DISSERTATION

IMMUNE-ACTIVATED CELLULAR THERAPIES FOR OSTEOARTHRITIS AND THE ROLE OF IMMUNE RECOGNITION OF JOINT ANTIGENS

Submitted by

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ABSTRACT

IMMUNE-ACTIVATED CELLULAR THERAPIES FOR OSTEOARTHRITIS AND THE ROLE OF IMMUNE RECOGNITION OF JOINT ANTIGENS

Osteoarthritis (OA) is a progressive, degenerative condition that affects over 550 million people worldwide – a 113% increase since 1990. Despite this high prevalence, there remains a lack of effective treatment options that improve quality of life without risk of adverse effects. Recent evidence supports that OA is a multifactorial condition in which the immune system plays a key role to perpetuate chronic inflammation. Cellular therapies to treat OA have emerged as an option, with mixed results reported in terms of efficacy. Heterogeneity within stromal cell populations has been proposed to be partially responsible for the observed variability in therapeutic responses, particularly in the context of variably inflamed recipient environments such as that seen in OA. Pre-activation, or 'inflammatory licensing' of mesenchymal stromal cells (MSC) through priming their respective ligands has been proposed as a means to generate a homogeneous population of immunomodulatory MSCs – thereby potentially improving their therapeutic consistency in the inflammatory environment of OA. The work in this defense addresses three primary aims: 1) to further investigation the role of the adaptive immune system in OA, investigating autoantibody production to synoviocytes and chondrocytes in OA progression, 2) to evaluate further mechanistically how innate immune pathway activation of mesenchymal stromal cell therapy modulates interactions of MSC with synovium and cartilage to mitigate OA progression, and 3) to examine alternate connective tissue sources of MSC for cell expansion as regenerative therapies. With the lifetime likelihood to develop symptomatic

knee OA currently 45% and increasing, the need to develop improved strategies towards disease-modification is critical.

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1.1 The role of inflammation in osteoarthritis

Osteoarthritis is the most prevalent joint disease and a leading cause of disability, with the lifetime likelihood to develop symptomatic knee OA currently 45% and increasing(1). OA has traditionally been viewed as a disorder that mainly affects articular cartilage(2), however it has more recently been established as a multifactorial disease(3). OA is characterized by the progressive degeneration of joint cartilage, remodeling of the underlying subchondral bone, and chronic inflammation. Pathological changes in OA joints include degradation and fibrillation of articular cartilage, the thickening of the subchondral bone with formation of osteophytes, synovial inflammation, and degeneration of ligaments and menisci. There are several cellular and molecular processes involved with these pathological changes including hypertrophy and death of chondrocytes, cartilage catabolism with a decrease in repair, osteoclastic remodeling of bone and the increase in inflammatory cells and inflammation in the joint. Commonly affected joints include knees, spine, hips, and fingers. Clinical features include pain, joint deformity and dysfunction. Risk factors associated with increased risk for OA include obesity, aging, previous joint trauma, genetics, smoking, and female sex(2). Current therapies for OA aim to address symptom alleviation with surgical interventions including lavage and debridement in more advanced cases.

Inflammation of the joint, including synovitis or inflammation of the synovial lining, is characteristic of OA (2–4). This synovitis is driven by the migration of inflammatory immune

cells into synovial tissue and their expression of inflammatory mediators(5,6). OA tissue and synovial fluid have increased levels of inflammatory cytokines, components of complement, and other inflammatory molecules produced by cells of the synovial joint as well as infiltrating immune cells(7,8). The inflammation seen in OA is somewhat analogous to rheumatoid arthritis but differs in intensity and cellular infiltration(9). Histology of human synovial tissue shows that inflammation in the synovium is less severe than that seen in RA in terms of cellular infiltration and inflammatory CD markers(10,11). Likewise, inflammatory molecules including cytokines IFN-γ, IL-1β, IL-6, TNF-α, and catabolic matrix metalloproteinases in both blood and synovial tissue are also lower in OA compared to RA(9,12–15). The number of immune cells, including synovial macrophages, T cells, and dendritic cells in synovium is elevated compared to healthy tissue, but lower in OA compared to RA(9,16–18). Effective treatments in RA that block the activity of inflammatory cytokines like TNF-α and IL-1β do not provide a similar benefit in OA(19–22). This demonstrates that although possessing limited similar characteristics, molecular mechanisms of inflammation are inherently different between the two diseases.

Trauma to the joint initiates a progressive increase in inflammation and resultant damage and tissue degeneration. The innate immune system uses pattern recognition receptors to respond and eliminate pathogens as a preliminary response. Cells respond to pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) through pattern recognition receptors (PRRs). These DAMPs and PAMPSs are produced from degenerating joint tissue and activate immune cells like macrophages to respond. Once activated, the innate immune cells release inflammatory signals that are usually followed by repair in normal tissue. However, in OA, this inflammatory state is typically destructive and initiates a cycle of chronic inflammation and tissue damage(23). DAMPs in OA include the products of

extracellular matrix breaking down, such as hyaluronic acid, fibronectin, and biglycan, plasma proteins released into blood vessels at sites of inflammation, like macroglobulins and fibrinogen and other particles released from cartilage including calcium phosphate and uric acid(24). Response to DAMPs involve cells expressing Toll-Like-Receptors (TLRs). Of the 10 TLRs found in humans, 8 have been found to be expressed by cells of the synovium (TLR1-7, 9)(25). Activation of TLRs has been implicated in the development and progression of OA through synovitis, cartilage degeneration, and other chronic inflammatory pathways.

1.1.1 Review of inflammatory cytokines, chemokines, and growth factors in OA

Inflammatory molecules implicated in OA progression include cytokines, chemokines, growth factors, adipokines, prostaglandins, and leukotrienes(24). Synovial joint cells responding to and producing these factors include synoviocytes, chondrocytes, mesenchymal stromal cells and immune cells such as macrophages(26). Cytokines reported to be elevated in OA and associated with disease progression include TNF-α, IL-1β, IL-6, IL-15, IL-17, IL-18, IL-21 (24). Cytokine elevation has been associated with cartilage degradation and inhibition of anabolic processes to support cartilage(27,28). IL-1β and TNF-α, mediated through NFκB and AP1 induces further expression of them in cells as well as producing other inflammatory and catabolic mediators. NO, PGE_2, IL-6, MMP-1, MMP-9, and MMP-13 are all induced and exhibit catabolic properties in the joint(29). However, blocking single cytokines like IL-1β(30) and TNF-α(21,22) have not elicited sufficient reduction in inflammation. This suggests that targeting single or even multiple cytokines may not be adequate to address the inflammation in OA. Similarly, determining which cytokines to target requires sufficient knowledge to understand resultant outcomes and mechanisms which have not been elucidated fully yet(31).

When considering roles of specific cytokines in OA progression, it has been reported that IL-1β is overexpressed in OA cartilage and synovium with a decrease in IL-1 receptor antagonist(32–34). Likewise, IL-1RI was upregulated in chondrocytes and synovial fibroblast making them more sensitive to IL-1β (35,36). IL-1β upregulates the expression of MMPs-1 -3 -13(37–39) and ADAMTS-4 -5(40) in chondrocytes and synovial fibroblasts. IL-1β also suppresses the expression of type II collagen and proteoglycans in chondrocytes(41–44). It is inhibited through the suppression of GlcAT-I, and enzyme involved in the synthesis of glycosaminoglycans(45). IL-1β has also been attributed to cause apoptosis through the increase of NO production through iNOS(46) or ROS(47,48) in chondrocytes.

TNF-α shows somewhat similar effects on cartilage and synovium and may act individually and collectively with IL-1β in OA. TNF-α is also overexpressed in OA cartilage and synovium(49,50), likewise, its receptor is also increased in cartilage and synovium in OA(51,52). TNF-α inhibits proteoglycan synthesis and causes resorption of cartilage(53). TNF-α also upregulates expression of MMPs-1 -3 -13(54,55). OA cartilage also has increased concentration of TNF-α converting enzyme which converts pro-TNF-α to mature TNF(56).

IL-6 plays a significant role in OA joint pathology and increased levels are associated with degradation of cartilage. IL-6 is upregulated in synovial fluid and serum of OA patients(57) (58). It is stimulated in part by IL-1β, TNF-α and specific growth factors(59). Interestingly, type II collagen stimulates IL-6 production in chondrocytes(60). It is also stimulated by PGE_2 in chondrocytes(61,62) as well as synovial fibroblasts(63). IL-6 has both catabolic and protective effects in cartilage, which are not completely understood. Confounding the role of IL-6 in cartilage, studies found IL-6 produced the upregulation of tissue inhibitor metalloproteinases in synoviocytes and chondrocytes(64) and increased proteoglycan synthesis human OA

chondrocytes(65), while others observed IL-6 upregulates MMP-1 and MMP-13 expression(66) inhibits the expression of type II collagen(67), and inhibits proteoglycan synthesis(68,69). This contradictory quality of IL-6 in OA is postulated to be related to the ability of IL-6 to initiate signal transduction through alternative modes of receptor activation(70).

Chemokines and their respective receptors are associated with the progression of OA including IL-8, CCL5, CCL19, CCR1, CCR2, CCR3, and CCR5(71–73). These chemokines are released and correlated with upregulation of matrix metalloproteinases in the joint and the following breakdown of extracellular matrix(74). However, some chemokines may be protective in OA, like SDF-1 which recruits mesenchymal progenitor cells to the tissue to promote tissue regeneration(75). The role of chemokines can also vary according to cell type, inflammatory state and the presence and interaction of other signaling molecules(76).

Growth factors such as TGF-β have been implicated in OA progression. TGF-β is commonly expressed in joint tissue including cartilage, bone and synovium(77). TGF-β maintains cartilage homeostasis but the dysregulation, including both up and downregulation, can lead to fibrosis and other tissue malformations(78). Fibroblast growth factor is also generally expressed in articular cartilage, mainly FGF-2 -8 and -18, and are also associated with cartilage homeostasis(79). The upregulation of VEGF promotes angiogenesis, which allows migration of immune cells and the ensuing inflammation in OA(80). NGF also regulates monocyte responses as well as the production of IL-1β, TNF-α, IL-6 and IL-8(81).

