stimulus persists, this feedback loop of pro- and anti-inflammatory cytokines is sustained, as demonstrated in our study by the continuous increases in gene expression and ISF concentrations of TNF-α, IL-6, and consequently IL-10, likely from continuously challenging BMNC by replenishing inflammatory culture medium (ISF) every 48 hours (91).

Increasing concentrations of IL-6 in ISF can be interpreted in different ways. Although IL-6 is classically known as a pro-inflammatory cytokine, there is substantial evidence that it plays both anti- and pro-inflammatory roles (92, 93). Early studies showed that IL-6 knockout mice are prone to development of inflammatory diseases and have increased neutrophilia and concentrations of TNF-α, macrophage inflammatory protein-2 α, GM-CSF, and interferon γ, in both local and systemic inflammatory responses (94, 95). Deficiency of the IL-6 receptor induces M1-like macrophage polarization in several tissues. IL-6 is required to prime macrophages for IL-4-mediated M2 activation by inducing expression of its receptor IL-4r (93).

IL-4 is involved in the signaling pathway leading to homeostatic functions in macrophages (96, 97). In our study, gene expression for IL-4r was upregulated both in SF and ISF cultures, but almost twice as much in ISF. Increased IL-6, associated with increased gene expression for IL-4r, and the overall findings of our study are in agreement with previous reports of the role of IL-6 in driving macrophages towards a homeostatic phenotype (92).

TNF-α has traditionally been associated with several types of arthritis, with defined roles in accelerating cartilage destruction through activating matrix metalloproteinases and aggrecanases (ADAMTS) (98). However, TNF-α also plays critical roles in both inciting and resolving inflammation (99, 100). TNF-α is involved in localizing harmful agents and amplifying local and systemic immune responses, and as observed in our study, coordinates myeloid cell proliferation to attend increased demands during recovery of inflammation-derived damage and tissue repair. These processes also involve other effects of TNF-α, such as regulation of apoptosis and efferocytosis (99, 100). In mouse models, TNF-α alone is not destructive and exerts its arthritogenic potential through IL-1 induction (101). As such, induction of arthritis in TNF-α deficient mice does not produce reduction in clinical, histological, and morphological joint abnormalities, suggesting that other cytokines, such as IL-1, may play a higher role in disease processes (86).

Despite the proposed central role of IL-1β as a main driver of OA (87, 89, 102), recent proteomic analyses of synovial fluid and genome-wide transcriptomic analysis of cartilage, comparing samples from OA and healthy joints, did not identify IL-1 (α or β) or TNF-α as central targets (103, 104). As a matter of fact, gene expression for IL-1β in our study was downregulated, particularly in ISF cultures. Therefore, higher ISF concentrations of IL-1β may be the result of accumulated cytokine produced *in vivo* prior to cell culture. On the other hand,

gene expression for IL-1α in our study was increased at 48 hours, more so in ISF, decreasing to baseline in both SF and ISF. Although these changes did not coincide with increases in IL-10, they were coincident with increases in IGF-1. Since culture of BMNC in SF does not represent an inflammatory environment, changes in gene expression for IL-1α and protein expression of IL-1β in SF cultures could result from cell stress induced by bone marrow harvesting, BMNC isolation, and *in vitro* culture (105). While early studies suggesting IL-1 as a central driver of OA emphasized the role of IL-1α (not IL-1β) (106, 107), we were not able to detect IL-1α in our validation/screening assay (minimum detection level 23.87 pg/mL) and therefore did not includ it in our custom designed kit.

MCP-1 is constitutively expressed by synovial macrophages and reported to increase in inflamed synovium in association with increased expression of classic pro- (IL-1β, IL-6) and anti-inflammatory (IL-4, IL-10) cytokines, as well as cartilage degradation (108-110). During synovial inflammation, MCP-1 contributes to recruitment, homing, and accumulation of mononuclear cells in the synovial fluid and membrane (synovial lining hyperplasia) (22), which is part of the homeostatic response against joint damage (12). As a consequence, MCP-1 deficient mice are unable to home macrophages to sites of injury and were therefore prone to infection and chronic inflammation (22, 110), demonstrating that MCP-1-driven macrophage chemotaxis is crucial for counteracting inflammation and promoting tissue homeostasis. The significantly increased concentrations of MCP-1 in conditioned ISF at all time points after culture may have contributed to the development of more prolific macrophage cultures in ISF.

In our study, higher SDF-1 concentrations in conditioned SF differs from previous reports (111, 112). SDF-1 is reported to be expressed proportionally to inflammation or disease activity, with higher concentrations in inflamed joints (111, 112). However, SDF-1 has

multifaceted roles in cartilage biology, including both homeostatic and pro-inflammatory functions (111-113). Our results showing lower SDF-1 concentrations in conditioned ISF are comparable to an additional study from our lab, where comparisons between normal and inflamed or osteoarthritic equine joints revealed higher SDF-1 concentrations in synovial fluid from normal joints (85). Even though SDF-1 gene expression was overall downregulated in relationship to fresh BMNC, it progressively increased in SF cultures after 6 days, while progressively decreasing in ISF, following the same pattern of protein expression. In injured tissues, SDF-1 is released from the injury site creating a concentration gradient that acts as a homing signal to circulating monocytes and hematopoietic stem cells, and guides their differentiation into macrophages (114, 115). Lower SDF-1 in acute and chronic synovial inflammation could suggest that mononuclear cell recruitment has already happened, which in this *in vitro* study is represented by introduction of BMNC to the culture systems, leading to marked decrease in SDF-1 production in both SF and ISF cultures at 48 hours as a negative feedback. Similarly, increased cell proliferation in ISF may have sustained this negative feedback keeping the SDF-1 gradient silent, while the reduction of cells in SF cultures due to lack of inflammatory/proliferative stimuli combined with efferocytosis may have re-activated the SDF-1 gradient to recover baseline concentrations (116, 117). These observations related to SDF-1 and the increased MCP-1 expression in ISF at 96 hours could be interrelated.

The current understanding that PGE_2 plays anti-inflammatory and anabolic roles, including inhibition of inflammatory cytokines, chondrocyte protection, and activation of pro-resolving macrophages (20, 118, 119), had replaced the long lasting concept of PGE_2 as a marker of joint inflammation (62, 120). In fact, PGE_2 generated during the early stages of the inflammatory response can induce resolution of inflammation by upregulating the synthesis of

other classes of bioactive lipids such as the specialized pro-resolving molecules (resolvins, maresins, and protectins), potent mediators of inflammation resolution and tissue repair (20). Increasing concentrations of PGE_2 in both SF and ISF cultures could result from phagocytic clearance of dying cells and metabolization of arachidonic acid, especially considering the absence of inflammatory or damage-associated triggers in SF. Insignificantly higher PGE_2 concentrations in ISF is likely an effect of higher cell density. Although measuring other oxylipids, such as the specialized pro-resolving molecules, could have provided information regarding this homeostatic response, their quantification was not part of the original scope of our study (16, 121). Nonetheless, the bone marrow niche is the richest source of these pro-resolving lipids and could have contributed to the outcomes observed in our study (16, 121-123).

Another exciting finding in our study was the increasing concentrations of IGF-1, ultimately higher in conditioned ISF. Similar to IL-10, even though IGF-1 gene expression was obviously higher in SF cultures at all times, the markedly higher cell proliferation in ISF cultures is likely the cause of higher concentrations of IGF-1 in conditioned ISF at 6 and 10 days. IGF-1 protects proteoglycans in cartilage from IL-1β- and TNF-α-induced breakdown, induces increased synthesis of type II collagen, hyaluronan, and proteoglycan in chondrocyte cultures, and most importantly improves the ability of transplanted chondrocytes to repair osteochondral lesions *in vivo* (82-84). Combined, these observations highlight another mechanism by which BMNC can provide benefits as a joint therapy.

Increased cell proliferation in ISF cultures is likely a combined effect of the cytokines analyzed. A defining characteristic of inflammation is the accumulation of innate immune cells in the tissue, through proliferation of both tissue-resident and bone marrow-derived macrophages. Primarily, tissue macrophages undergo rapid *in situ* proliferation, but monocyte

progenitors can also be recruited from the blood stream. This is a key feature of the inflammatory process in order to increase population density, and have major implications for the mechanisms underlying a pro-resolving response (96, 100, 124), in agreement with observations from this *in vitro* and our previous *in vivo* study (85). IL-4 signaling is a key component of regulatory inflammation and can induce macrophage proliferation, enough to drive accumulation of tissue macrophages through self-renewal without a destructive inflammatory response. Both IL-4R-dependent and -independent mechanisms contribute to macrophage proliferation (12, 96, 125). Macrophage self-renewal in most tissues also relies on the CSF-1 receptor (CSF1R), and CSF-1 is required for both steady state maintenance of resident macrophages and recovery of the resident population by proliferative expansion following an inflammatory response (126). IL-4R-independent proliferation is controlled by a rise in local CSF-1 concentrations (125). Gene expression for both IL-4r and CSF-1 were upregulated in both SF and ISF cultures and were more marked in ISF cultures, in agreement with observed cell proliferation, and is likely associated with the IL-6-driven activation of the IL-4r promoter.

The regulatory response of BMNC was also demonstrated by flow cytometry, where BMNC cultured in both SF and ISF ultimately developed equivalent profiles to that seen in native cells from normal synovial fluid. Although this was only observed at 10 days, it was at 48 hours that intracellular expression of IL-10 was significantly higher in BMNC cultured in ISF, showing an early response to the inflammatory insult. In contrast to the more clearly defined macrophage phenotypes proposed by most *in vitro* studies (54, 61, 62, 127), defining macrophage phenotypes *in vivo* is challenging. *In vivo,* macrophages exist in a hybrid state, oscillating surface marker expression (and thus nuances of phenotypes) according to stimuli and stage of inflammation (18, 24, 128). The concept of macrophage activation as either

inflammatory (M1) or suppressive/healing (M2) originated from monocyte-derived macrophages treated in cultures systems with standard culture medium, FBS and defined and overwhelming cytokine stimuli, which does not represent *in vivo* conditions at all (62, 63, 72, 127). Our findings of macrophage marker expression *in vitro* match those observed following BMNC injection into joints, and broadly agree with *in vivo* studies regarding the expression of mixed features of so-called macrophage phenotypes, showing an overall regulatory response (18, 35, 128). Collectively, these findings suggest that events translating immune responses, as well as cell therapies aimed at clinical use, should not be primarily investigated in standard cell culture systems, but in systems similar to the one used in our study where the native body fluid is used as the growth medium.