Adipokines, cytokines secreted by adipose tissue, represent a link between obesity and some metabolic syndromes with an increased risk for OA(82,83). Adipokines have dichotomous immunomodulatory properties based on environmental and cellular properties. Produced in the joint by infrapatellar fat pads as well as synovium and chondrocytes, this other source of

inflammatory signaling plays a role in proteoglycan production, expression of MMPs, and upregulation of NGF and PGE_2(84). Lipid mediators have been detected in the OA joint and are likely involved in the progression of OA(85). Leukotrienes and prostaglandins, specifically PGE_2, contribute to OA progression increasing inflammation, apoptosis, and angiogenesis. LTB4, a leukotriene acts as a chemoattractant for immune cells and is involved with stimulating IL-1β and TNF-α production in synovium(86,87). The mechanism for these prostaglandins involves production from arachidonic acid through the cyclooxygenase pathway where COX2 is the rate limiting enzyme. Inflammatory articular cartilage has an increase in COX2 levels, and its expression is induced by inflammatory cytokines (IL-1β, TNFα, IL-6) and also TLR4 stimulation(88,89). Prostaglandin E synthase, the terminal enzyme for PGE_2 production is also upregulated in OA cartilage(90), where it can be induced by IL-1β and TNF-α(91).

Nitric oxide is also involved in inflammatory processes and can be induced by cytokines through the iNOS pathway(92). Elevated levels of NO as well as high levels of nitrite have been detected in OA cartilage, synovial fluid, and serum. NO increases expression of MMPs, inhibits collagen and proteoglycan synthesis, induces apoptosis, and increases PGE_2 levels(93,94). Though NO also has been shown to have protective effects in OA and can inhibit inflammatory pathways(95).

Finally, neuropeptides associated with pain response pathways are also involved in inflammation and the production of inflammatory cytokines(96). Substance P is detected in synovium of OA patients and causes the activation of immune cells, stimulates cytokine production, and causes production of PGE_2 and collagenase in synovium. Bradykinin can also contribute to OA through inflammation and cytokine secretion(97).

1.1.2 The role of the adaptive immune system in OA

The infiltration of immune cells in synovial tissues is observed in OA patients(98–100). Infiltrating lymph cells expressed early, intermediate, and late activation markers suggesting an active adaptive immune response in OA tissue(101). Likewise, oligoclonal populations of T cells in OA tissue were observed and an indication of active immune response to antigens within the synovium(101). Increased autoimmunity to type III collagen and proteoglycan was observed when rabbits had a partial meniscectomy(102). Similarly, higher levels of aggrecan recognizing antibodies were seen in OA patients when lymphocytes interacted with cartilage link protein and proteoglycan(103). This increased immune response to cartilage derived antigens suggests an adaptive immune response to extracellular matrix molecules of the OA joint. B cells are also in the population of infiltrating immune cells in the OA synovium(104). Similarly to T cells, these B cells are oligoclonal, suggesting activation by antigens(105). Autoantibodies produced against cartilage components have been commonly reported, cartilaginous proteins that showed autoantibody reactivity include osteopontin(106), YKL-39(107), fibulin-4(108), and collagen(109). Auto-reactivity to post-translational modifications of proteins is also seen in a proportion of OA patients, with anti-cyclic citrullinated proteins being detected, although at a lower rate than in RA patients(110).

The presence of low-grade chronic inflammation in OA has been well-established; however, relatively recently the focus has shifted to the critical role this inflammation plays in the pathogenesis of OA. Contrary to the perception that OA degradation was due to simple cartilage degradation, OA has more recently been viewed as a complex systemic and local disease process involving the entire joint. The complexity of the inflammatory environment and its role in OA makes treatment extremely difficult, necessitating future work to further define the

role of inflammation. Treatment for OA has largely focused on symptomatic relief, and the lack of available disease modifying OA drugs requires novel approaches in therapeutic treatments. Regenerative therapies, including mesenchymal stromal cells, have emerged as a potential therapeutic alternative, with immunomodulatory properties that may be applicable in OA.

1.2 Biological Function of Mesenchymal Stromal Cells (MSC)

1.2.1 Discovery and definition of MSC: cell origin, cell morphology

The first evidence to support investigation of mesenchymal stromal cells (MSCs) dates back to 1867 where the pathologist Julius Cohnheim observed nonhematopoietic cells present in bone marrow(111) that could be the source of fibroblastic cells in wound healing. In 1970, Friedenstein was able to isolate and culture these nonhematopoietic cells(112) from the bone marrow of guinea pigs. Friedenstein observed these cells forming colonies, capable of self-replication and differentiation into chondrocytes, adipocytes, and osteocytes(113). The discovery of these cells in humans occurred between 1985 and 1992, with Ashton (114) and Davies(115) characterizing human cells with similar cellular characteristics to the isolated rodent cells. Haynesworth contributed further by identifying human cells that had exhibited osteochondral potential in 1992(116), a factor previously missing from Ashton and Davies work. The term mesenchymal stem cell was proposed by Caplan(117) in 1991 to identify these progenitor cells isolated from bone marrow, but recently mesenchymal stromal cell (MSC) has gained popularity as a more suitable term(118–120) with the International Society for Cell & Gene Therapy (ISCT) officially endorsing it in 2019.

As MSC research, therapy and applications rapidly increased, the ISCT defined minimum criteria for what constitutes an MSC(121). In 2006 they proposed three requirements:

1: MSCs need to plastic adherent when cultured in vitro

2: MSCs must express CD73, CD90, and CD105 on their cell surface, while showing the absence of CD34, CD45, CD14 or CD11b, CD79α or CD19, and HLA-DR expression.

3: MSCs must be able to differentiate into 3 different cell types, adipocytes, chondrocytes, and osteoblasts when cultured in certain in vitro conditions.

In 2019, the ISCT recommended to further categorize MSCs by adding three additional recommendations: 1) Source of the MSCs to highlight tissue specific properties, 2) clarification that MSCs are 'stromal' instead of 'stem' cells unless evidence could suggest stemness, and 3) that "a robust matrix of functional assays" be utilized to edify therapeutic mechanisms of action. MSCs of bone marrow origin are most commonly isolated and studied, but MSCs have been isolated from adipose tissue(122), endothelium from the umbilical vein(123) and cord blood(124), synovium(125), ligaments(126) and dental pulp(127). This further characterization is necessary to provide contextual evidence due to the heterogeneity of MSC isolation and culture methods, and the uncertainties of MSC identity and function(128). MSC plasticity, self-renewal capacity, homing and immune regulation make them promising therapeutic targets for many diseases including OA.

1.2.2 Migration, cell proliferation and differentiation in tissue

MSCs function in vivo through cell proliferation, multipotency potential, trophic cellular effects, homing and migration, and immunosuppression/modulation. Early research into MSCs hypothesized that MSCs mainly repair tissues though their ability to proliferate and take the place of damaged or dead cells in vivo. MSC migration is thought to involve the expression of integrins, selectins, and chemokine receptors on MSCs(129) that allow them to target and home

in on tissues expressing receptors upregulated in specific tissues and injury. MSCs then migrate across the endothelium where they interact with and cause changes to the cellular environment(130,131). Regulated by and responding to the microenvironment of local tissues, differentiation of MSCs is a complex and intricate process that is not understood well. Current knowledge suggests that MSCs elicit effects through two differing mechanisms. They can differentiate into tissue specific cells necessary to replace damaged or dead cells, for example, Gojo et al showed that direct injection of MSCs into cardiac tissue can cause them to differentiate into cardiomyocytes, endothelial cells, and pericytes(132), and alternatively, MSCs respond to and alter the inflammatory microenvironment through trophic and immunomodulatory effects(133).

Along with bone marrow, adipose, and embryonic tissue derived MSCs, tissue resident MSCs have been discovered in most or all organs. The anatomical distribution and organization of these resident MSC populations are established during development and are maintained and driven by distinctive local tissue signals. These tissue resident MSCs have embryonic origins and populate organs at different times during development. These tissue resident MSCs share phenotypic similarities, however distinct differences in epigenetics are present(134,135). In vivo, MSCs have a unique phenotype caused by signals and cells in the tissue in which they reside(136). Recent analysis of connective tissue lineage capabilities indicated that progenitor cells residing in skeletal tissues can generate osteoblasts or chondrocytes in vivo, while progenitor cells from non-skeletal tissue can't. Transcriptomic analysis also identified differences between tissue-source MSCs, where the increased expression of TGF-β receptor 2, signal transducer and activator of transcription 2, FGF-18 was observed in bone marrow MSCs while skeletal muscle MSCs had elevated levels of bone morphogenetic protein 2, insulin growth

factor 2, and FGF-13(137). These tissue specific MSCs consistently reside next to blood vessels(138).

The multipotential and proliferative properties of MSCs show how valuable they could be for tissue repair. However, many studies observed that treatment with injected MSCs produced poor survival outcomes for the cells, challenging the concept that migration and differentiation into tissue was the main cause of regeneration of damaged tissue(139–142). Research found that many positive outcomes in MSC treatments lacked sufficient quantity of injected MSCs to directly cause the observed improvements(143–146). This led to examination of the paracrine activity for injected or migrated MSCs and how the MSC secretome may exert an effect on tissue regeneration.

Therefore, investigation of the functional properties of MSC has shifted away from the proliferation and replacement of damaged cells as their primary mechanism of action, and towards the paracrine effects they elicit in target cells and tissues. The secretome of a cell consists of its secreted factors, including but not limited to cytokines, growth factors, bioactive lipids, extracellular vesicles and their contents, and microRNA. These secreted factors work to elicit changes in the microenvironment in vivo and through conditioned media in vitro. Therapeutic benefits of MSCs are now associated more with their secretory components(147–151). Due to this, research has expanded into characterizing the MSC secretome, as these components can vary dramatically in composition as well as concentration depending on many factors including MSC phenotype and origin, cellular stimuli and conditions(152,153). Because the secretome depends on these properties, directing the MSC secretome and its reparative qualities can be accomplished through various intrinsic and extrinsic factors. The MSC

secretome can be broadly characterized as having anti-inflammatory and immunomodulatory effects(154).

1.3 Immunomodulation by MSCs

1.3.1 Innate immune regulation by MSCs

The body's immune system maintains homeostasis through immune surveillance, defense and regulatory functions. Injuries, infections and immune dysfunction can cause or exacerbate damaging conditions and lead to an excessive immune response. MSCs modulate the immune system's innate and adaptive pathways through direct cell contact and paracrine effects of their secretome. MSCs interact with natural killer cells (NK cells), macrophages, and dendritic cells. Activated NK cells release cytotoxic factor, perforin and inflammatory cytokines that elicit cytotoxicity(155,156). MSCs have been shown to suppress NK cell proliferation as well as inhibit the secretion of their inflammatory cytokines(156). MSCs also alter the polarization and differentiation of macrophages. Macrophages differentiated into the M1 or inflammatory phenotype, highly express inflammatory factors including TNF-α and IL-1β as they set about phagocytosing and destroying dead cells and invading pathogens. M2 polarized macrophages secrete anti-inflammatory cytokines to control the inflammatory immune response and help repair tissue. MSCs have been shown to inhibit macrophage differentiation into M1 phenotype while encouraging them towards the M2 phenotype. MSCs affect macrophages predominantly through signaling and secretion of cytokines. Proposed mechanisms for this include the increased activity of indoleamine 2,3-dioxygenase (IDO) and its depletion of tryptophan and production of kynurenines, and binding PGE_2 and EP2/EP4 receptors on the surface of macrophages, which causes M2 phenotype shift and an inhibitory effect on inflammation(157). MSC induced polarization towards M2 macrophages is accompanied by the increase in anti-inflammatory IL-

10 and arginase-1 as well as a decrease in the production of pro-inflammatory mediators including TNF-α, IL-12 and IL-1β(158,159). These M2 macrophages then inhibit T cell responses and cause induction of regulatory T cells(160,161) promoting supplementary immunosuppression and effects of MSCs. MSCs have also been shown to hinder the differentiation and effects of monocytes. A reduction in HLA-DR/DP/DQ and CD86 in MSC treated monocytes and macrophages was seen, as well as a functional decrease in phagocytosis and antigen presenting capability(162). When MSCs themselves were phagocytosed, monocytes increased IL-10 and PD-L1 expression in mice(163). Likewise, immunosuppressive effects of MSCs were also observed through apoptosis induced by cytotoxic immune cells(164,165) where the induction of IDO and depletion of phagocytes was observed.

Dendritic cells induce T cell responses and are main contributors of inflammatory cytokines(166,167). These contributors to inflammation can lead to various immunopathological diseases and autoimmunity. MSCs have been shown to inhibit dendritic cell differentiation from hematopoietic stem cells as well as monocytes(168,169). This is accomplished through the downregulation of cyclin D2 delaying monocyte cell cycle progression. MSCs also impair development of DCs and reduce their capacity to activate T cells through decreased expression of HLA-DR(170,171), CD40, OX40L, CD80, CD83, CD86 and increased PD-L1 expression(172). MSCs also shift the expression of CD cytokines on dendritic cells from inflammatory to immunoregulation. This change has been linked to effects mediated by PGE_2, TSG6(173–175), and M-CSF.

1.3.2 Adaptive immune regulation by MSCs

T helper and cytotoxic T cells play a central role in autoimmune and inflammatory disorders. Activated cytotoxic T cells destroy cells and secrete inflammatory cytokines including

IFN-γ and TNF-α. Stimulated T helper cells support dendritic cells to induce cytotoxic T cells through CD40 receptor and ligand interaction. Studies have shown that MSCs can reduce the proliferation of T helper and cytotoxic T cells by cell-cell contact and paracrine mechanisms(176,177). They accomplish this through the expression of Fas ligand and the secretion of monocyte chemotactic protein 1 where T cells are recruited for FasL regulated apoptosis(178). MSCs also mediate T cells through cyclin-dependent kinase inhibitor 1B upregulation and subsequent down regulation of cyclin-dependant kinase 2(179). Based on IDO, MSCs also cause a decrease in secretion of IFN-γ, IL-17, and IL-6, and increase IL-4 and IL-10 by T helper and regulatory T cells(180–182).

Antibody production in the adaptive immune response is mainly from activated B cells. These B cells also regulate immune responses by the secretion of cytokines. MSCs inhibit the proliferation, maturation and antibody secretion of B cells(183). This has been shown to be accomplished through secretion of PGE_2, TGF-β(184), and IL-1RA(185). The suppression of antibody production in B cells by MSCs varies depending on the level of B cell stimulation. When B cells were stimulated to produce antibodies, for example through LPS induction or IFN-γ stimulation, the effects are reduced by MSC treatment(186–188). IDO has been shown to be a mediator here as well, but also includes the effects of secreted IL-35(189) and secretory vesicles. Extracellular vesicles induced negative regulation of the PI3K/AKT pathway involved in proliferation and survival of B cells(190).

MSCs show a great capability for immune regulation and modulation of the functional and phenotypical properties of immune cells. They can inhibit the function and maturation of macrophages and dendritic cells and also cause a shift towards M2 macrophages and regulatory

dendritic cells. These immunomodulatory capabilities indicate their potential for immunosuppressive and healing effects in immune related diseases and injuries(191).

1.4 Activation of immunomodulatory capacity of MSCs

1.4.1 MSC response to in vivo signals in damaged tissues

When tissues are damaged, MSCs home to the site of damage and when they reach the target tissue they secrete a variety of regenerative molecules in the microenvironment. A benefit for MSC based therapies are their ability to home to a site of injury. Tissue resident, circulating, and injected MSCs home to the site of injury by a chemokine gradient. MSCs in the bloodstream utilize a number of receptors and enzymes to attach and migrate across the endothelial layer. MSCs migrate towards numerous signals including insulin like growth factor, RANTES, MDC, and SDF-1(192). When exposed to TNF-α, MSCs increase their migration towards specific chemokines by upregulating their CCR2, CCR3, and CCR4 receptors. IL-8 is also thought to draw MSCs to injured sites(193).

Immunomodulatory factors including TGF-β, HGF, IDO, HLA-G5, PGE_2, IL-6, IL-10, TSG-6 are some of the soluble factors that MSCs have been shown to secrete. TGF-β and HGF inhibit T cell proliferation when stimulated(194). TGF-β also promotes the maturation of naïve T cells into Treg cells to support immune tolerance(195). MSCs respond to IFN-γ by secreting IDO to dampen the T cell response to antigens(196). IDO catalyzes conversion of tryptophan to kynurenine impeding T cell proliferation(197). IFN-γ responding MSCs also secrete PD-L1 that has an inhibitory effect towards adaptive immune response(198). MSCs also secrete IL-6, NO(199), LIF(200), galectin-1, and semaphorin 3a(201) to inhibit T lymphocyte proliferation and maintain T regulatory cells(202). The immunomodulatory effects of MSCs have been

detected during antigen recognition and presentation, and T cell activation stages of the immune response.

MSCs mediate these immune responses through multiple mechanistic pathways. Depending on the cellular microenvironment, MSCs can be either immunosuppressive or immunostimulating. For example, the level of NO production works as a switch in MSC immunomodulation where MSCs promote T cell proliferation when iNOS is blocked. MSC function (i.e. immunosuppressive vs stimulating) also depends on the ratio of iNOS to IDO(203), and can fluctuate between inflammatory states and IFN-γ and IL-2 secretion(204).

The angiogenic properties of MSCs can contribute to healing in ischemic or otherwise damaged tissue repairs(205–207). This complex multi-step process is responsible for forming new blood vessels, requiring growth factors like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), HGF, PGF, MCP-1, SDF-1 and angiopoietin-1(208–214). IL-6, MCP-1, and VEGF inhibited apoptosis. IL-6 directly promotes angiogenesis via survival of endothelial cells(215). MCP-1 is a chemoattractant necessary for angiogenesis(216). VEGF also promotes endothelial cell migration and differentiation through the MAPK PI3K/AKT, Src, and Rac pathways(217). MSCs also stimulate angiogenesis via SDF-1/CXCR4 axis(218). Additional soluble factors promote angiogenesis and restore blood to ischemic tissues but the mechanisms are more uncertain(219).

MSCs can secrete B cell lymphoma 2, survivin, VEGF, HGF, IGF-1, stanniocalcin-1, TBFb, FGF and granulocyte-macrophage stimulating factor that inhibit apoptosis and work to restore tissue homeostasis(220–225). MSCs have been shown to ameliorate hepatic ischemia reperfusion injuries through the MEK/ERK pathway. Likewise, MSCs down regulate Bax, FAS and caspase 3 levels inhibiting apoptosis(226). Indirect inhibition of apoptosis is also seen where

MSC secreted factors have enhanced cell survival. VEGF, HGF, IGF-1, FGF and GM-CSF secreted into MSC conditioned culture media are found when exposed to hypoxic conditions(227).

Reactive oxygen species (ROS) are involved in the regulation of many signaling pathways involving cell proliferation, survival and inflammation(228,229). Imbalance of ROS levels can occur in tissues via aging, carcinogenesis, immune disorders, inflammation, and disease(230). MSCs modulate ROS levels through secretion of STC1, HO-1, and GDNF(231–233). MSCs have been shown to secrete unique antioxidative factors in different microenvironmental settings and tissues and in disease.

MSCs can also exert effects through direct cell-cell contact. MSCs modulate T lymphocytes through the expression of integrins, intercellular adhesion molecules, vascular cell adhesion protein-1, CD72 and CD58 on their cell surface(234,235). They can also express PD-1 ligand on their surface to inhibit T helper cell differentiation, while the expression of galectin-1 and -3 can inhibit T helper and cytotoxic T cells(236). Regulatory T cells can be induced by TLR activated MSC expression of Delta-like 1, a Notch ligand(237). MSCs can also reduce natural killer T cell cytotoxicity through CD73 expression(238). When exposed to inflammatory macrophages, MSCs enhanced TSG-6 production to increase their immunomodulatory effects on macrophages and T cells(239). MSCs also exhibit effects when in contact with endothelial cells, which induces MSC differentiation toward a pericyte phenotype conducive for angiogenesis(240).

Mitochondria play important roles regulating oxidative phosphorylation generation of ATP and cellular apoptosis. When mitochondria become dysfunctional, ROS production increases causing excess oxidative damage in cells(241). Recent evidence suggests that MSCs

work to regenerate damaged cells through mitochondrial transfer. This can occur through cell fusion, gap junctions, tunneling nanotubes, microvesicles and transfer of isolated mitochondria(242–246). This transfer has demonstrated benefits in lungs, allergic diseases, cardiomyocytes, corneal epithelial cells, kidneys, ischemic tissue and spinal cord injury(247–255).

Part of the secretome, extracellular vesicles (EVs) are classified into exosomes, microvesicles and apoptotic bodies based on size and biogenesis. EVs, mostly exosomes, have been the topic of interest for new research. MSC derived EVs have been shown to have myriad effects in many diseases and tissues. The limitations that MSC based therapies possess, including immune rejection, malignant transformation, deleterious differentiation, and cell death, are largely eliminated with EVs. EVs also have the capacity to cross the blood brain barrier to treat neurological disorders(256). EVs can also be modified by MSCs to contain specific biologically active molecules according to their phenotype and response to stimuli. MSC EVs have been shown to be immunomodulatory through the induction of anti-inflammatory cytokines, inhibition of B lymphocytes, mobilization of neutrophils, and polarization of macrophages(257–259). MSCs regulate the microenvironment through multiple secreted molecules and have a large effect on the immune response in tissues.