Interpreting CD86 and CD206 expression in macrophages requires attention to the functions of these receptors, rather than simply considering them as classical M1 and M2 markers (7, 24, 127). Although CD86 expression has a relevant role in the development of arthritis and has become a therapeutic target (56, 57), it is constitutively expressed at low levels in resting macrophages and circulating monocytes. Increased CD86 expression is observed in the bone marrow during commitment to the monocytic lineage and is required for macrophage activation and survival (129). These findings are consistent with our observation that cells native to normal synovial fluid substantially expressed CD86, in a context that does not relate to inflammation. For example, even in native cells from ISF at 0 hours (peak inflammation), expression of CD86 was obviously lower than that of CD206 (1% vs. 6%). Moreover, increased expression of CD86 has been reported as a feature of pro-resolving macrophages (18). Similarly, CD206 also has a key function in host defense, efferocytosis and clearance of tissue debris, remodeling repair tissue, and resolving inflammation (130). It is undeniable that the mannose

receptor (CD206) is a defined marker for *in vitro*-produced M2 macrophages (61-63). In fact, CD206 deletion is reported to favor development of M1-like cells with higher expression of inflammatory cytokines in a mouse model of obesity (59). In a similar fashion to CD86, CD206 is constitutively expressed in mononuclear phagocytes and the intensity of expression is associated with demands for clearance of damage-associated molecular patterns and secretion of anabolic cytokines (103, 130). Therefore, in the face of inflammation, expression of both CD86 and CD206 increase as a result of higher macrophage recruitment and response to injury, as corroborated both by our flow cytometry and gene expression data. Major increases in CD14, CD206, and CD86 expression, with steady expression of IL-10, as detected over time by flow cytometry, support our observations that macrophages in BMNC became activated and established an overall homeostatic response.

The homeostatic response observed in both culture media, but especially in ISF, was also confirmed by pairing the findings from cytokine quantification and flow cytometry to that from gene expression. Genetic markers traditionally assigned to M2 macrophages were overall upregulated, while those generally attributed to M1 macrophages were mostly downregulated (63, 72). The decreased expression of CD34 combined with progressively increasing expression of CD68 and CD14, more so in ISF, demonstrate that hematopoietic stem cells in BMNC (CD34$^+$ cells) committed to a myelomonocytic lineage (CD68) and matured (CD14) to establish a regulatory immune response. Such response is proportionate to the inflammatory insult, similar to that previously described in mouse models of inflammation (35, 131).

The horse is a well-established model to study naturally occurring and experimentally-induced OA (1), and develops inflammatory reactions and immune responses that more closely represent those in people than do murine models (73, 132). As a result, our study in horses

closely translates joint inflammation in people and agrees with findings from a controlled clinical

trial of BMNC therapy for OA-affected human patients (45). The synovitis model used to

generate inflamed synovial fluid in our study is widely used to investigate mechanisms of early

joint pathology and evaluate joint therapies aimed at synovitis and OA (133-137). Although this

is a model of acute synovitis, it has been reported to induce significant synovial fluid increases in

general MMP activity (MMP-1, MMP-8, MMP-13) and biomarkers of type II collagen and

proteoglycan degradation (C2C and 3-B-3) and synthesis (CPII and CS-846) that last for at least

1 week (119, 133, 138). Thus, our findings, in agreement with our *in vivo* study treating acute

synovitis with BMNC (85), represent an accurate translation from *in vitro* to *in vivo* models. The

in vitro model used in this study should be considered for future studies assessing cellular

responses and mechanism of articular cell biology, pathobiology, and drug testing.

Inflammation resolution is an active process, largely modulated by macrophages, and

involves inflammatory mediators produced during the acute inflammation. Therefore, the idea of

inhibiting inflammation as a joint therapy through the use of anti-inflammatory drugs and intra-

articular corticosteroids requires revision (20). An alternative way of approaching the treatment

of OA, potentially with a much better safety profile, could be the stimulation of the endogenous

resolution of inflammation by maximizing the innate homeostatic mechanisms of joint

physiology. The parameters investigated in our study, conventionally termed pro- or anti-

inflammatory, are components of a complex immune response aimed at homeostasis and should

be cautiously interpreted together, with attention to the phases of the inflammatory response,

including its resolution. Production of both pro- and anti-inflammatory mediators increases

simultaneously after injury, decreasing when inflammation is efficiently resolved (20, 91, 139).

The overall decreasing ISF concentrations of IL-1β, in association with increasing IL-10 and

IGF-1, suggests that pro-resolving mechanisms were preserved over time, and were potentially favored by progressive macrophage proliferation. Collectively, our data suggest that BMNC therapy could increase numbers of macrophage progenitors in the joint, potentiating the macrophage- and IL-10-associated mechanisms of joint homeostasis lost during progression of OA. In addition, BMNC therapy preserves the production of cytokines, oxylipds, and growth factors required for tissue repair, such as IL-10, PGE_2, and IGF-1, dampened by the frequently used intra-articular corticosteroids. Optimizing the functions of synovial macrophages by intra-articular injection of BMNC has the potential to resolve joint inflammation and re-establish an anabolic synovial environment, substantially benefiting equine and human patients affected by inflammatory joint diseases.

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AUTHOR CONTRIBUTIONS

B. C. Menarim, S. R. Werre, X. Luo, C. R. Byron, S. H. Barrett, J. N. MacLeod and L. A. Dahlgren designed studies. K. H. Gillis, A. Oliver and C. Mason assisted with data collection and assembly. B. C. Menarim performed research, and developed analytic tools under supervision from L. A. Dahlgren. T. S. Kalbfleisch and J. N. MacLeod generated gene expression data. S. R. Werre analyzed data. B. C. Menarim and L. A. Dahlgren analyzed and interpreted data, and wrote the paper.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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CHAPTER 4: AUTOLOGOUS BONE MARROW MONONUCLEAR CELLS MODULATE JOINT HOMEOSTASIS IN AN EQUINE *IN VIVO* MODEL OF SYNOVITIS.

ABSTRACT

Osteoarthritis is characterized by macrophage-driven synovitis. Macrophages promote synovial health, but become inflammatory (M1) when their regulatory (M2) functions are overwhelmed. Bone marrow mononuclear cells (BMNC) are a rich source of macrophages progenitors used for treating chronic inflammation and produce essential molecules for cartilage metabolism. This study investigated the response to autologous BMNC injection in normal and inflamed joints. Synovitis was induced in both radiocarpal joints of 6 horses. After 8 hours, one inflamed radiocarpal and one normal tarsocrural joint received BMNC injection. Contralateral joints were injected with saline. Synovial fluid was collected at 24, 96, and 144 hours for cytology, cytokine quantification, and flow cytometry. At 144 hours horses were euthanatized, joints evaluated, and synovium harvested for histology and immunohistochemistry. Four days after BMNC treatment, inflamed joints had 24% higher macrophage counts with 10% more IL-

10^+ cells than saline-treated controls. BMNC-treated joints showed gross and analytical improvements in synovial fluid and synovial membrane, with increasing regulatory macrophages and synovial fluid IL-10 concentrations compared to saline-treated controls. BMNC-treated joints were comparable to healthy joints histologically, which remained abnormal in saline-treated controls. Autologous BMNC are readily available, regulate synovitis through macrophage-associated effects, and can benefit thousands of patients with osteoarthritis.

INTRODUCTION

Osteoarthritis (OA) is the leading cause of physical disability and is a major cause of medical expenditures in horses and people (1, 2). Chronic synovitis is a major facet of OA and is often the single driver of related catabolic processes (3-7)}. Synovial macrophages are the central drivers of the inflammatory response and cytokine production in the OA-affected synovium (8, 9). Although synovial fibroblasts further amplify the inflammatory reaction, they cannot induce it in the absence of macrophages (5, 8-10). In fact, synovial macrophage depletion strikingly controls joint inflammation, as corroborated in patients with rheumatoid arthritis (RA). Depletion of synovial macrophages is associated with decreased expression of MMP-1, MMP-3, MMP-9, MMP-13, IL-6 and IL-8, known participants in the process of cartilage degradation in OA (8, 9, 11-14). Moreover, activation of macrophages in the synovium of OA-affected patients is directly related to disease activity, severity, and pain (15), reinforcing that synovial macrophages can be a key therapeutic target.

Remarkably, synovial macrophages are also key drivers of synovial health through phagocytic clearance and secretion of anti-inflammatory cytokines, chemokines, enzymes, and growth factors (16-18). Following injury, synovial macrophages proliferate to form a protective immunological barrier in the synovial lining, providing a tight-junction-mediated shield for intra-articular structures (19). When these regulatory functions are overwhelmed, synovial macrophages upregulate inflammation signaling to monocytes and other leukocytes (e.g., neutrophils and lymphocytes) to counteract the increased demands for tissue repair and restore homeostasis (8, 20). However, sustained joint damage impedes resolution of the inflammatory process, and cytokines and catabolic enzymes resulting from chronic inflammation adversely

affect joint homeostasis, leading to degeneration (8, 21). Resolution of inflammation is required

for interrupting the degenerative processes in OA and to provide an environment favorable for

the repair of synovial tissues (22, 23).

In response to environmental stimuli, macrophages display a spectrum of different

responses, ranging from inflammatory (M1) to suppressive/healing (M2) phenotypes (24, 25).

Macrophages play a fundamental role in resolving inflammation and promoting tissue repair,

such that impaired macrophage chemotaxis or macrophage depletion results in inefficient healing

or chronic inflammation (26-28). Inflammatory macrophages (M1) isolated from the synovium

of osteoarthritic joints impede chondrogenic differentiation of progenitor cells *in vitro* (29). On

the other hand, healthy (M2) macrophages are absolutely required for efficient chondrogenesis

and lead to reduced clinical and histological signs of joint disease in a mouse model of arthritis

(17, 30). In addition, inflammation and neutrophil influx into arthritic joints are dampened by

macrophage-derived MMP-12, favoring resolution of joint inflammation (18). Macrophages are

clearly involved in both the aggravation and resolution of joint inflammation and have therefore

become the focus of significant research efforts to understand how they participate in these

opposing physiologic effects (8, 9, 11, 17, 29).

The complexity of the inflammatory response resulting in OA presents a significant

treatment challenge. There is an urgent need for new therapies that, instead of simply blocking

inflammation, can favor an inflammatory reaction with efficient clearance of its triggers leading

to its resolution (23, 31). Biologicals such as platelet rich plasma, autologous conditioned serum,

and mesenchymal stem cells (MSC) are reported to provide modest benefits in treating OA and

as a result gained popularity (32-37). However, these and other current therapies provide only

partial and/or temporary improvements. Importantly, corticosteroids can have detrimental

effects on cartilage metabolism by antagonizing common cellular pathways that are innately required for efficient tissue homeostasis and repair (e.g., IL-10, prostaglandins, and NF-kB) (38, 39). Joint therapy would greatly benefit from the development of approaches that carefully resolve synovial inflammation, while preserving physiological function.

Bone marrow mononuclear cells (BMNC) are a rich source of macrophage (>50% naïve macrophages) and hematopoietic progenitors (25%) readily isolated from bone marrow aspirate (40). Macrophages in bone marrow by default promote a regulatory response, counteracting the deleterious effects of inflammation *in vitro* and *in vivo,* and therefore are considered biased M2 progenitors (25, 40). Macrophages in BMNC reduce inflammation in association with increased production of IL-10 (39, 41), a key cytokine in chondrocyte metabolism and recovery from injury (42, 43). These homeostatic effects of macrophages in BMNC suggest that increasing the numbers of cells responsible for maintenance of joint health may restore joint homeostasis by tipping the balance back toward an anabolic state. BMNC provide potent anti-inflammatory and regenerative effects in injured tissues, including those with minimal regenerative capacity such as the pancreas, central nervous system, and osteochondral defects (44-49). BMNC can also induce resolution of inflammation in chronically inflamed tissues refractory to other treatments, such as joints and the respiratory system (39, 50). Macrophage progenitors are the primary cells in BMNC responsible for downregulating inflammation, while other hematopoietic and mesenchymal stem cells contribute, but to a lesser extent (40, 51).