1.4.2 Characteristics and effects of common activation factors

MSC properties are influenced by biological, biochemical, and biophysical factors, which combine to regulate MSC phenotype, function and survival(260) through interactions between cells, extracellular matrix and soluble molecules. The major current challenge in MSC therapy is to develop in vitro culture methods to culture expand MSCs while exposing them to proper signals that will restore tissue homeostasis and repair damaged tissue. Cell priming or activation

was one of the first approaches where MSCs were exposed to inflammatory factors. Activating MSCs has expanded into several distinct categories including inflammatory factors, hypoxia, pharmacological drugs, biomaterials and culture conditions, and other effective molecules. When activating MSCs with inflammatory stimulators, the goal is to improve MSCs immunosuppressive function prior to in vivo application through the secretion of anti-inflammatory immunomodulating factors.

Interferon gamma was one of the first inflammatory stimulators to be used for activating MSCs. When exposed to IFN-γ stimulation, MSCs have been shown to upregulate expression of IDO, PGE2, HGF, TGFb, CCL2, HLA molecules, and co-stimulatory molecules(261). The type of MSC as well as culture conditions also influence how they will respond to these molecules. Whartons jelly derived MSCs stimulated with IFN-γ causes an upregulation of IDO, HLA, CXCL9, CXCL10, CXCL11, VCAM-1, and ICAM-1(262). INF-γ stimulated MSCs decreased production of IFN-γ and TNF-α, increased IL-6 and IL-10 and decreased T helper cell frequency when co cultured with activated lymphocytes(262). IFN-γ also caused MSCs to inhibit T cell effector functions by upregulating PDL-1 and IDO(263). NK cell activation was suppressed by IFN-γ-MSCs and hindered IFN-γ secretion by NK cells through IDO and PGE2. IFN-γ also increased MSC expression of class I HLA molecules and activating ligand NKG2D to be less susceptible to NK cytotoxic cells(264). Murine MSCs activated with IFN-γ showed an upregulation in STAT1/STAT3 and also inhibited mTOR activity associated with immunoregulation(265). Likewise, inhibition of T cell proliferation and cytotoxic T cell degranulation were accomplished through increased IDO secretion(266). In experimental colitis IFN-γ caused MSCs to increase migration to inflammatory tissues to reduce the amount of inflammation and tissue damage(267).

TNF-α activation has been shown to upregulate immunomodulation through PGE_2, IDO and HGF, however it is less pronounced than IFN-γ priming(268). That has led to the introduction of multiple cytokines to stimulate MSCs. When activating with both TNF-α and IFN-γ, MSCs showed an increase in factor H(269). Likewise, coactivating causes MSCs to produce an M2 polarization in monocytes/macrophages, which then ultimately amplify the immunosuppressive effects by suppressing T cell proliferation through IL-10 secretion(270). TNF-α and IFN-γ co activation inhibited hypersensitivity responses in vivo(271).

IL-17 has also been used for activating MSCs, resulting in enhanced proliferation in both human and murine BM-MSCs in a dose dependent manner(272–274). This increase happened via generation of ROS through ACT-1 and TRAF-6. Likewise, IL-17 caused greater migration and osteoblastic differentiation of MSCs(275). However, other studies indicate conflicting data, with IL-17 demonstrated to promote osteogenic differentiation by enhancing IL-6 and IL-8 expression(276). Conflicting studies support that osteogenic differentiation is both suppressed through IκB kinase and NFκB(277) or does not have an effect on differentiation potential of MSCs(278). IL-17 also increased class I MHC expression, inhibited T cell secretion of inflammatory cytokines and promoted the generation of T regulatory cells(279). This shows that the effects of inflammatory cytokines may also be dependent on the source of MSCs, the phenotype and culture conditions, and homogeneity of MSC cultures.

Inflammatory cytokines and growth factors have been used to activate MSCs isolated from various tissues and species. In general, inflammatory activation has been shown to increase the immunosuppressive effects of MSCs by increasing the secretion of anti-inflammatory and immunomodulatory factors. However, it is important to note that variable responses to these signals is commonly seen(280). The inflammatory modulation of MSCs is due to the detection

and response to pathogenic infectious agents. When these pathogen associated molecular patterns (PAMPS) are recognized by the host's pattern recognition receptors (PRRs), the initiation of the immune system happens rapidly to destroy them. When PAMPs are recognized by cells that have PRRs, including neutrophils, macrophages, and dendritic cells(281–283), complex signaling pathways are elicited to upregulate the immune system to find and eliminate pathogens. Several classes of PRRs are known with Toll like receptors being the most widely studied and considered the primary sensors for detecting pathogens during infections.

TLRs are transmembrane glycoproteins with extracellular domains that are responsible for recognizing PAMPs. 10 human TLRs have been detected and designated depending on where they are located in the cell as well as which PAMPs they can detect. There are cell surface TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10) which are expressed on the cell surface and recognize mainly microbial components including lipids, lipoproteins and proteins. There are also intracellular TLRs (TLR3, TLR7, TLR8, TLR9) that are expressed in intracellular compartments like endosomes, lysosomes and the endoplasmic reticulum that detect viral nucleic acids. TLRs have primarily been thought to have been evolved as sensors of exogenous stimuli however the recognition of endogenous stimuli, like intracellular RNA and DNA, is now seen as playing an important role in regulating inflammation. These endogenous signals can be seen in injury and non-infectious dangerous tissue as well(284). TLR signaling has been implicated in immunomodulation including inflammatory diseases where inflammation is initiated or perpetuated due to continuous TLR signaling(285–287). Alternatively, some TLR signaling can also downregulate inflammation through specific TLR pathways(288).

Signaling pathways activated by TLRs can be generally classified as MyD88 dependent and MyD88 independent pathways. Most TLRs use the MyD88 dependent signaling and activate

NFκB to upregulate inflammatory cytokines(289). However TLR4 uses both dependent and independent pathways while TLR3 only uses MyD88 independent pathway which activates IRF3 to induce type I interferon transcription.

TLRs are expressed by innate and adaptive immune cells(290–292) as well as fibroblasts, endothelial cells, epithelial cells and MSCs which may contribute to their ability to protect against infection(293,294). The expression of TLRs in MSCs was first discovered in mice, where TLRs 1-8 were expressed, but TLR9 was not(295). Likewise, human MSCs also express varying levels of TLRs, based on the origin and environmental conditions of the MSCs. TLR1-6 are generally consistently expressed in MSCs but TLR7-10 have been shown to have varying levels of expression(296–301). The low function and expression of some TLRs seem to be characteristic for early developmental stage MSCs. Hypoxia also increases expression of some TLRs(302). Infection with viral vectors upregulated TLR3 expression and increased its signaling(303). The effect of environmental stimulants significantly modulates and activates TLRs in cells that can respond to those signals.

The recruitment of MSCs to the site of injury is necessary to elicit strong effects. TLR3 stimulation with poly I:C showed a robust response for cell migration(304). TLR3 and TLR4 activation (LPS) cause chemotaxis and increase MSC expression of IL-1β, IL-6, IL-8, CCL5, IP-10, and monocyte chemotactic protein through NFκB signaling(305). TLR2, stimulated by peptidoglycan also increased MCP-1, granulocyte chemotactic protein 2, IL-1β, and macrophage inflammatory protein-3a. Once MSCs have been established at the site of injury, modulating the migration of immune cells to the tissue is important for immunomodulation(306). TLR4 activation caused the secretion of CXCL1, IL-6, IL-8, and CCL2 while TLR3 activation increased only IL-6 and macrophage migration inhibitory factor(307). For leukocyte binding,

MSCs also responded differently to TLR3 and TLR4 stimulation where TLR3 increased leukocyte binding through hyaluronic acid expression and TLR4 increased VCAM-1 and ICAM1 to bind leukocytes(307).

With immune cells at the site of infection/damage TLR activation causes further immunomodulation through various mechanisms. However, the mechanisms and results are highly variable and confounding, even having contradictory results between studies. Alternate polarization states of TLR signaling have been proposed with TLRs that activate and produce inflammatory mediators, and TLRs that produce anti-inflammatory mediators. These states are largely influenced by pathway specific mechanisms as well as strength of activation but there needs to be more research to understand the mechanisms more in depth.

STimulator of Interferon Genes (STING) is another PRR that initiates potent immune responses when recognizing intracellular dsDNA leading to induction of type I interferon pathway responses. This intracellular presence of dsDNA is detected by the cyclic-GMP-AMP synthase (cGAS) which produces the second messenger 2'3'cyclic-GMP-AMP(308) that ultimately activates the protein STING. This activation pathway through IRF3 leads to induction of type I interferons(309). In addition to type I interferon induction, STING also induces NFκB signaling(310). STING provides an alternative to TLR induction of type I interferons, and its pathways with respect to immune activation have not been elucidated completely. Further investigation and comparison of these immune licensing agents is warranted in the context of OA.

1.5 Conclusions

The complex multifactorial pathogenesis of OA, involving innate and adaptive immune mechanisms, remains incompletely understood. Regenerative therapies such as mesenchymal stromal cells (MSC) have therapeutic potential to modulate inflammation associated with OA. Pre-activation of MSC with inflammatory licensing agents may represent one mechanism to direct MSC therapies in the context of specific disease processes of OA, which warrants further investigation mechanistically in vitro and in animal models in vivo.

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CHAPTER 2 - IDENTIFICATION OF AUTOANTIBODIES TO SYNOVIOCYTE AND CHONDROCYTE ANTIGENS IN NATURALLY OCCURRING OSTEOARTHRITIS IN HORSES

2.1 Summary

Investigators exploring mechanisms of osteoarthritis have previously detected autoantibodies in humans with osteoarthritis. These antibodies were found to be specific to cartilage degradation proteins and to extracellular matrix derived proteins. However, there have not been studies done previously to evaluate the presence of autoantibodies to cellular proteins expressed by stromal cells in the joint, including chondrocytes or synoviocytes. Though the pathogenesis of OA and rheumatoid arthritis is very different, there may be some overlap in terms of the role of autoantibodies in the disease processes. For example, a recent study in patients with RA revealed the presence of autoantibodies that recognized antigens produced by joint stromal fibroblasts, in addition to the citrullinated proteins already known to be targets in RA. Thus, the potential overlap in OA and RA immune responses provided the rationale to determine whether autoantibodies to joint stromal cell antigens were present in horses with naturally occurring OA. Therefore, the objective of Chapter 2 was to use immunoassays to determine whether autoantibodies to joint stromal cells were present in plasma and synovial fluid of horses with OA, and to help identify the target antigens.