Despite their high therapeutic potential, the mechanisms by which macrophage progenitors may achieve a therapeutic response are not yet fully elucidated. The aim of this study was to harness the anti-inflammatory and anabolic effects of macrophages in BMNC to resolve synovitis, establishing the synovial response of normal and inflamed joints to BMNC

injection using a lipopolysaccharide (LPS) model of equine synovitis. We hypothesized that intra-articular injection of BMNC would reduce clinical and pathological signs of synovitis and would cause only transient, self-limiting inflammation in normal joints.

METHODS

Study design

Six adult Thoroughbred horses (3-9 years of age, median 5 years; 4 castrated males and 2 females) donated to the Veterinary Teaching Hospital for reasons unrelated to OA or severe systemic inflammation were used under IACUC approval (#17-052) and oversight. A validated model of synovitis was used to increase homogeneity compared to naturally occurring cases. General clinical exams, as well as hematological analysis (complete blood count) were performed to ensure general health and absence of systemic inflammation disturbing the bone marrow niche. Lameness examinations were performed including joint palpations, flexion, and rotation aimed at identifying synovial effusion or pain originating from the joint. Radiographs were used to rule out boney changes to the joint. An *in vivo* model of synovitis was created by injection of LPS into each radiocarpal joint. Tarsocrural joints served as normal controls (PBS-injected in place of LPS). After 8 hours, normal and inflamed synovial fluid was harvested as baseline controls. At the same time, one inflamed radiocarpal joint and one normal tarsocrural joint were randomly treated with BMNC injection, while the contralateral joints received PBS as controls. Synovial fluid was collected at 24, 96, and 144 hours following treatment. After obtaining the final synovial fluid samples, horses were humanely euthanatized by intravenous barbiturate overdose. Macroscopic evaluation of the joints was performed and synovial

membrane biopsies were harvested for histology. Macrophage phenotype activation (flow cytometry and immunohistochemistry) and quantification of synovial fluid cytokines and growth factors (immunoassays) were used to characterize the response to BMNC injection in normal and inflamed joints.

Bone marrow aspiration and BMNC isolation

Bone marrow aspirates were performed immediately prior to induction of the synovitis model to prevent potential changes in the myeloid niche as a consequence of LPS injection. Horses were sedated, and the sternum aseptically prepared and locally anesthetized. Bone marrow aspirates (25 mL each; selected to minimize peripheral blood contamination (52)) were collected from the 4th and 5th sternebrae using an 8 gauge Komiyashiki needle and a heparinized 60 mL syringe (15,000 IU/aspirate) (52). In a laminar flow hood, bone marrow aspirates were filtered (blood administration set), gently layered over Ficoll-Paque™ Plus (GE Healthcare Life Sciences, St. Louis, MO), and centrifuged for 30 min at 500g and 4C°. The resulting supernatant plasma was removed up to 1.5 mL above the Ficoll ring, in order to prevent disturbing the mononuclear cell layer overlying the Ficoll. Mononuclear cells on Ficoll were aspirated, transferred to a sterile tube, washed twice in PBS, centrifuged for 10 min at 300g and 4C°, resuspended ($4x10^4$ cells/µL PBS), and preserved at 4C° until injection.

Induction of synovitis

Since studies using the LPS model of synovitis consider carpal joints (53, 54), we followed a similar approach for comparative purposes. Therefore, synovitis was induced in both radio-carpal joints, and both tarsocrural joints were used as normal controls.

Lipopolysaccharide was reconstituted according manufacturer instructions in silanized vials, using low retention pipette tips (02-717-156; Fisher Scientific, Pittsburg, PA) to prevent LPS adsorption, and stored at -20°C. Synovitis was induced by LPS injection (0.5 ng in 2 mL PBS/joint; *E coli* strain 055:B55; Sigma-Aldrich, St Louis, MO) in both radiocarpal joints (55). Eight hours after model induction (0 hour time point), when inflammation was expected to be at its peak, synovial fluid samples were collected and processed as described below, as a reference of baseline inflammation. Because the LPS model is reported to be short-lived (55-57), a second dose of LPS was combined with the experimental treatments (BMNC or PBS) immediately prior to joint injection and LPS+BMNC or LPS+PBS was injected into radiocarpal joints in order to prolong synovitis. A second dose of PBS was injected in both tarsocrural joints at the same time as the experimental treatments for continuity of models.

Clinical evaluation

Attitude, appetite, temperature, pulse, and respiratory rate were monitored twice daily. Carpi and tarsi were assessed for heat, swelling, and response to flexion (pain classified as none, mild, moderate, severe) at -8, 0 (baseline), 24, 96, and 144 hours post-injection. Gait was only assessed at the walk on a flat surface and horses were not subjected to lameness evaluation at the trot to prevent induction of hemarthrosis, and to mimic limited exercise following joint injection as recommended for horses and people.

Synovial fluid collection and cytology

Horses were sedated, the radiocarpal and tarsocrural joints aseptically prepared, synovial fluid collected at -8 hours previous to model induction, at 0 (baseline), 24, 96, and 144 hours

(~2.0 mL). Samples were aliquoted for cytology (EDTA tubes; Virginia Tech Animal

Laboratory Services, Blacksburg, VA), flow cytometry and cytokine and growth factor

quantification (13-698-795; Protein LoBind®; Eppendorf®, Westbury, CT). Synovial fluid

samples for cytology (total nucleated cell count (TNCC), total protein (TP)) were analyzed using

an automated processor (ADVIA 2120 hematology analyzer, Siemens Healthcare Diagnostics,

Inc., Tarrytown, NY). Differential cell counts were performed by a veterinary clinical

pathologist (SHB). EDTA-free samples were centrifuged (12,000g; 10 min; 4°C), the

supernatant stored at -80°C for cytokine and growth factor quantification, and the cell pellet

stored at 4°C for flow cytometry.

Joint treatment

Following synovial fluid collection at 0 hours, inflamed radiocarpal joints were injected

with 2 mL PBS containing 0.5 ng LPS ± $20x10^6$ BMNC, mixed immediately before injection.

Normal tarsocrural joints were injected with 2 mL PBS ± $20x10^6$ BMNC. Treatments for

radiocarpal and tarsocrural joints were first assigned to the right joint by simple randomization

using a coin flip (heads assigned to BMNC and tails assigned to PBS treatment). Following

assignment of the right joint, the left joint received the remaining treatment option.

Cytokine and growth factor quantification

Growth factors and anti- and pro-inflammatory cytokines and chemokines (FGF-2, GM-

CSF, IL-1B , IL-6, MCP-1, IL-10, TNF-A , SDF-1, IGF-1, IL-1ra and PGE$_2$) were quantified by

equine multiplex bead-based assay (EQCTTMAG-93K; Milliplex MAP Equine

chemokine/cytokine; Luminex 200 plate reader; Millipore; St. Louis, MO) with manufacturer

modifications to include IGF-1, SDF-1, and IL-1ra and to validate inclusion of one additional

point at the lower end of the standard curve to maximize detection of low analyte concentrations.

PGE$_2$ was quantified by ELISA (KGE004B; R&D Systems, Minneapolis, MN; SpectraMax M5

plate reader; Molecular Devices, San Jose, CA).

Synovial fluid samples were thawed and digested with 10uL hyaluronidase solution (100

IU testicular hyaluronidase in acetate buffer [LS005474; Worthington Biochemical Corporation,

Lakewood, NJ) in 200 µL of synovial fluid and incubated for 30 minutes at 37°C. Samples were

then centrifuged for 10 mins at 12,000 x g and 4°C to remove any particulate matter and the

supernatant was recovered (Protein LoBind microfuge tubes, Eppendorf®). Based on our

previous experience and limitations caused by interfering factors in cytokines detection in

synovial fluid (58, 59), spike-and-recovery assays were performed for the PGE$_2$ ELISA and 4

representative serially-diluted targets in the bead-based MILLIPLEX assay (IL-1β, IL-6, IL-10,

TNF-α). Based on the results, a dilution of 1:2 was selected for PGE$_2$ quantification and no

dilution was deemed necessary for the MILLIPLEX assay. Following hyaluronidase digestion,

PGE$_2$ samples were subjected to solid-phase extraction. Synovial fluid (500 µL) was mixed with

500 µL of 100% reagent ethanol and 10 µL of glacial acetic acid, the mixture homogenized and

incubated at room temperature for 5 min, centrifuged for 8 min at 2,500 X g and room

temperature, and the supernatant harvested and assayed. The remaining analytes were measured

using hyaluronidase-digested samples without any additional processing.

Flow cytometry

Synovial fluid cells were washed with PBS, fixed in 2% paraformaldehyde at room

temperature for 20 min, washed and stored in PBS for up to 14 days at 4°C for batch staining and

cytometry. Cells were stained for 30 min, washed and resuspended in PBS, and analyzed (FACSAria Cell Sorter and FlowJo v10, Tree Star, Inc; BD Biosciences; San Jose, CA). Staining solution included cell permeabilization buffer (00-8333-56, eBioscience™; Santa Clara, CA) and the following primary antibodies: CD14 for mature monocyte/macrophages (0.1:100; Wagner Lab, Cornell University, equine specific); CD206 for M2 macrophages (0.02:100; mouse anti-human, COIM2741, 3.29B1.10 clone; Beckman Coulter; Brea, CA); CD86 for M1 macrophages (3:100; mouse anti-human, 555666, IT2.2 clone; BD Biosciences); and IL-10 (0.25:100; Wagner Lab; Cornell University). Markers were selected based on the literature to replicate as best possible similar work done in other species [CD14 (60-64), CD 86 (65-68), CD206 (69-74) and IL-10 (42, 75-78)], within the confines of specificity or validated cross reactivity to equine samples (79-83). Gates were set and auto fluorescence compensated using unstained cells. All antibodies were re-validated with positive and negative controls using cells known to express such markers or not. Antibody dilutions were determined by individual titration assays (82).

Gross pathology and synovial membrane histology and immunohistochemistry

Following euthanasia, joints were evaluated for macroscopic signs of inflammation (e.g., synovial hyperemia and hematomas), as well as intra- and peri-articular hemorrhage and edema. Two synovial membrane biopsies from each joint were obtained using a 6 mm dermal biopsy punch at sites adjacent to where radiocarpal joints most often show cartilage degeneration (84). Biopsies were fixed in AZF Fixative® (1009B, Newcomer Supply, Middleton, WI) at room temperature for 24 hours, rinsed and stored in PBS at 4°C until processing for histology and immunohistochemistry. Paraffin-embedded samples were sectioned at 5 µm and H&E stained.

Semi-quantitative scoring of synovitis based on the OARSI histopathology guide included cell infiltration, vascularity, hyperplasia, and edema. Synovial fibrosis was ignored from the OARSI scoring system due to the short time frame of the study and resulting lack of fibrosis. A composite score was calculated based on the sum of the scores of each parameter assessed (84).

For immunohistochemistry, tissue sections were baked at 66°C overnight, deparaffinized, and incubated in antigen recovery solution at 95°C for 10 min (Antigen Retrieval Citra Plus Solution, HK087-20K BioGenex, Fremont, CA). Slides were stained (Super Sensitive[TM] Polymer-HRP IHC Detection System, QD420-YIKE, BioGenex) using antibodies targeting the following markers: pan macrophage (equine CD14; Wagner Lab, Cornell University); M1 (mouse anti-human CD86 [555663, clone 2331(FUN-1); BD Biosciences]; and M2 (CD 206 [clone ab64693; Abcam; Cambridge, UK]), and IL-10 (equine IL-10; Wagner Lab, Cornell University). Markers were selected using the same criteria as described for flow cytometry. Staining distribution throughout the section was also scored as limited to the synovial lining (1), reaching the sub synovial lining (2), and throughout the entire synovial villus (3). Composite scores for each marker were compared between groups and were used to assess the relative macrophage polarization.

Statistical analysis

Sample size (n=6) was determined using estimated 2-fold differences in expected gene expression and SD=0.5 based on gene expression and flow cytometry on preliminary data (α=0.05; β=0.80), in agreement with previous studies using the same model (54, 85). Normal probability plots were used to assess data normality. Data analysis was performed using General Estimating Equations (GEE) to assess the effects of the different joints sampled as well as the

effects of disease (normal vs. inflamed). Least square means adjustment for multiple comparisons was performed using the Tukey-Kramer test. All analyses were performed by SW using commercial software (SAS version 9.4, SAS Institute, Inc, Cary, NC) at the Study Design & Statistical Analysis Lab. Significance was set at $P<0.05$. Immunohistochemistry data were scored to provide a semi-quantitative comparison, but were not analyzed due to the lack of a validated scoring system.

RESULTS

Clinical exam

Injection of BMNC caused no adverse effects. Selected joints from both normal and inflamed groups developed mild to moderate synovial effusion following injection of either LPS or PBS. Mild heat was palpable over some joints, most commonly if treated with LPS. These findings were inconsistent and followed no pattern based on model (normal or inflamed) or treatment (BMNC or PBS). Also, heat was also noticed over some normal joints following repeated arthrocentesis.
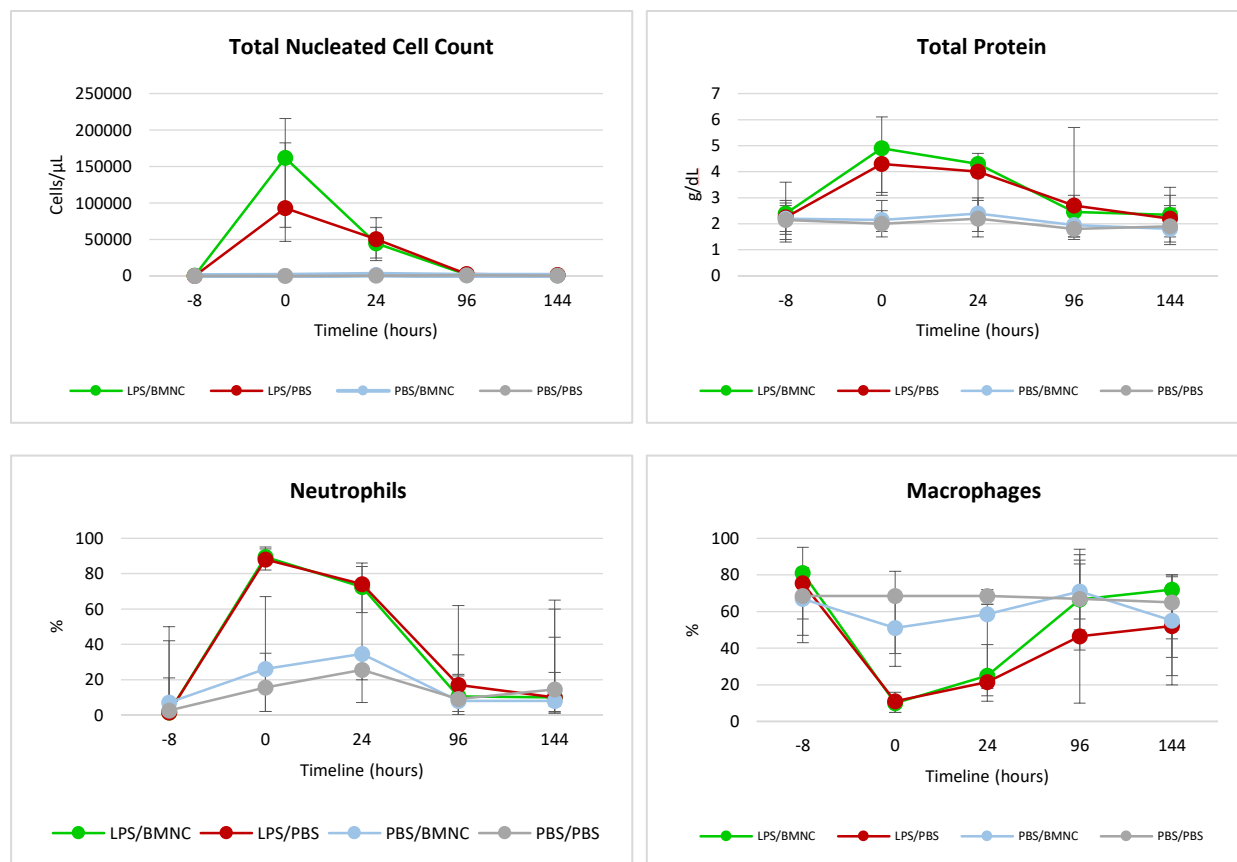
Synovial fluid cytology

As expected, following induction of the synovitis model (at 0 hour), synovial fluid TP and TNCC were increased compared to samples from the -8 hour time point, with the increased TNCC largely composed of neutrophils (**Fig. 1**). In normal tarsi from the PBS model, synovial fluid TP and TNCC were unchanged in joints receiving either BMNC or PBS. Inflamed joints randomly assigned to receive BMNC had much higher median TNCC (161,745 cell/µl) at 0 hour

(prior to treatment) compared to those receiving PBS (93,065 cell/µl); however, differences were not statistically significant ($P=0.0567$). Despite this marked difference prior to treatment, by 24 hours inflamed joints treated with BMNC had slightly lower TNCC (44,595 cells/µL) compared to their inflamed counterparts treated with PBS (50,355 cells/µL; $P=0.9724$). Total protein was not different between these same joints, regardless of treatment ($P=0.6386$).

The effects of BMNC on inflamed joints became most apparent at 96 hours, when gradual recovery of macrophage counts occurred earlier in BMNC-treated joints and were 20% higher than in PBS-treated joints at both 96 ($P=0.2339$) and 144 hours ($P=0.9834$) (**Fig. 4-1**). By comparison, at 96 hours in normal joints, only a 4% increase in macrophage counts was observed following treatment with BMNC compared to PBS. At 96 hours, TNCC and TP were markedly decreased in both groups (BMNC and PBS), but were lower in the BMNC-treated group (TNCC: 1,580 vs. 2,360 cells/µL [$P=0.2867$]; TP: 2.4 vs. 2.7 g/dL [$P=0.7272$]) and were associated with gross improvement in synovial fluid from BMNC-treated joints (**Fig. 4-4**). Large mononuclear cells were often identified, individually or in clumps, in smears of synovial fluid from normal and inflamed joints treated with BMNC, but not in PBS-treated joints (**Fig. 4-4**). In normal joints, PBS injection for model induction resulted in mildly increased neutrophil counts at 0 hours in all horses, with higher neutrophil counts in BMNC-assigned (26%) compared to PBS-assigned joints (16%) ($P=0.0257$). At 24 hours, neutrophil counts increased slightly in PBS-treated (1.6 times to 25%) and BMNC-treated (1.3 times to 34%) joints ($P=0.0755$).

Figure 4-1: Synovial fluid cytology from normal and inflamed joints treated with BMNC or PBS. Differences in synovial fluid cytology between BMNC- and PBS-treated joints were most remarkable at 96 and 144 hours. Neutrophils decreased faster in inflamed joints treated with BMNC compared to PBS-treated controls, favoring earlier recovery of macrophages baseline counts (20% higher in BMNC-treated joints). Median (95% confidence interval).
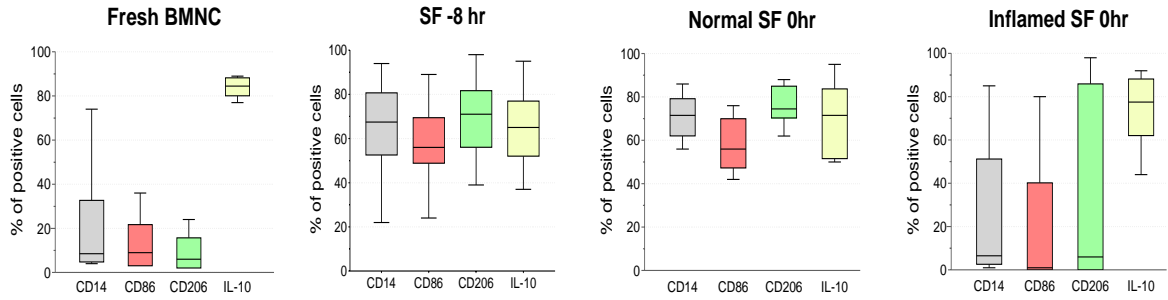
Flow cytometry

Freshly isolated BMNC exhibited very low median expression for CD14 (8.5%), CD86 (9.0%), and CD206 (6.0%), though IL-10 expression was consistently high, even at this early myeloid stage (**Fig. 4-2, top**). In contrast, most cells from normal synovial fluid collected before model induction (-8 hours) expressed CD14 (68%), CD86 (56%), CD206 (66%), and IL-10

(61%), with no differences between radiocarpal and tarsocrural joints. Injection of PBS for

model induction (0 hours) caused increased expression of CD14 (77%), CD86 (60%), CD206

(89%), and IL-10 (79%). Contrarily, expression of macrophage markers in LPS-inflamed joints

was very low (8%, 5%, and 9% for CD14, CD86, and CD206, respectively), as a result of a

predominance of neutrophils dominating the flow cytometry counts. Surprisingly, 81% of those
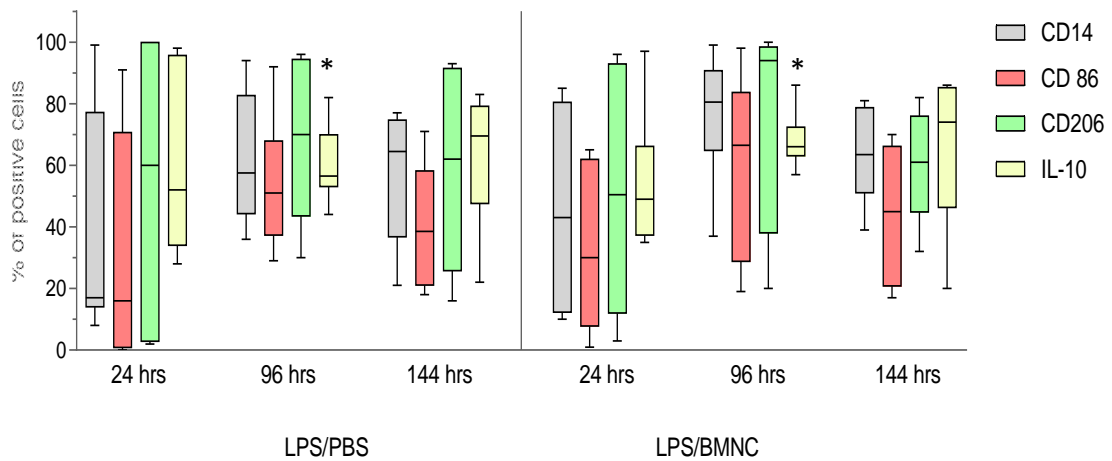
cells were IL-10$^+$.