2.2 Introduction

Osteoarthritis (OA) is a common degenerative joint disease and a leading cause of disability in humans, with the lifetime likelihood to develop symptomatic OA currently 45% and increasing (1). Naturally occurring OA is also commonly exhibited in horses, estimating to affect up to 80% of horses in their lifetime(2). Similar anatomical and mechanical joint characteristics to humans lend translational relevance to the equine model of OA(3). Traditionally, OA was viewed as a degenerative disease resulting from routine wear and tear of the body. However, it is now known to be a multifactorial disorder centered around chronic inflammation. Epidemiological studies have revealed inflammatory synovitis is connected to increased pain, cartilage degradation and progression of OA in joints(4,5). Previous studies investigating the role of the immune system in OA have focused primarily on innate immunity in the synovial joint. Infiltrating cells of the adaptive immune system have also been detected in synovial tissue(6) as well as immunoglobulins in synovium (7,8), suggesting that they play a significant role in the continuation of chronic inflammation in the joint. Antibodies against extracellular matrix derived proteins have been detected in OA that can exert degenerative cytotoxic effects(9).

Recently, antibody recognition of stromal cell antigens has been recognized as a source of the adaptive immune response in rheumatoid arthritis (RA). Although RA and OA pathologies are largely different, some similarities between RA and OA necessitate the investigation into cellular autoantibodies to determine if antibody recognition of joint cell antigens exist in OA. In OA, synovial fibroblasts undergo a phenotypical shift to an activated and inflammatory state comparable to that described in RA(10). Likewise, chondrocytes produce inflammatory mediators and altered protein expression(11). These changes in the function and secretion of key

inflammatory mediators by chondrocytes and synoviocytes, as well as OA driven apoptosis(12) suggest cells may contribute to local autoimmunity by acting as a source of autoantigens similar to RA(13).

Therefore, the purpose of the study was to detect and identify auto-antibodies to joint cell antigens through immunoassay and proteomic analyses using the relevant equine large animal model of OA. Specifically, the first aim was to determine if horses with OA had a detectable adaptive immune response to cellular antigens through measurement of antibodies in plasma or synovial fluid. The second aim was to characterize, isolate, and identify those cellular antigen(s). We hypothesized that antibodies against chondrocyte and synoviocyte antigens would be increased in osteoarthritic horse plasma and synovial fluid. The long-term goal of this study is to further the field's understanding of the adaptive immune response in osteoarthritis towards development of disease modifying treatments for OA.

2.3 Methods

Study Overview – the Institutional Animal Care and Use Committee at Colorado State University (No. CSU IACUC #5672) approved tissue collection for this study. All methods were conducted according to the national guidelines under which the institution operates and NIH Guidelines for the Care and Use of Laboratory Animals (8th edition). One healthy donor horse provided synoviocytes and chondrocytes which were used to identify plasma and synovial fluid autoantibody reactivity from healthy donor horses (n=10) and osteoarthritic horses (n=9). ELISA, flow cytometry, immunocytology, western blot, and proteomics analysis were used to characterize and identify the cellular antigens (Figure 1).

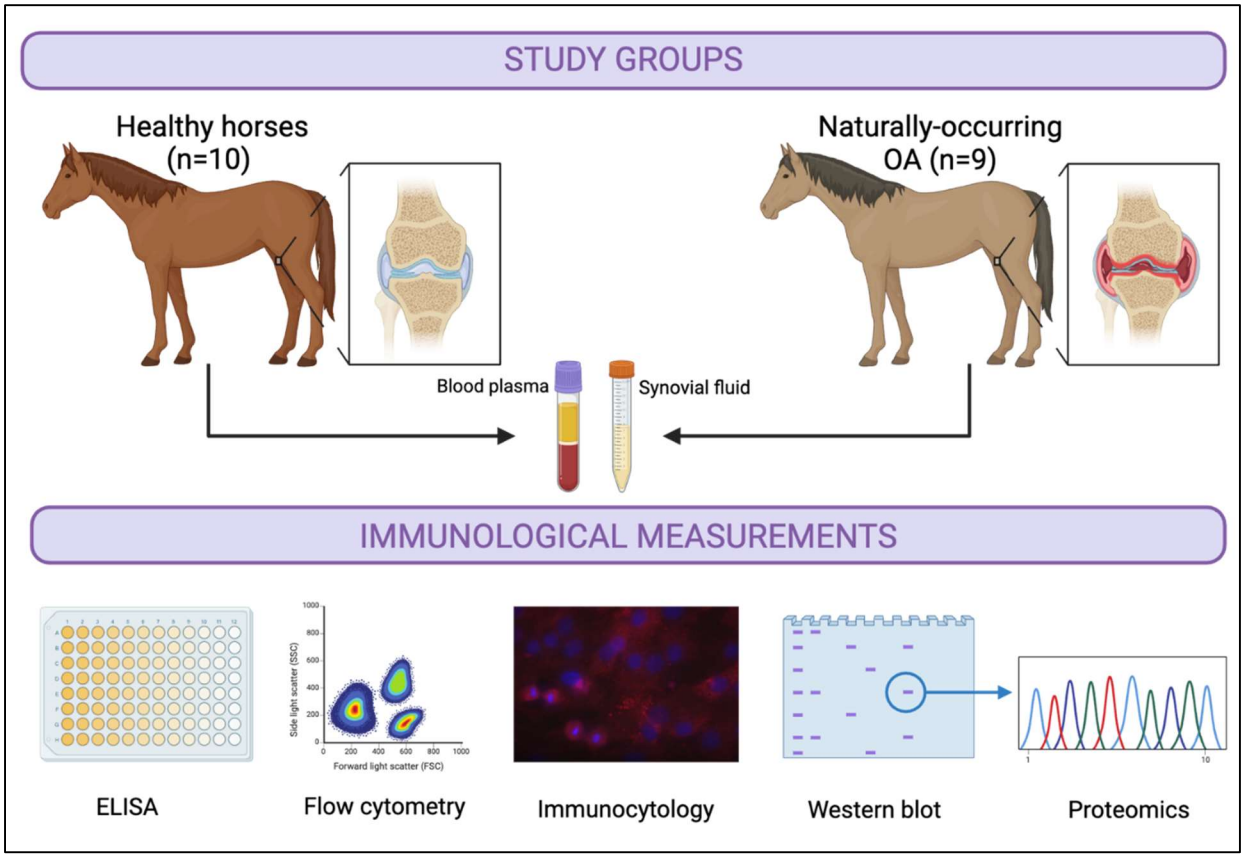


Figure 2.1 – Study overview. Horses (n=10 healthy, n=9 ostoarthritic) were donors of plasma and synovial fluid utilized in immunoassays. Isolated chondrocytes and synoviocytes for these assays were donated by one unrelated healthy horse. Cells and plasma/synovial fluid were utilized in ELISA, flow cytometry, immuncytology, western blot, and proteomics for the characterization of osteoarthritic autoantibody response in vitro.

Chondrocyte and synoviocyte cell culture – Chondrocytes and synoviocytes were donated from one unrelated healthy donor horse. Chondrocytes were isolated from articular cartilage collected from the distal aspect of the femur and caudal surface of the patella. Specimens were minced and digested in complete growth media (Dulbecco's Modified Eagle Medium with 1g/L glucose, 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/ml), 1M HEPES) with type II collagenase (0.75 mg/g cartilage)(Thermo Fisher Scientific, MA, US) overnight at 37 °C and 5% CO_2. Digested tissue was passed through 70 µM and 40 µM

cell strainers (Greiner Bio-One, Austria) and centrifuged at 400 g for 10 minutes. Chondrocytes were counted and plated at 10,000 cells/cm^2 and expanded in complete growth media to second passage where they were detached via trypsinization and cryopreserved in liquid nitrogen at $5x10^6$ cells/ml freeze media (90% FBS, 10% DMSO) for future use(14).

Synovial tissue was acquired from the synovial lining of the femoropatellar joint in 4 sections, dorsomedial, dorsolateral, plantaromedial and plantarolateral aspects of the joint. Tissue was digested as for chondrocytes for 4 hours at 37 °C and 5% CO_2, filtered, centrifuged and counted. Synovial cells were plated at 10,000 cells per cm^2, expanded in culture to second passage and frozen in freeze media at $5x10^6$ cells/mL in liquid nitrogen for future use(14). To generate chondrocytes and synoviocytes for assays, cells were thawed from cryopreservation quickly in 37 °C water bath and cultured 24 hours in complete growth medium under standard incubation conditions (37°C, 5% CO_2).

Plasma and synovial fluid collection - Plasma was collected from healthy donor horse (n=10), osteoarthritic horse (n=9), and OCF model of induced PTOA horse (n=4) whole blood. Osteoarthritis was diagnosed through physical examination and lameness evaluation by board-certified veterinary surgeons and radiographic examination of joints. After blood was drawn in EDTA tubes (BD, NJ, US) plasma was separated from erythrocytes and buffy coat through centrifugation at 2000g for 15 minutes, collected and frozen (-20°C) for future use.

Synovial fluid (SF) was obtained from healthy(n=10) and donor horse (n=9) tibiotarsal joint using an 18-gauge needle and extension set. After collection, SF was digested with hyaluronidase (Sigma-Aldrich, 30ug/ml) for 15 minutes at 37°C, 5% CO_2. SF was centrifuged for 500g for 10 minutes to pellet and remove cells, then frozen at -20°C for future use.

ELISA for detection of chondrocyte and synoviocyte antigens - An ELISA was used to measure the reactivity of autoantibodies against chondrocytes and synoviocyte lysates in healthy and OA plasma. Cell protein lysate was collected from cultured cells lysed with lysis buffer (mammalian protein extraction reagent, 2% SDS, 1mM NaOVa, 1mM PMSF). Quantification of protein concentration was performed with Pierce BCA protein assay kit (Thermo Fisher Scientific, MA, US). Briefly, 1 μg of cell lysate protein was loaded into each well of a 96-well Nunc MaxiSorp ELISA plate (Nalge Nunc International, NY, US) for 12 hours. Wells were washed with ELISA wash buffer (Thermo Fisher Scientific) and then blocked with 5% normal goat serum (Jackson ImmunoResearch, PA, US) in PBS overnight. After blocking, wells were washed with ELISA wash buffer and plasma (diluted 1:100 in PBS, with 1% normal goat serum) samples were added for 2 hours. Plasma samples were discarded, and wells were washed with ELISA wash buffer. Peroxidase conjugated Goat Anti-horse IgG antibody (Jackson ImmunoResearch) was then added (diluted 1:5000 in PBS and 1% normal goat serum). After 1 hour, this antibody was removed, and wells were washed with ELISA wash buffer again. TMB substrate (Sigma-Aldrich) was added and incubated for 10 minutes. TMB stop solution (Sigma-Aldrich) was then added and absorbance values were measured in a spectrophotometer (Agilent Technologies, CA, US) at 450nm.