In inflamed joints treated with BMNC, expression of all markers was overall higher at all

time points (**Fig. 4-2, bottom**). Similar to that observed for cytology, the effect of BMNC on

inflamed joints was more evident at 96 hours, at which time CD206 and IL-10 expression were

24% (*P*=0.9608) and 10% (*P=0.0431*) higher in BMNC-treated joints compared to those treated

with PBS. No differences were observed between normal joints treated with BMNC or PBS at

any time point. No differences were observed in the mean fluorescence intensity expressed for

CD86 and CD206 for any of the experimental conditions.

Cytokine and growth factor quantification

Values were within detectable limits for 7 of the 11 analytes assayed. MCP-1, SDF-1,

IL-10, and PGE$_2$ were detectable in almost all samples. IL-1β, IL-6, and TNF-α were detected in

only approximately half of the samples from inflamed joints at early time points, and in very few

samples from normal joints, precluding statistical analysis. The remaining analytes were below

detectable limits for all samples (FGF-2, 11.5 pg/mL; IGF-1, 0.3 pg/mL; GM-CSF, 3.7 pg/mL;

IL-1ra, 0.02 pg/mL). IL-10 was markedly increased in synovial fluid from inflamed joints at 0

and 24 hours, and was notably higher in joints treated with BMNC compared to those treated

with PBS (**Fig. 4-3**; *P*=0.1784). Concentrations of PGE$_2$ and MCP-1 were not different between

Figure 4-2: Top: Flow cytometry from fresh BMNC, normal (SF) and inflamed synovial fluid. In fresh BMNC (left) only ~10% of cells were positive for macrophage markers, yet 83±4% were IL-10$^+$. In normal synovial fluid (middle), there was a correlation between baseline counts of macrophages (65±4%) and IL-10$^+$ cells (71±3%) not observed in synovial fluid from inflamed joints (right). **Bottom**: Flow cytometry on SF from inflamed BMNC- and PBS-treated joints. At 24 and 96 hours, macrophage (CD14$^+$) counts in synovial fluid from BMNC-treated joints were higher than from control joints. At 96 hours, the number of IL-10$^+$ cells were higher in BMNC-treated joints compared to controls (*p = 0.043). The overall ~10% more M2 cells (CD14$^+$CD206$^+$CD86$^+$IL-10$^+$) in BMNC-treated joints was comparable to the values quantified in normal joints in our study (data not shown). Synovial fluid from BMNC- and PBS-treated joints reached baseline values at 6 days. Median (95% confidence interval).

normal and inflamed joints, or between BMNC- and PBS-treated joints. SDF-1 concentrations

were initially lower in inflamed joints; however, they were not affected by treatment. In

inflamed joints, both IL-1β and TNF-α were higher than in normal joints at 0 hour and

substantially decreased at 24 hours, becoming nearly undetectable by 96 hours, with no

differences between BMNC or PBS treatment. In normal joints, concentrations of IL-1β, IL-6

and TNF-α were below detectable limits in the majority of samples.

Gross pathology

Inflamed joints were prone to subcutaneous hemorrhage following arthrocentesis, which

was clearly more evident in joints treated with PBS (**Fig. 4-4**). Also, inflamed joints treated with

 PBS were characterized by swollen, dark orange to light brown synovium. In contrast, the

synovium of inflamed joints treated with BMNC was less swollen and light orange to pink in

color (**Fig. 4-4**). The above differences in gross inflammation between BMNC and PBS

treatments were marked for 3 horses, moderate for 2 horses, and mild in 1 horse. In normal

joints subcutaneous hemorrhage was mild and confined to the sites of arthrocentesis. No

macroscopic changes were detected in the articular cartilage surfaces of any horses, reinforcing

the initial clinical assessments of joint health prior to model induction.

**Histology and immunohistochemistry**

Inflamed joints treated with BMNC had lower histological scores (closer to normal) than

PBS-treated joints for each of the parameters evaluated (**Fig. 4-5**); however, differences only

reached significance for vascularity ($P<0.001$). Scores for cell infiltration, vascularity, and

intimal hyperplasia were also lower for normal joints treated with BMNC compared to PBS
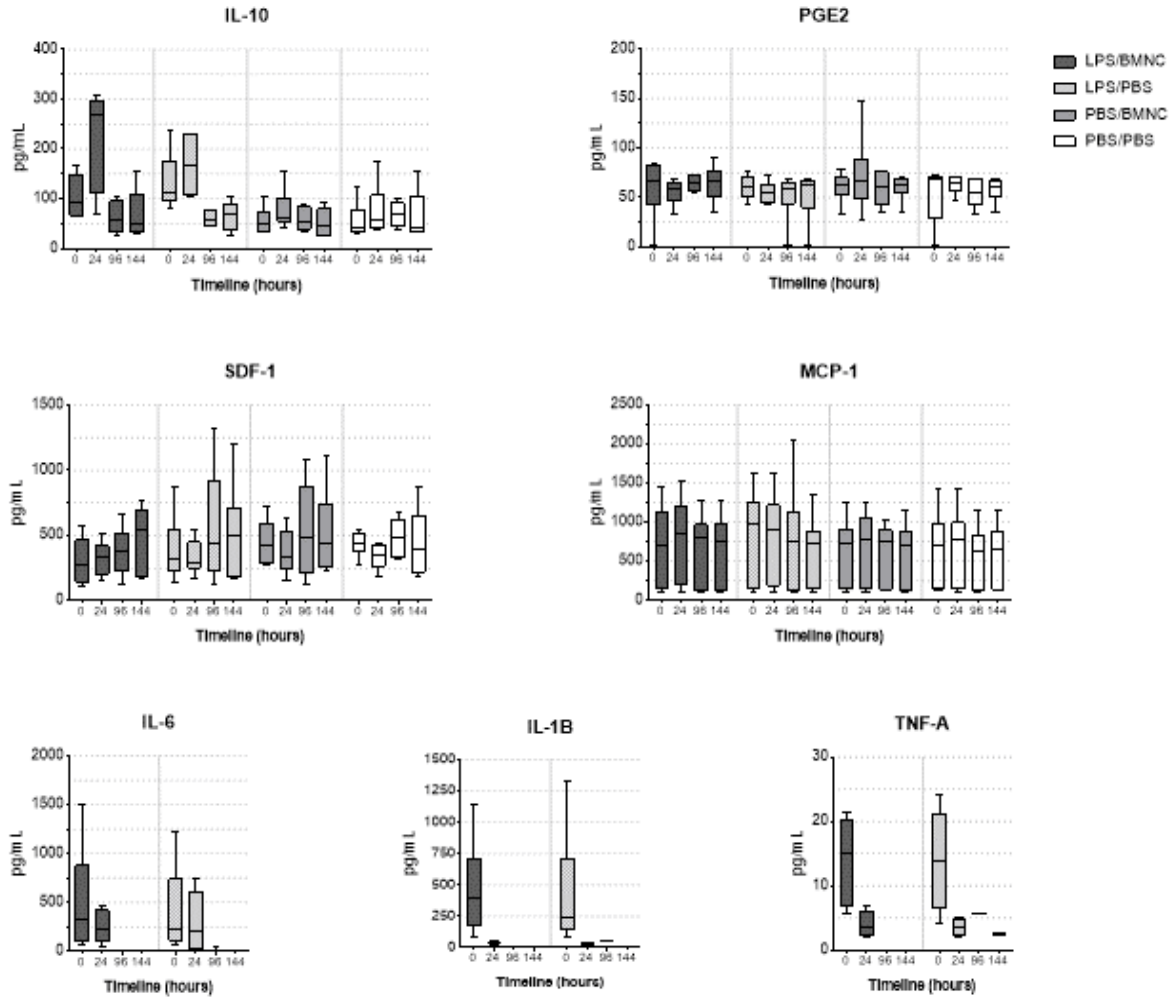
Figure 4-3: Synovial fluid concentrations of cytokines and chemokines in normal and inflamed joints treated with BMNC or PBS. There was a marked increase in synovial fluid IL-10 concentrations at 24 hours in inflamed joints, which was much higher in BMNC-treated joints as compared to PBS-treated joints. Concentrations of SDF-1 were initially lower in inflamed joints compared to normal joints, becoming ultimately similar; however, they did not change as an effect of treatment. Differences between treatment groups and conditions were not significant at any time point for any analyte assayed. Detection of IL-6, IL-1β, and TNF-α was only possible in inflamed joints during the earlier time points, markedly decreasing regardless of treatment. Median (95% confidence interval).

joints, although not statistically significant. Interestingly, the highest scores for cell infiltration and intimal hyperplasia were observed in normal joints treated with PBS. Composite scores were significantly lower in inflamed joints treated with BMNC compared to those treated with PBS (**Fig. 4-5**; *P<0.001*).
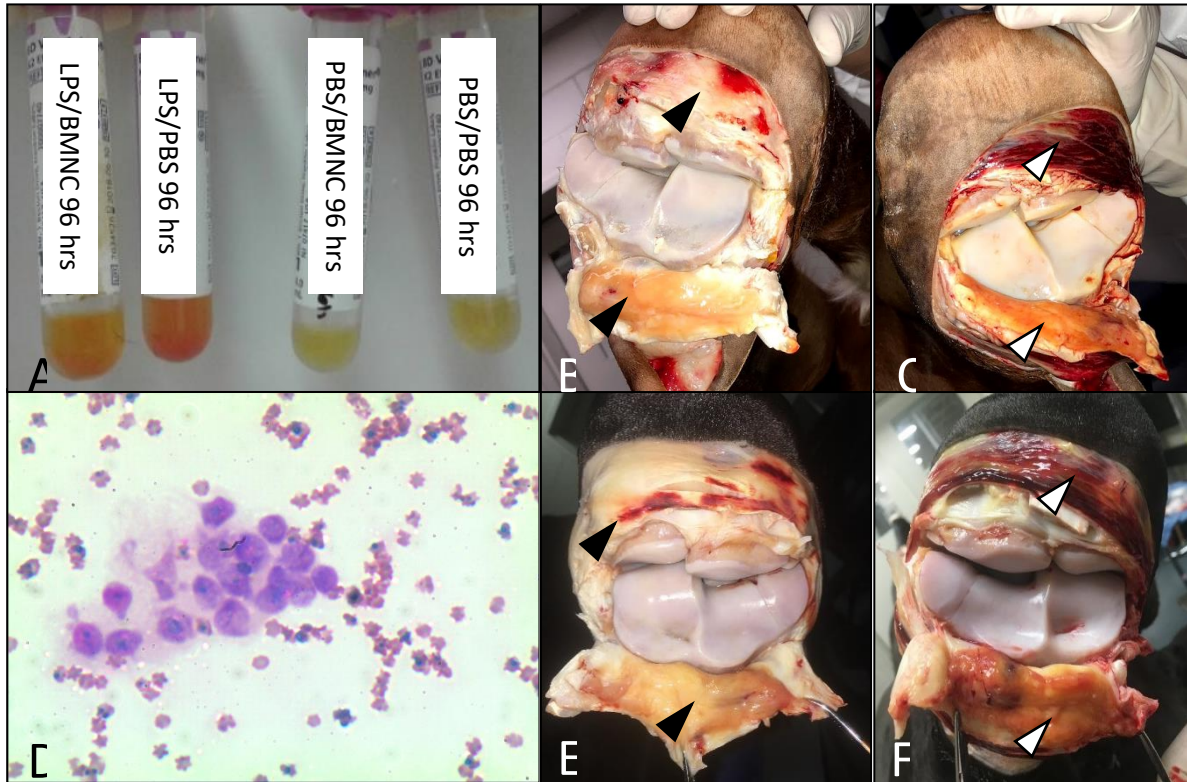
Figure 4-4: Inflamed joints treated with BMNC showed gross improvements in synovial fluid with decreased cellularity and red cell contamination at 96 hours compared to PBS-treated controls (A). Similar improvements were observed in the synovium characterized by less swelling and peri- and intra-articular hemorrhage in joints treated with BMNC (B, E; white arrowheads) compared to PBS (C, F; black arrowheads). Abundant large mononuclear cells were identified only in the synovial fluid of BMNC-treated joints at all time points (D).
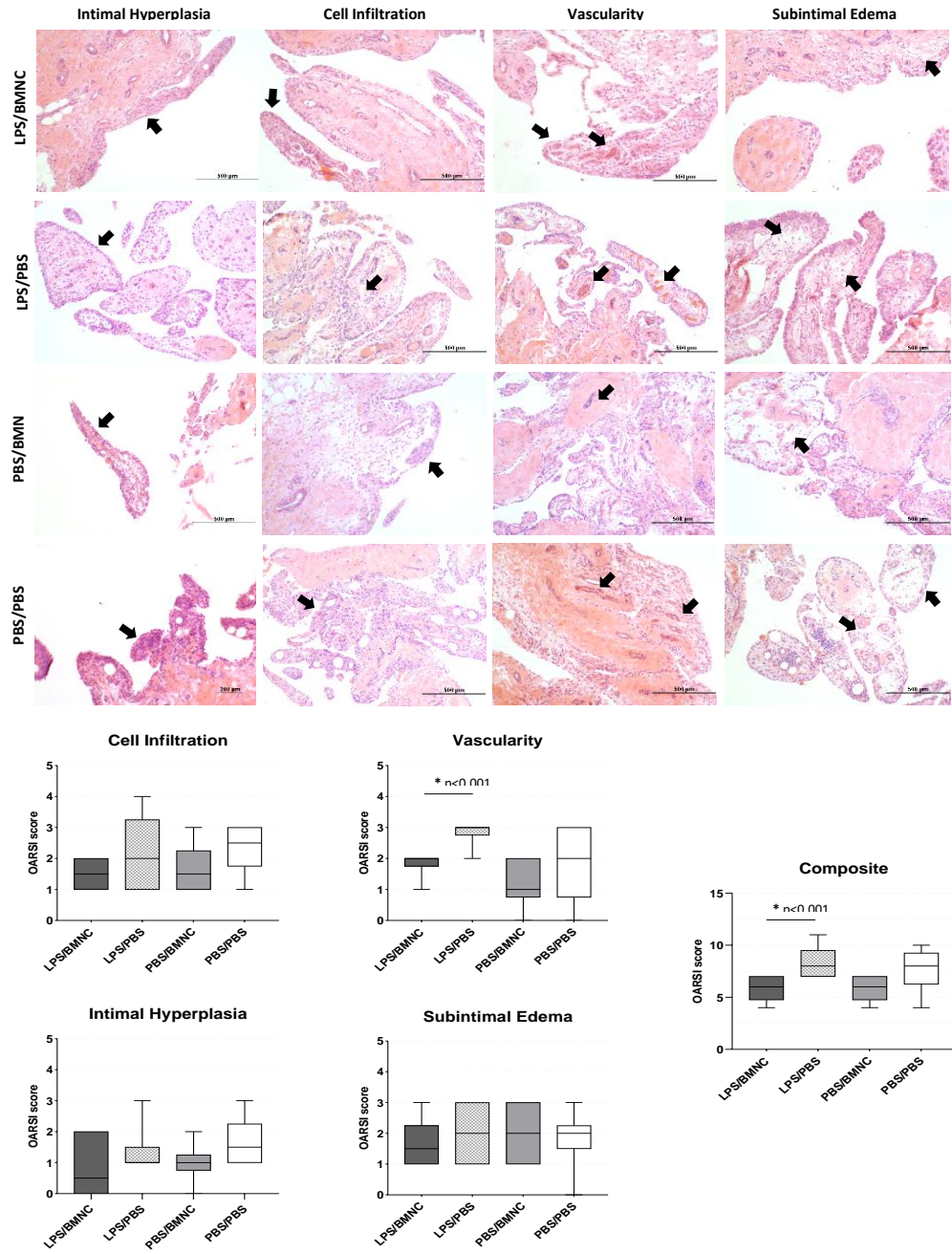
Figure 4-5: Representative H&E images depicting differences in histological parameters (arrows) between normal and inflamed joints treated with BMNC or PBS (A) (scale bar = 500µm). BMNC-treated joints exhibited lower scores for all histological aspects of inflammation, although these were only significant for vascularity and the composite score (B). Median (95% confidence interval).

While the median expression of CD14 was overall consistent among all experimental groups, differences in the expression of other markers varied according to inflammation or as an effect of treatment. CD86 expression was higher in inflamed compared to normal joints, regardless of treatment (**Fig. 4-6**). Expression of CD206 was higher in inflamed joints treated with PBS compared to those treated with BMNC. All markers were consistently expressed among all horses in inflamed joints treated with BMNC, which was not observed in synovial samples from any of the three other groups. In normal joints, the most relevant difference in marker expression was the markedly higher expression of IL-10 in BMNC-treated joints compared to those treated with PBS.

DISCUSSION

Joint injection with BMNC was not associated with adverse reactions in either the normal joints or those with synovitis and provided measurable, anti-inflammatory effects. This is the first controlled experimental study investigating autologous BMNC injection as a joint therapy and with a focus on the role of the macrophage component of BMNC. In our study, BMNC injection resulted in decreased gross, laboratorial, and histological markers of inflammation (cellular and vascular), in association with higher concentrations of IL-10-expressing macrophages in the synovial fluid. Inflamed joints treated with BMNC were comparable to healthy joints regarding histological patterns, which remained abnormally high in PBS-treated controls. Autologous BMNC are readily available, regulate synovitis through macrophage-associated effects, and can benefit thousands of patients with osteoarthritis.
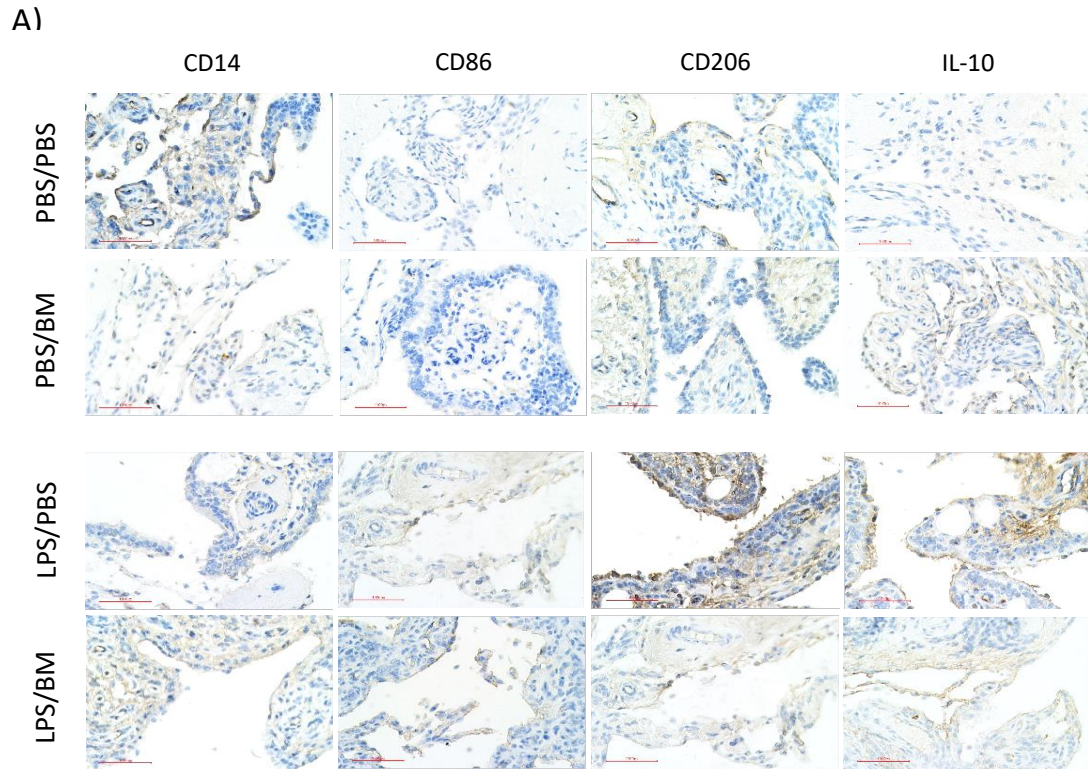
Figure 4-6: (A) Representative immunohistochemical sections (40X magnification- scale bar = 50 µm) from normal (top 2 rows) and inflamed (bottom 2 rows) equine joints treated with PBS or BMNC, denoting the median staining scores for markers CD14, CD206 [M2], CD86 [M1] and IL-10 [M2] (scale bar = 150µm).; (B) Composite staining scores for the same markers. Median (95% confidence interval).

Consistent with published reports, the anti-inflammatory effects of BMNC in inflamed joints were readily obvious in each of the 6 horses in our study, despite variations between horses (39, 50). A single dose of BMNC provided significant clinical improvement in 96% of people with knee OA, which lasted more than 12 months and was superior to 3 weekly injections of hyaluronan (50). BMNC delivered by tracheal infusion resulted in marked resolution of inflammation in clinical cases of equine airway inflammation, comparable to the effects provided by corticosteroids (39). Similar increasing concentrations of macrophages and IL-10 in the broncho-alveolar lavage fluid were noted in these horses with airway inflammation as in the joints of our horses. Significant anti-inflammatory effects were also observed using BMNC to treat a murine model of airway disease (40). In that same study, the clinical, laboratory, and histological improvements observed following BMNC treatment were abolished when the BMNC were depleted of macrophages prior to treatment, providing further evidence of the potent anti-inflammatory potential of BMNC-derived macrophages (40). Collectively, these studies reinforce the important role of IL-10-producing macrophages found in BMNC in modulating inflammation resolution (31).