Flow cytometry for assessment of cell surface antigens - Flow cytometry assessed whether cellular antigens were expressed on the cellular surface. Chondrocytes and synoviocytes cells were grown to 80% confluency in standard culture conditions and then trypsinized and counted. Cells were pelleted by centrifugation for 5 minutes at 500g to remove supernatant, and 50,000 cells per reaction were resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS with 2% FBS and 0.05% sodium azide). Cells were washed with FACS buffer, then sample

plasma was added (diluted 1:100 in FACS buffer with 5% normal goat serum) for 30 minutes. Plasma was removed and cells were washed with FACS buffer, then Cy3-conjugated Goat Anti-horse IgG antibody (Jackson ImmunoResearch) was added (diluted 1:500 in FACS buffer and 5% normal goat serum). After 20 minutes, cells were washed and resuspended in FACS buffer. Samples were then processed on a Gallios flow cytometer (Beckman Coulter, CA, US) and data were analyzed using FlowJo v10 (FlowJo, LLC, OR, US).

Immunocytology for detection of intracellular antigen targets - Immunocytology was used to examine intracellular antigen presence and distribution throughout chondrocytes and synoviocytes. Cells were plated at 80% confluency on 8-well Falcon culture slides (Corning, NY, US) in normal culture conditions. After 24 hours, cells were fixed with 4% paraformaldehyde (Santa Cruz Biotechnology, CA, US) for 10 minutes. Fixed cells were washed with ELISA wash buffer and incubated overnight in a permeating blocking buffer (PBS with 5% normal goat serum, 0.2% Triton X-100). Cells were then washed with ELISA wash buffer and plasma samples were added to the slides (diluted 1:100 in PBS with 5% normal goat serum, 0.2% Triton X-100). After 2 hours, the slides were washed and Cy3-conjugated goat anti-horse IgG antibody (diluted 1:500 in PBS with 5% normal goat serum and 0.2% Triton X-100)(Jackson ImmunoResearch) was added. After 1 hour, slides were washed and stained with DAPI (Sigma Aldrich) for 3 minutes to visualize nuclei. Slides were washed with PBS, dried and mounted using Prolong Diamond antifade mount (Invitrogen, MA, US). Slides were imaged using an IX-83 microscope (Olympus, Japan) fitted with an ORCA 2 camera (Hamamatsu Photonics, Japan) using CellSens Dimension v4.1 (Olympus).

Immunoprecipitation for cellular antigens - Immunoprecipitation of the antigen of interest was performed with Dynabeads linked to protein G (Invitrogen, MA, US). Then 1 µl of

healthy plasma (n=1) or OA plasma (n=1) was applied to Dynabeads linked to protein G (Invitrogen, MA, US). After a 10-minute incubation, the Dynabeads were washed with Dynabead wash solution (Invitrogen) and then 50 μg of chondrocyte protein lysate was applied to the Dynabeads. After 10 minutes, Dynabeads were washed and transferred to a clean microtube. A Dynabead elution buffer (Invitrogen) was used to elute and capture the antigens. For gel electrophoresis, the eluent was mixed with Laemmli sample buffer (Bio-Rad, CA, US) with 10% 2-mercaptoethanol (Bio-Rad) and incubated for 5 minutes at 95 °C. The eluent was then added to Mini-PROTEAN TGX pre-cast gels (Bio-Rad). A power-pac power supply (Bio-Rad) applied 150v to the gel for roughly 60 minutes. Gels were removed from cassettes and were stained with Gel-Code Blue Stain (Thermo Fisher Scientific) according to manufacturer's directions to visualize total protein content.

Western blotting for cellular antigens - Western blot was used to identify the molecular weight of the cellular antigens. Chondrocytes and synoviocytes were grown to 80% confluency, then lysed with lysis buffer as above. For gel electrophoresis, 25 μg of cell lysate was mixed with Laemmli sample buffer (Bio-Rad) with 10% 2-mercaptoethanol (Bio-Rad) and incubated at 95 °C for 5 minutes. The cell lysate was then added to Mini-PROTEAN TGX pre-cast gels (Bio-Rad). Precision plus protein standards (Bio-Rad) were also run alongside lysates. A power-pac power supply (Bio-Rad) applied 150v to the gel for roughly 60 minutes. Gels were removed from their cassette and placed on top of PVDF membranes (Bio-Rad), where they were transferred using a Trans-Blot Turbo transfer system (Bio-Rad) according to manufacturer's specifications. Membranes were washed and blocked with 5% normal goat serum in TBS/T (Bio-Rad) for 1 hour. Membranes were then washed and sample plasma was added at a 1:50,000 dilution in TBS/T with 5% normal goat serum for 2 hours. These membranes were washed with

TBS/T and then a peroxidase-conjugated goat anti horse IgG antibody (Jackson ImmunoResearch) was added at a 1:50,000 dilution in TBS/T, 5% normal goat serum for 1 hour. The antibody was washed off with TBS/T and Clarity Western ECL substrate (Bio-Rad) was added to the membranes. Chemiluminescence was visualized with a ChemiDoc Imaging System (Bio-Rad) using Image Lab (Bio-Rad).

Proteomic analysis of proteins identified by western blotting and immunoprecipitation - To identify the antigen of interest, two procedures were used to isolate proteins of interest. First, gel electrophoresis and western blotting were used concurrently to isolate proteins of a specific weight. 25ug of chondrocyte protein was prepared as in the western blot protocol above. After electrophoresis, half of the lanes for the gel were used for a western blot as above to pinpoint molecular weight and migration of the antigen in the gel. The remaining lanes of the gel were stained with Gel-Code Blue Stain (Thermo Fisher Scientific) according to manufacturer's directions. Visualized protein bands in the gel were aligned with western blot results, and were cut out of the gel, frozen and sent for protein sequencing. The second method employed involved immunoprecipitation. Then 1 µl of healthy plasma (n=1) or OA plasma (n=1) was applied to Dynabeads linked to protein G (Invitrogen, MA, US). The Dynabeads were washed with wash solution (Invitrogen) and then 50 µg of chondrocyte protein lysate was applied to the Dynabeads. After washing again, an elution buffer (Invitrogen) was used to capture the antigens. This eluent was frozen and sent for protein sequencing. Protein sequencing was carried out on a Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) coupled to an EASY-nLC 1200 (Thermo Fisher Scientific) through a nanoelectrospray liquid chromatography–mass spectrometry (LC–MS) interface. Samples were loaded onto a 20 µL loop using the autosampler. The analytical column was then switched on-line at 400 nL/min over an in-house-made 100 µm

i.d. × 150 mm fused silica capillary packed with 2.7 μm CORTECS C18 resin (Waters, MA, US). LC mobile phase solvents consisted of 0.1% formic acid in water (buffer A) and 0.1% formic acid in 80% acetonitrile (buffer B, Optima LC/MS, Fisher Scientific). After 22 μL of sample loading at a maximum column pressure of 700 bar, each sample was separated on a 120 min gradient at a constant flow rate of 400 nL/min. Instrument control and data acquisition were performed using Xcalibur (version 4.5) software. The following parameters were utilized for data-dependent acquisition in positive ion mode: mass range m/z 375–1600, higher energy collisional dissociation (HCD) MS/MS (30% collision energy) using the standard automatic gain. control (AGC) target and a 35 ms maximum injection time with an isolation width of 1.6 m/z. MS1 and MS2 detection at resolutions of 120,000 and 50,000, respectively. Dynamic exclusion was 45 s. Singly charged ions were excluded from HCD selection.

Antigenic analysis of HSP60 with immunocytology and ELISA - Immunocytology was used to examine intracellular HSP60 presence and distribution throughout chondrocytes. Cells were plated at 80% confluency on 8-well Falcon culture slides (Corning, NY, US) in normal culture conditions. After 24 hours, cells were fixed with 4% paraformaldehyde (Santa Cruz Biotechnology, CA, US) for 10 minutes. Fixed cells were washed with ELISA wash buffer and incubated overnight in a permeating blocking buffer (PBS with 5% normal donkey serum, 0.2% Triton X-100). After blocking, wells were washed with ELISA wash buffer and rabbit anti-HSP60 (diluted 1:500 in PBS, with 1% normal donkey serum, Atlas Antibodies, Sweden) samples were added for 2 hours. Plasma samples were discarded, and wells were washed with ELISA wash buffer. Cells were then washed with ELISA wash buffer and Cy5-conjugated donkey anti-rabbit IgG antibody (diluted 1:500 in PBS with 5% normal donkey serum and 0.2% Triton X-100)(Jackson ImmunoResearch) was added. After 1 hour, slides were washed and

stained with DAPI (Sigma Aldrich) for 3 minutes to visualize nuclei. Slides were washed with PBS, dried and mounted using Prolong Diamond antifade mount (Invitrogen, MA, US). Slides were imaged using an IX-83 microscope (Olympus, Japan) fitted with an ORCA 2 camera (Hamamatsu Photonics, Japan) using CellSens Dimension v4.1 (Olympus).

An ELISA was used to measure the reactivity of healthy and OA plasma antibodies to HSP60. 0.1µg of Human recombinant HSP60 (Sino Biological, PA, US) was coated on a 96-well Nunc MaxiSorp ELISA plate (Nalge Nunc International, NY, US) for 12 hours. Wells were washed with ELISA wash buffer (Thermo Fisher Scientific) and then blocked with 5% normal goat serum (Jackson ImmunoResearch, PA, US) in PBS overnight. After blocking, wells were washed with ELISA wash buffer and plasma (diluted 1:100 in PBS, with 1% normal goat serum) samples were added for 2 hours. Plasma samples were discarded, and wells were washed with ELISA wash buffer. Peroxidase conjugated Goat Anti-horse IgG antibody (Jackson ImmunoResearch) was then added (diluted 1:5000 in PBS and 1% normal goat serum). After 1 hour, this antibody was removed, and wells were washed with ELISA wash buffer again. TMB substrate (Sigma-Aldrich) was added and incubated for 10 minutes. TMB stop solution (Sigma-Aldrich) was then added and absorbance values were measured in a spectrophotometer (Agilent Technologies, CA, US) at 450nm.