Lower inflammation in our BMNC-treated joints was demonstrated by gross and histological signs of decreased vascularity and lower composite histology scores. These findings are similar to those reported following treatment with a non-steroidal anti-inflammatory drug, phenylbutazone, in the same model of equine synovitis (86). Horses in that study also exhibited fewer vascular changes that led to subintimal hemorrhage compared to controls. Decreased vascularization is most likely the reason for lower intra- and peri-articular hemorrhage following arthrocentesis in our BMNC-treated horses, and suggests that the anti-inflammatory effects of BMNC are not restricted to the intra-articular environment, but also extend to adjacent tissues.

BMNC therapy favored earlier recovery from damage, as confirmed by flow cytometry data at 96 hours that was comparable to that seen in healthy joints prior to model induction. These findings were also associated with significantly higher IL-10-expressing cells and faster recovery of relative macrophage counts on synovial fluid cytology. Recovery of relative values of macrophages within synovial fluid following injury is an indicator of resolution of acute inflammation (57, 87). Since relative changes in macrophage counts following BMNC injection into normal joints were minimal (only 4% increase), the increased macrophage counts (20%) seen in inflamed joints treated with BMNC on cytology at 96 and 144 hours suggest that BMNC therapy favored inflammation resolution and was not only an effect of injecting the joint with a macrophage-rich cell product. Collectively, these findings suggest that the effect of BMNC in joints follows a similar pattern to that observed in other tissues, induction of endogenous anti-inflammatory and homeostatic effects (39, 40, 47, 49, 88).

In contrast to the more clearly defined macrophage phenotypes proposed by *in vitro* studies (65, 72, 73, 89), defining macrophage phenotypes *in vivo* is challenging. Similar to other studies, our findings suggest that *in vivo*, macrophages more likely exist in a hybrid state, oscillating surface marker expression (and thus phenotype) according to stimuli and stage of inflammation (29, 90, 91). The concept of macrophage activation as either inflammatory (M1) or suppressive/healing (M2) originated from monocyte-derived macrophages treated *in vitro* with defined and overwhelming cytokine stimuli, which do not represent *in vivo* conditions (24, 73, 74, 89). As a result, despite substantial literature supporting the use of CD86 (65-68, 72) and CD206 (69-74) as M1 and M2 markers respectively, interpreting expression of CD86 and CD206 requires attention to the functions of these receptors, rather than simply considering them classical M1 and M2 markers (9, 29, 89, 92).

Although there is compelling evidence of the role of CD86 as a cornerstone for the development of arthritis, thus making it an interesting therapeutic target (67, 68), CD86 is constitutively expressed at low levels in monocytes and resting macrophages, and increased expression is observed during commitment to the monocytic lineage in the bone marrow and during macrophage activation and survival (93). This is consistent with our observation that samples from normal synovial fluid substantially expressed CD86 and that its expression did not follow a classically-described inflammatory phenotype at any time point in our study. For example, even in inflamed synovial fluid at 0 hours (peak inflammation), expression of CD86 was clearly lower than that of CD206 (1% vs. 6%). Similarly, CD206 has a pivotal function in host defense, clearance of tissue debris, remodeling in wound healing, and resolution of inflammation (homeostasis) (88). The mannose receptor (CD206) is an undeniable M2 marker for artificially (*in vitro*) produced M2 macrophages (72-74), and its deletion favors development of M1-like cells with higher expression of inflammatory cytokines in a mouse model (70). However, CD206 is constitutively expressed in mononuclear phagocytes and the intensity of expression is associated with demands for anabolic cytokine secretion and clearance of damage-associated molecular patterns (DAMPs) (88, 94). In summary, the expression of both CD86 and CD206 increases with inflammatory stimuli as a result of increased macrophage recruitment and response to injury.

Macrophage phenotype activation should therefore be carefully analyzed in consideration of other indicators of the overall disease process. Although our study considered a limited panel of markers for the so-called macrophage phenotypes, their expression in BMNC, synovial fluid and synovial membrane macrophages was interpreted in the lights of findings including gross pathology, synovial fluid cytology and cytokine profiling, and histopathology of the synovial

membrane. Altogether these indicators define the states of health, damage and recovery from injury in a far superior fashion then a set of cell surface markers.

The mild increases in expression of all markers following PBS injection for induction of the control model are a result of increased macrophage recruitment and activation in the face of modest loss of homeostasis (28, 88, 93, 95). Intra-articular injection of balanced electrolyte solution has been shown to cause mild to moderate synovitis, (96), inducing resident macrophage activation and proliferation in response to temporarily disruption of homeostasis (19). On the other hand, the low expression of CD14, CD86, and CD206 in samples from acutely inflamed synovial fluid at 0 hours is an effect of disproportionately high relative neutrophil counts, which generally do not express CD86 and CD206. There were few macrophages in the cells counted for flow, diluting marker expression at that time point. Although macrophages and monocytes are considered the main source of IL-10 (97, 98), neutrophils also express IL-10 during acute inflammation (99), as observed by our flow cytometry (0 hour) and cytokine quantification (0 and 24 hours) in samples from inflamed synovial fluid.

The increased concentration of synovial fluid IL-10 in inflamed joints at 24 hours post-treatment (BMNC or PBS) has two possible explanations. Based on our preliminary studies, major changes in flow cytometry, clinical, and pathological parameters were expected to happen after 48 hours post-treatment. Since the LPS model of synovitis is considered to be short-lived (12-48 hours) (56, 57), a second dose of LPS was given in conjunction with BMNC or PBS treatment (at 0 hour) to extend the duration of the model. Following injury, the acute inflammatory response is characterized by increases in inflammatory cytokines such as TNF-α, IL-1 (α and β), and IL-6. In response, proportional increases in IL-10 occur as a negative feedback control, inducing decreased production of these pro-inflammatory cytokines, as

observed in our study (43, 97, 100). Should the inflammatory stimulus persist, this loop of

cytokine feedback is sustained, as shown by increases in IL-10 concentrations following each

injection of LPS. The increased IL-10 likely prevented IL-1β, TNF-α, and IL6 from

continuously increasing. Should this be one of the reasons for increases in IL-10, BMNC therapy

clearly contributed to it.

Increased synovial fluid concentrations of IL-10 in BMNC-treated joints could also be

produced directly by macrophage, hematopoietic, and mesenchymal progenitors within the

BMNC itself. Based on their majority proportion in BMNC (>50%), macrophage progenitors

are the most likely source of direct IL-10 production. Hematopoietic progenitors (~25-30%) (40)

and B and T cells (~10%) make up a smaller proportion of cells. Our preliminary studies ruled

out the participation of T regulatory cells as a source of IL-10 in this context, based on the

absence of FOXP3 expressing cells. Although mesenchymal progenitors in BNMC could

contribute, to a lesser extent, for increased IL-10 concentrations in BMNC-treated joints, their

contribution is unlikely to be substantial based on their very low proportion in BMNC (0.001-

0.02%) (52, 101, 102). Depletion of the macrophage and hematopoietic stem cell components

from BMNC abolish its beneficial effects, which are associated with increased production of IL-

10 (39, 40). As revealed by our flow cytometry findings, freshly isolated BMNC were over 80%

IL-10$^+$, a proportion that likely comprises the hematopoietic and mesenchymal progenitors as

well as the macrophage-committed components. We did not evaluate the expression of early

stage monocyte/macrophage markers (CX3CR1 or CD68) or hematopoietic stem cell markers

(CD34 and SCA-1), due to an absence of antibodies that cross react with horses. Moreover, the

direct production of IL-10 by mesenchymal stem cells is reported to be limited to individual

horses (103). In our study, IL-10 concentrations markedly increased in inflamed joints of all

horses following treatment with BMNC. In addition, it has been reported that mesenchymal

stem cells produce little IL-10 themselves. Instead they induce macrophages to increase their IL-

10 production by increasing homeostatic activation in response to stem cell-derived PGE_2 (104,

105). Although neutrophils also express IL-10 during acute inflammation (99), the differences

observed between BMNC- and PBS-treated joints is undeniable. Therefore, considering all of

the aforementioned, the increases in IL-10 following BMNC injection into inflamed joints

observed in our study most likely correspond to the response of BMNC-derived macrophages

and hematopoietic progenitors, which the latest also ultimately differentiate into macrophages

during inflammation (106).

Higher synovial fluid IL-10 concentrations provide a higher capacity to counteract joint

inflammation after injury, similar to the effect observed following treatment of inflammatory

airway disease with BMNC (39, 40). Moreover, marked increases in IL-10 production by LPS-

challenged macrophages are associated with a protective effect against the development and

progression of human OA (100), highlighting the benefits of BMNC for equine and human joint

therapy (50).

Although PGE_2 is conventionally used as a marker of joint inflammation (107, 108), it

also plays anti-inflammatory and anabolic roles, including inhibition of inflammatory cytokines,

chondrocyte protection, and activation of macrophages. In fact, PGE_2 generated during the early

inflammatory response can also induce resolution of inflammation by upregulating the

transcription of enzymes required for the synthesis of other classes of bioactive lipids that are

potent mediators of resolution and tissue repair (31, 73, 104). The lack of differences observed

in PGE_2 concentrations between the experimental groups in our study is likely because PGE_2 is

involved in both inciting and resolving inflammation and its concentrations in synovial fluid vary

with the stage of response to injury. Thus, the use of PGE_2 as a marker of inflammation in OA may not be as effective as previously reported (107, 109). Quantifying other oxylipids, such as the specialized pro-resolving molecules (resolvins, maresins and protectins), may provide more information regarding stage of inflammation and likelihood of its resolution (23, 110). Remarkably, the bone marrow niche is the richest source of these and other pro-resolving lipids of interest in the resolution of joint inflammation (110-112).

Higher SDF-1 concentrations in synovial fluid from normal compared to inflamed joints in our study is inconsistent with previous reports (113, 114). SDF-1 has multifaceted roles in cartilage biology, including both homeostatic and pro-inflammatory functions, such as chemotaxis of immune cells and direct SDF-1/CXCR4 signaling of chondrocytes, synovial fibroblasts, and macrophages (113-115). SDF-1 is expressed proportionally to inflammation or disease activity, with higher concentrations reported in inflamed joints (113, 114). Our results showing lower synovial fluid SDF-1 concentrations during the peak of inflammation is comparable to two additional ongoing studies from our lab, where comparisons between normal and osteoarthritic equine joints revealed higher SDF-1 concentrations in synovial fluid from normal joints. Lower SDF-1 in acute and chronic inflamed joints could suggest impaired signaling mechanisms for chemotaxis and macrophage recruitment through the SDF-1 gradient (26).

Although IL-1β and TNF-α are considered the main drivers of disease processes in joint inflammation and OA (116-121), both were detected in fewer than half of our samples from inflamed joints, with no differences between BMNC- and PBS-treated joints. Detection of IL-1 in synovial fluid is long-reported to be limited (58, 59), even in samples from highly inflamed joints such as in rheumatoid arthritis (122). Such observations, combined with recent proteomic

and genomic studies comparing samples from OA and healthy joints, suggest that IL-1 and/or TNF-α may not be the central drivers of joint inflammation and disease as they have classically been reported to be (94, 123).

Co-expression of CD14, CD86, CD206, and IL-10 in synovial membrane samples from normal and inflamed joints at similar intensities has been reported for human osteoarthritic joints (29) and has been observed in another ongoing study in our lab. Although differences in expression of each marker between groups were subtle, lower expression of CD86 in normal joints reflects lower macrophage recruitment or activation in the synovium in the absence of inflammatory stimulus (93, 95), and suggest resolution of the arthritic process (67, 68). Increased expression of CD206 in inflamed joints treated with PBS suggests that inflammatory stimuli such as LPS and other DAMPs were still present, while BMNC treatment more efficiently cleared these inflammatory stimuli (88, 92, 124). Moreover, higher IL-10 expression in normal joints treated with BMNC compared to those treated with PBS could result from the interaction of IL-10-expressing BMNC with normal synovium. Although engraftment of BMNC to the synovium could theoretically occur adding to the protective effects of synovial macrophages, our study was not designed to assess the source of macrophages identified on histology.

The synovitis model considered in this study has been used to investigate mechanisms of early joint pathology and evaluate joint therapies aimed at synovitis and OA for over 30 years (55, 85, 86, 119, 125-131). The first described model of synovitis-induced OA showed that synovitis sustained through weekly stimulation for up to 12 weeks induced hallmark features of OA in the synovium and cartilage, in addition to clinical and radiographic changes, comparable to naturally occurring OA (132, 133). Further, it was shown that synovitis sustained for 18 days

by joint injections with LPS every 5 days, induced early cartilage degeneration characterized by superficial cartilage fibrillation and increased expression of IL-1β, TNF-α, and their receptors in cartilage and synovium, leading to chondrocyte hypertrophy and increased synovial fluid 3-B-3 (a marker of proteoglycan breakdown) (119). More recently, other authors using the same protocol used in our study reported that intra-articular injection of a single dose of 0.5 ng LPS alters joint physiology through significant increases of synovial fluid total protein and TNCC as early as 6 hours after induction. At 24 hours, the model induces significant increases in synovial fluid general MMP activity (MMP-1, MMP-8, MMP-13) and biomarkers of type II collagen and proteoglycan degradation and (C2C and 3-B-3) and synthesis (CPII and CS-846) that last for at least 1 week, a similar timeline in which gross pathology was still clearly evident in our study (54, 57, 134). In addition, others have also demonstrated significantly increased gene expression for MMP-1, ADAMTs-5, and ADAMTs-4 in the synovium and cartilage (53). A major catabolic effector in OA, MMP-13 is highly expressed in arthritic cartilage (1, 135). One study assessing cartilage expression of MMP-13 in the LPS model failed to detect significant changes compared to controls (53). In that study, cartilage biopsies were obtained at 8 hours post model induction. At such an early time point, chondrocyte catabolism does not cause cartilage damage inducing mechanical stress of chondrocytes that is required to activate the HTRA1-DDR2-MMP-13 axis inducing changes in MMP-13 expression and activity (54, 135). Collectively, these studies emphasize that this acute and reversible model of synovitis represents the early events of cartilage degradation in response to inflammation, making it suitable for the evaluation therapies targeting early arthritic events such as those induced by inflammation. Although the LPS model is commonly described as short-lived (12-48 hours), based on the duration of changes in clinical signs and synovial fluid cytology (53, 55-57, 125), we saw marked signs of synovial

inflammation in PBS-treated joints even at 6 days following model induction as previously reported by other authors (128).

In summary, BMNC therapy resulted in marked reduction of acute inflammation and did not cause adverse reactions in normal joints. The profound anti-inflammatory effects of BMNC therapy were likely achieved by optimizing the functions of synovial macrophages, including increased production of IL-10 through the addition of an exogenous source of macrophages capable of maximizing their homeostatic response following injury. The minimal cell manipulation involved in processing BMNC, combined with its autologous origin comply with the US Food and Drug Association (FDA) restrictions for cell therapy in people. The horse is a well-established model to study naturally occurring and experimentally induced OA (1), with inflammatory reactions and immune responses that more closely represent that in people than murine models (136, 137). As a result, our study in horses closely translates inflammatory events in people, and agree with findings from a prospective randomized controlled clinical trial of intra-articular BMNC therapy for OA-affected human patients (50). Therefore, our findings in the context of a clinically relevant model of joint inflammation, and considered in light of existing literature, provide further support for the clinical use of BMNC in equine and human patients affected by inflammatory joint diseases.

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AUTHOR CONTRIBUTIONS

B. C. Menarim, S. R. Werre, X. Luo, C. R. Byron, and L. A. Dahlgren designed research. K. H. Gillis, A. Oliver, C. Mason, and Y. Ngo assisted with data collection and assembly. B. C. Menarim performed research, and developed analytic tools. S. H. Barrett performed synovial fluid cytology. S. R. Werre analyzed data. B. C. Menarim and L. A. Dahlgren analyzed and interpreted data, and wrote the paper.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

Clinical and scientific knowledge generated over the last 50 years have provided remarkable contributions to our current understanding of the pathobiology of articular tissues, dramatically advancing the fields of arthritis and rheumatology. However, there is limited information regarding the biology of synovial macrophages, the main drivers of the inflammatory process that is important for both joint health and joint disease. There is also a remarkable gap of knowledge concerning overall mechanisms of sterile inflammation. Inflammatory mechanisms have been mostly investigated in the context of infection and autoimmunity. Moreover, molecules and events involved in the sterile inflammatory process have often been inadvertently seen as causative of inflammation and thus were assigned a detrimental effect. Inflammatory mediators involved in inciting inflammation are also necessary to induce the synthesis of mediators required to effectively drive endogenous resolution of the inflammatory process, or can even act as such mediators themselves.

Substantially fewer investigations have explored the inflammatory mechanisms necessary for the natural recovery from inflammation following injury, a process called inflammation resolution, which is required for re-establishing homeostasis. Both inflammation and its resolution are processes largely mediated by macrophages. The acute phase of inflammation following injury is a process of macrophage recruitment to the injury site, not only to fight aggressors and clear tissue debris and apoptotic cells, but most importantly to orchestrate repair of the damaged tissue through cellular and paracrine effects. Once tissue repair has been efficiently established, macrophages coordinate resolution of inflammation, eventually promoting homeostasis.

Since macrophages are involved in both inflammatory and pro-resolving responses, they quickly became designated as inflammatory (M1) or suppressive (M2). However, in the complexity of biological systems, including the joint, clear identification of such macrophage phenotypes *in vivo* is not observed as proposed by *in vitro* studies. Our studies, combined with current evidence, suggest that, *in vivo,* macrophages combine these responses, predominating the facet most required at each phase of the inflammatory response, overall targeting homeostasis.

Chronic inflammation suggests that the inflammatory response has not efficiently achieved its purpose, and thus inflammation could not be resolved. Chronic synovitis, as seen in OA, is evidence of a failed attempt to clear aggressors and guide tissue repair, perpetuating the phlogistic phase of the inflammatory response. There is growing evidence that synovial macrophages in OA are overwhelmed, preventing inflammation resolution. Blocking inflammatory mechanisms to treat OA provides symptomatic relief, but does not favor recovery of homeostasis, which depend on pro-inflammatory mediators. The idea of inhibiting inflammation as a therapy requires revision. 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, largely attributable to macrophages. The reason why endogenous recruitment from myeloid macrophages to the joint is not efficient enough to recover homeostasis is not yet known. It could be related to the tight-junction shield formed by synovial lining macrophages during inflammation, preventing efficient signaling from the synovial environment to the myeloid niche. However, this is a field that certainly requires further investigation.

Our findings from *in vitro* and *in vivo* studies of BMNC showing markedly decreased gross and analytical signs of synovitis and decreases in synovial fluid concentrations of IL-1β,

combined with increasing IL-10 and IGF-1 concentrations, show that classic markers of joint disease were inhibited, while key factors for chondrocyte and overall joint homeostasis were enhanced. Altogether, these findings support our hypothesis that re-establishing macrophage-derived mechanisms of joint homeostasis induces production of molecules intrinsically involved in endogenous resolution of synovitis and tissue repair, otherwise inhibited by the use of non-steroidal anti-inflammatory drugs and corticosteroids. Maximizing macrophage-mediated joint homeostasis can recover a healthy synovial environment and overall joint health and is the basis of another ongoing clinical research project using BMNC to treat horses with naturally occurring OA.

Information generated in these studies also uncovered new methods to study cellular responses to the normal and inflamed synovial environments *in vitro.* Our methods more closely mimic the environment into which the cells will ultimately be injected for treatment of clinical problems. Importantly, the *in vitro* methods we used avoid the artifacts produced in typical *in vitro* models that artificially induce nonspecific immune responses, biasing the overall interpretation of the results. Investigating the use of this model to assess other scientific questions related to joint inflammation is an avenue to be explored. This model can remarkably impact the field of rheumatology by providing *in vitro* information of much higher biological value, decreasing the bias introduced by currently used *in vitro* models using culture media supplemented with serum and unnatural concentrations of cytokines. Moreover, while the inclusion of multiple cell types to *in vitro* experiments can increase the complexity of experimental design, synovial macrophages and fibroblast should always be incorporated in studies assessing the response of chondrocytes and cartilage explants to articular inflammation.

Without synovial macrophages and fibroblasts, a key piece of the inflammatory and pro-resolving response would be missing.

Most *in vitro* models of joint inflammation use stimulation with IL-1β, given its proposed central role in driving OA process. Our findings on these 3 studies, in agreement with most of the published literature, shows inconsistent detection of IL-1β, even in severely inflamed joints with OA and rheumatoid arthritis, questioning the proposed role of IL-1β. This concept was shaped based on limited studies, often assessing IL-1-like activity, or using a dramatically restricted set of samples, and with experimental designs that were scoped and prone to identify IL-1 related events. Such observations led to many studies that often solely assed the effects of suprapathological concentrations of IL-1, indulging interpretation of disease mechanisms in an inadvertently biased fashion. More refined scientific techniques available today have revisited such fundamental concepts and do not support them. As such, this could be a reasonable explanation for the failed clinical use of IL-1ra-based therapies. Overall these findings highlight the need for broader scope studies identifying a larger set of key drivers of disease processes in joint disease.

Based on the roles of bone marrow aspirate concentrate (BMAC) on cartilage repair, and the overall role of macrophages in tissue repair, the transmutation of macrophages into fibrocytes at advanced stages of healing, the role of BMNC in improving osteochondral repair, similar to that provided by microfracture or BMAC, should be assessed. It is highly suggestive that BMNC hold the same potential to improve osteochondral repair as these other therapies, with the advantage of not containing red blood cells and therefore decreasing the pro-inflammatory response caused by ferritin in the breakdown of hemoglobin, which is known to negatively affect

the quality of tissue repair. These observations combined with the practicality to cryopreserve BMNC for future use, make it an attractive alternative to be explored.

A more thorough understanding of how macrophages exert paracrine function on other cells in the synovial environment is a very much needed knowledge to advance therapeutic approaches that can account for the complexity of joint inflammation and degeneration. Tissue macrophages can be identified in nearly every organ and tissue. Therefore, comprehending mechanisms involved in this crosstalk of tissue cells with macrophages, while inciting and resolving inflammation, can revolutionize the development of therapies that more efficiently approach the complexity of the biological mechanisms required to treat inflammatory conditions in a multitude of organs and systems.

Finally, identifying specific molecules that increase macrophage recruitment into the intra-articular environment or directly drive joint homeostasis, similarly or more efficiently than BMNC, could eventually provide off-the-shelf therapies to treat arthritic conditions.