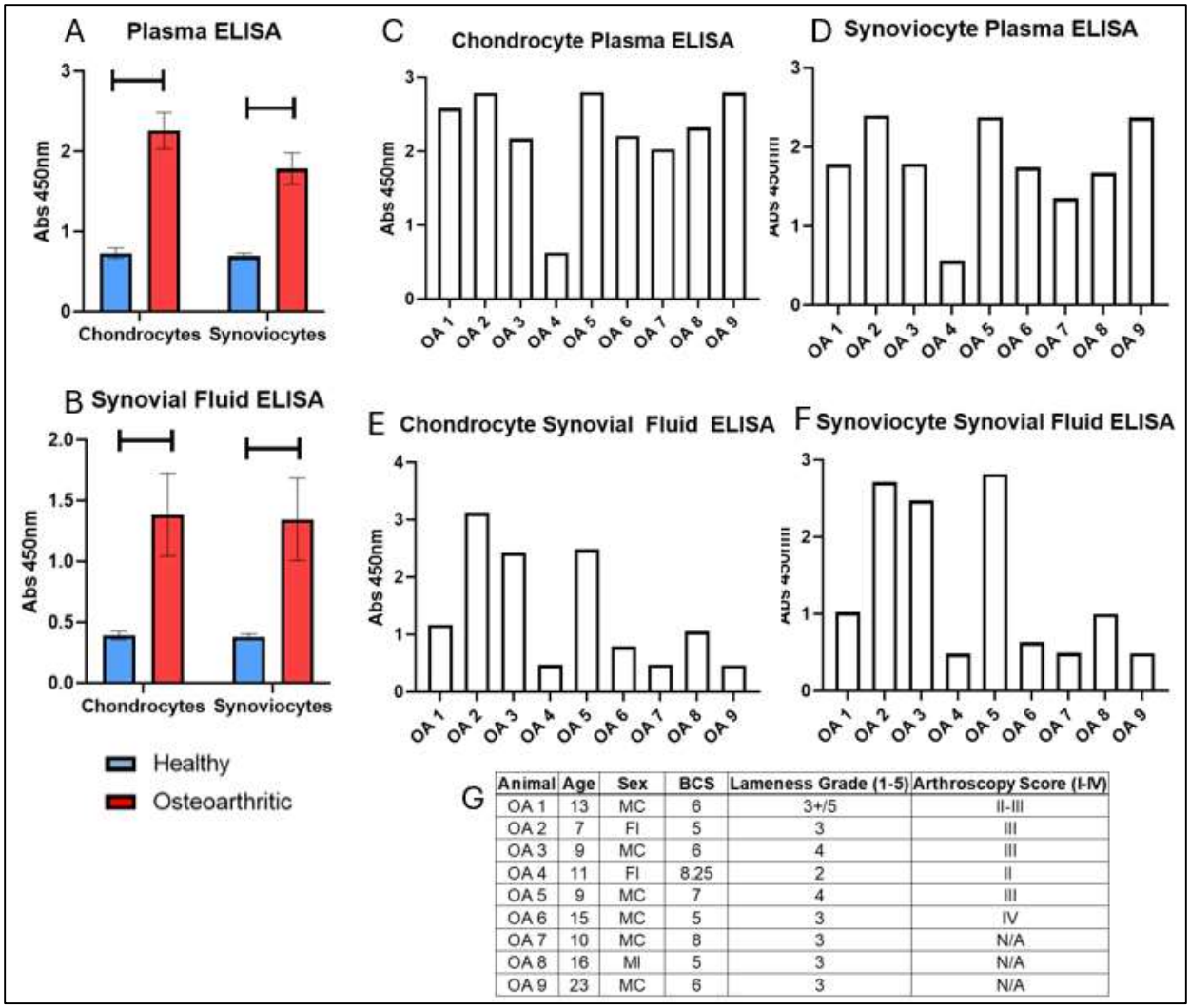
Data Analysis - Data was assessed for normality via Shapiro-Wilk tests, and visual assessment of diagnostic plots. The effect of osteoarthritis on antibody detection in ELISA was evaluated by two-sample t-tests. Correlation between arthritic scoring and ELISA results was assessed via Spearman rank correlation. Statistical analyses, graph analyses and graphical representations were performed using Prism Software v9.1.1. Statistical significance was established as $p<0.05$.

Proteomics data was analyzed on MSFragger. Precursor and fragment tolerance were set at 10 and 15 ppm, respectively. Data was searched against the appropriate UniProt database. Database was adapted with 50% decoys and MSFragger common contaminants. All searches were carried out with trypsin enzymatic cleaveage. Variable modifications were set as the following: methionine oxidation (15.9949), N-terminal modification (42.016), Gln->pyro-Glu (N-term Q). Fixed modification was set as cysteine (57.021465). Match between runs was utilized within MS1 quantification. Searches were performed with all replicates from each sample condition grouped to obtain protein coverage across all analyzed replicates. Results were filtered to 1% FDR at the peptide and protein level. Post processing for proteomics was performed to remove non-equine proteins, proteins with less than 0.99 protein probability, and excluded proteins less than 46 kDa and greater than 67 kDa.

2.4 Results

Antibodies specific for chondrocyte and synoviocyte cellular antigens are present in osteoarthritic horse plasma, as detected by cell antigen ELISA - To screen for the presence of cellular autoantibodies in horses with OA, we first used a cell antigen ELISA for detection of antibodies to either chondrocytes or synoviocytes. This assay was designed to identify antibodies that recognized the total array of cell antigens, potentially both cell surface and intracellular, produced by chondrocytes and synoviocytes, using cell lysates to coat the ELISA screening plates. Using these assays, we found that indeed, autoantibodies to both chondrocyte and synoviocyte antigens were present in horses with OA, but not in healthy horses. In plasma, osteoarthritic horses (n=9) showed a significant increase towards both chondrocyte cell lysate ($p<0.001$) as well as synoviocyte cell lysate ($p<0.001$) (Figure 2.2 A). Moreover, when antibody titers in the individual OA horses were depicted (Figure 2.2 C, D), we found that the plasma

antibody titer may be related to lameness grade and arthroscopy score. Spearman rank correlation for healthy and osteoarthritic horses showed significant correlation in both lameness grade(Figure 2.3A) and arthroscopy score(Figure 2.3B) for chondrocyte ($p=0.023$, $p<0.001$ respectively) and synoviocytes ($p=0.006$, $p<0.0001$). Plasma from equine induced model of post traumatic OA (n=4) were screened at 5 time points (pre-surgery, and 4 times post-surgery up to 126 days). In contrast to naturally occurring OA, plasma from these PTOA horses did not increase reactivity above healthy levels at any timepoint and remained consistent from pre-surgery up to 126 days post-surgery (Figure 2.4).



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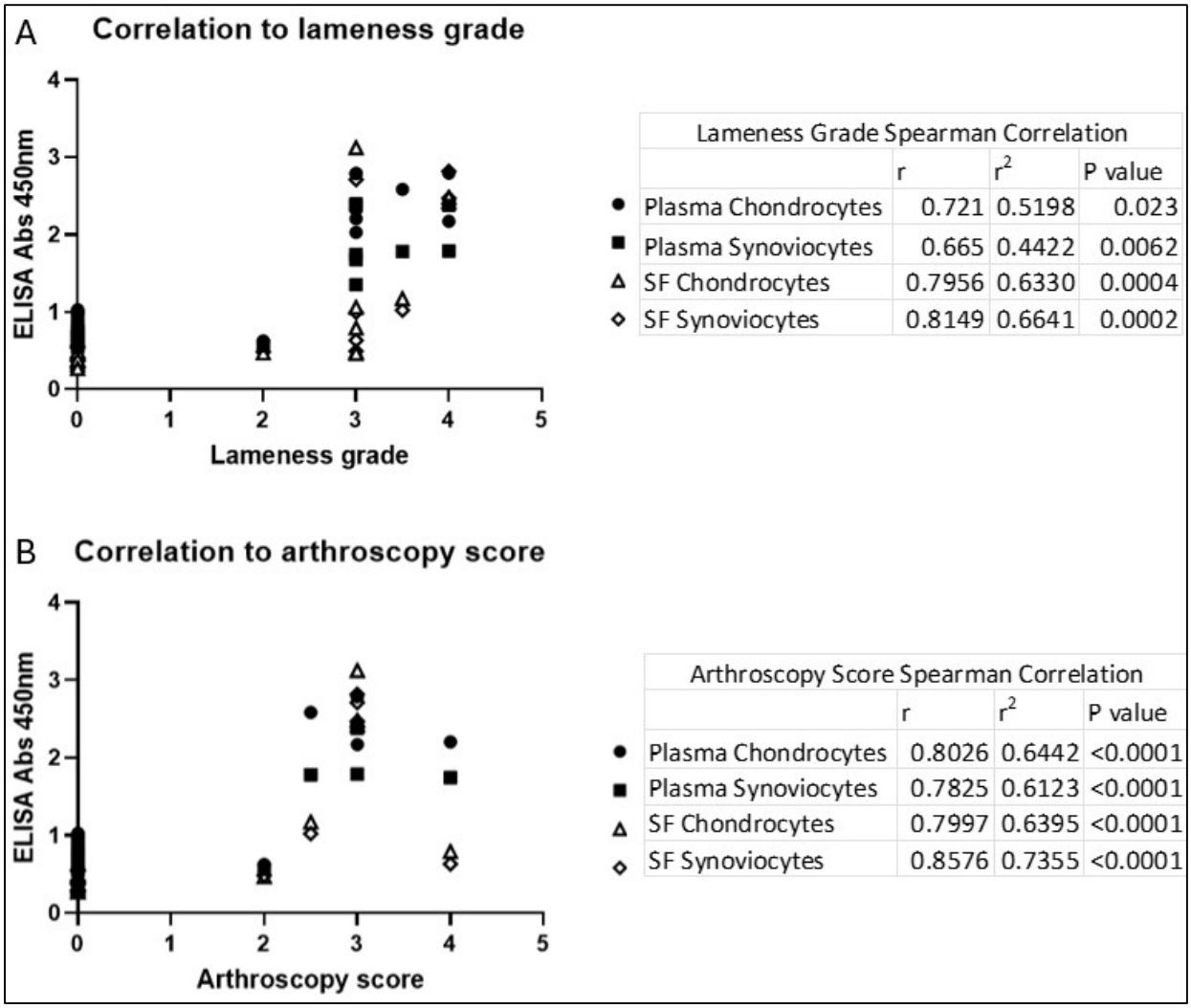
Animal	Age	Sex	BCS	Lameness Grade (1-5)	Arthroscopy Score (I-IV)
OA 1	13	MC	6	3+/5	II-III
OA 2	7	FI	5	3	III
OA 3	9	MC	6	4	III
OA 4	11	FI	8.25	2	II
OA 5	9	MC	7	4	III
OA 6	15	MC	5	3	IV
OA 7	10	MC	8	3	N/A
OA 8	16	MI	5	3	N/A
OA 9	23	MC	6	3	N/A

Figure 2.2 – ELISA results of chondrocyte and synoviocyte cell lysate with naturally occurring OA plasma and synovial fluid. A) Plasma ELISA results show osteoarthritic (n=9) upregulated detection of cell lysate antigens compared to healthy controls (n=10). Both chondrocyte ($p<0.001$) and synoviocyte ($p<0.001$) levels were elevated. B) Synovial ELISA results show osteoarthritic upregulated detection of chondrocyte ($p=0.007$) and synoviocyte ($p=0.008$) C,D) Individual levels of plasma autoreactivity in osteoarthritic animals show variable upregulation. E,F)) Individual levels of synovial fluid autoreactivity in osteoarthritic animals show variable upregulation. G) Osteoarthritic measures of clinical severity for OA horses. Horses OA 7-9 were not measured for arthroscopy score (N/A)



Lameness Grade Spearman Correlation			
	r	r^2	P value
Plasma Chondrocytes	0.721	0.5198	0.023
Plasma Synoviocytes	0.665	0.4422	0.0062
SF Chondrocytes	0.7956	0.6330	0.0004
SF Synoviocytes	0.8149	0.6641	0.0002

Arthroscopy Score Spearman Correlation			
	r	r^2	P value
Plasma Chondrocytes	0.8026	0.6442	<0.0001
Plasma Synoviocytes	0.7825	0.6123	<0.0001
SF Chondrocytes	0.7997	0.6395	<0.0001
SF Synoviocytes	0.8576	0.7355	<0.0001

Figure 2.3 – Correlation results between ELISA and lameness grade and arthroscopy score analyzed by spearman rank correlation. A) Correlation of ELISA to lameness grade show significant correlation to ELISA results when examining plasma in chondrocyte lysate, plasma in synoviocyte lysate, synovial fluid in chondrocyte lysate and synovial fluid in synoviocyte lysate. B) Correlation of ELISA to arthroscopy score also show significant correlation to ELISA in all 4 analyzed groups. Healthy donors (n=10) had lameness and arthroscopy scores of 0. All osteoarthritic donors were analyzed for lameness (n=9) but not arthroscopy (n=6).

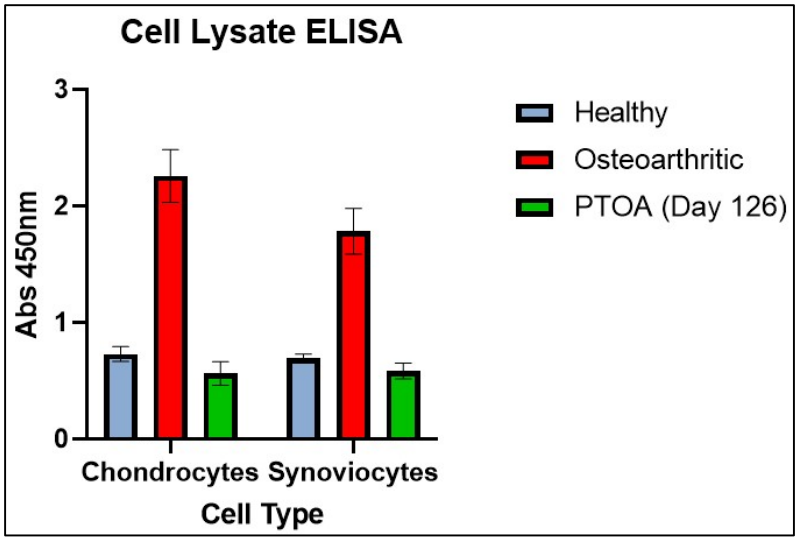


Figure 2.4 – ELISA results of chondrocyte and synoviocyte cell lysate with PTOA model plasma. A) Plasma ELISA results show osteoarthritic (n=9) upregulated detection of cell lysate antigens compared to healthy controls (n=10) and PTOA model (n=4) at 126 days post-surgery. Level of reactivity to chondrocytes and synoviocytes in PTOA model were not significantly different compared to healthy horses.

Antibodies specific for joint stromal cell antigens also present in synovial fluid of horse with OA - The preceding studies determined that autoantibodies were present in blood of osteoarthritic horses, which could result from production by plasma cells present in lymphoid organs (e.g. the spleen) but might also reflect local production within the joint itself. To address this question, we next evaluated SF for the presence of chondrocyte and synoviocyte antibodies with an ELISA as described above. Synovial fluid detection of cellular antigens also showed a significantly increased response in the osteoarthritic group compared to healthy in both chondrocyte cell lysates ($p=0.007$) and synoviocyte cell lysate ($p=0.008$) (Figure 2.2B). When analyzing synovial fluid antibodies in individual horses with OA (Figure 2.2E, F) we found a significant correlation between synovial fluid antibody titers in OA to lameness grade (Figure 2.3A), and arthroscopy score (Figure 2.3B) in chondrocyte ($p=0.0004$, $p<0.0001$) and synoviocyte lysates ($p=0.0002$, $p<0.0001$) similar to plasma. With the discovery of the

upregulation of antibodies specific to chondrocytes and synoviocytes in osteoarthritic plasma as well as synovial fluid, we will want to determine the localization of the antigen in the cell.

Osteoarthritic autoantibodies do not recognize cell surface antigens expressed by chondrocytes and synoviocytes - To further characterize potential antigens recognized by autoantibodies in horses with OA, we used flow cytometry to assess cell surface binding of the antigen. Live chondrocytes and synoviocytes were exposed to plasma (diluted 1:100) and a fluorescent secondary antibody anti-horse IgG (diluted 1:500). The absence of a permeating agent in this assay limited the antigen detection to the cell surface. We saw no increase in fluorescence for either chondrocytes (Figure 2.5A) or synoviocytes (Figure 2.5B) for osteoarthritic plasma, compared to healthy controls and an experimental control. No increase in fluorescence was observed with an increased concentration of plasma (1:10 and no dilution) or increased secondary concentration (1:100, 1:50) (data not shown). The lack of a response to cell surface antigens necessitates the examination of intracellular antigens.

Intracellular antigens are recognized in chondrocytes and synoviocytes by osteoarthritic antibodies - With the absence of cell surface antigen recognition, we next used immunocytology to determine whether OA antibodies detected intracellular antigens in chondrocytes and synoviocytes. Chondrocytes and synoviocytes were cultured on cell culture microscope slides, fixed, permeated and exposed to healthy and osteoarthritic plasma and synovial fluid. In both chondrocytes (Figure 2.6A) and synoviocytes (Figure 2.6B), osteoarthritic plasma detection of intracellular antigens was elevated compared to healthy. Likewise, synovial fluid autoantibody detection is increased in both chondrocytes (Figure 2.6C) and synoviocytes (Figure 2.6D). Visual assessment shows the presence of an intracellular antigen with areas of scattered density throughout the cytoplasm with no nuclear or cell membrane involvement.

Staining patterns were consistent between cell types and antibody source (plasma, synovial fluid). The visualization of an intracellular antigen to autoantibodies in OA compels further studies to identify and isolate this antigen.

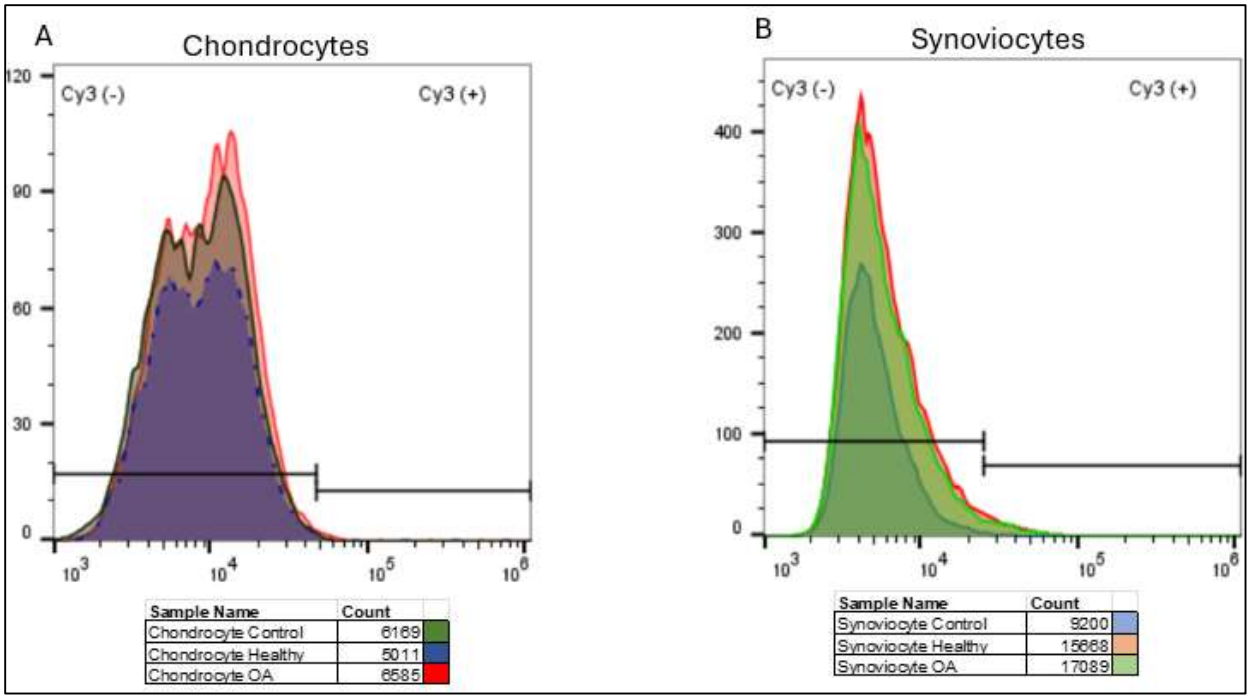


Figure 2.5 – Flow cytometry results for the detection of cell surface antigens in chondrocytes and synoviocytes. Plasma autoantibody reactivity (diluted 1:100) towards chondrocyte (A) and synoviocyte (B) cell surface antigens were analyzed by cell fluorescence detected by Cy3 secondary antibodies anti-horse IgG (diluted 1:500). An experimental control consisting of cells reacting with secondary antibody only was used for gating settings. Experimental groups started with 50,000 cells per reaction, and flow cytometry was stopped when the final number of cells analyzed was >5,000. Percent of positive cells was less than 0.1% for healthy and OA plasma in both chondrocytes and synoviocytes.

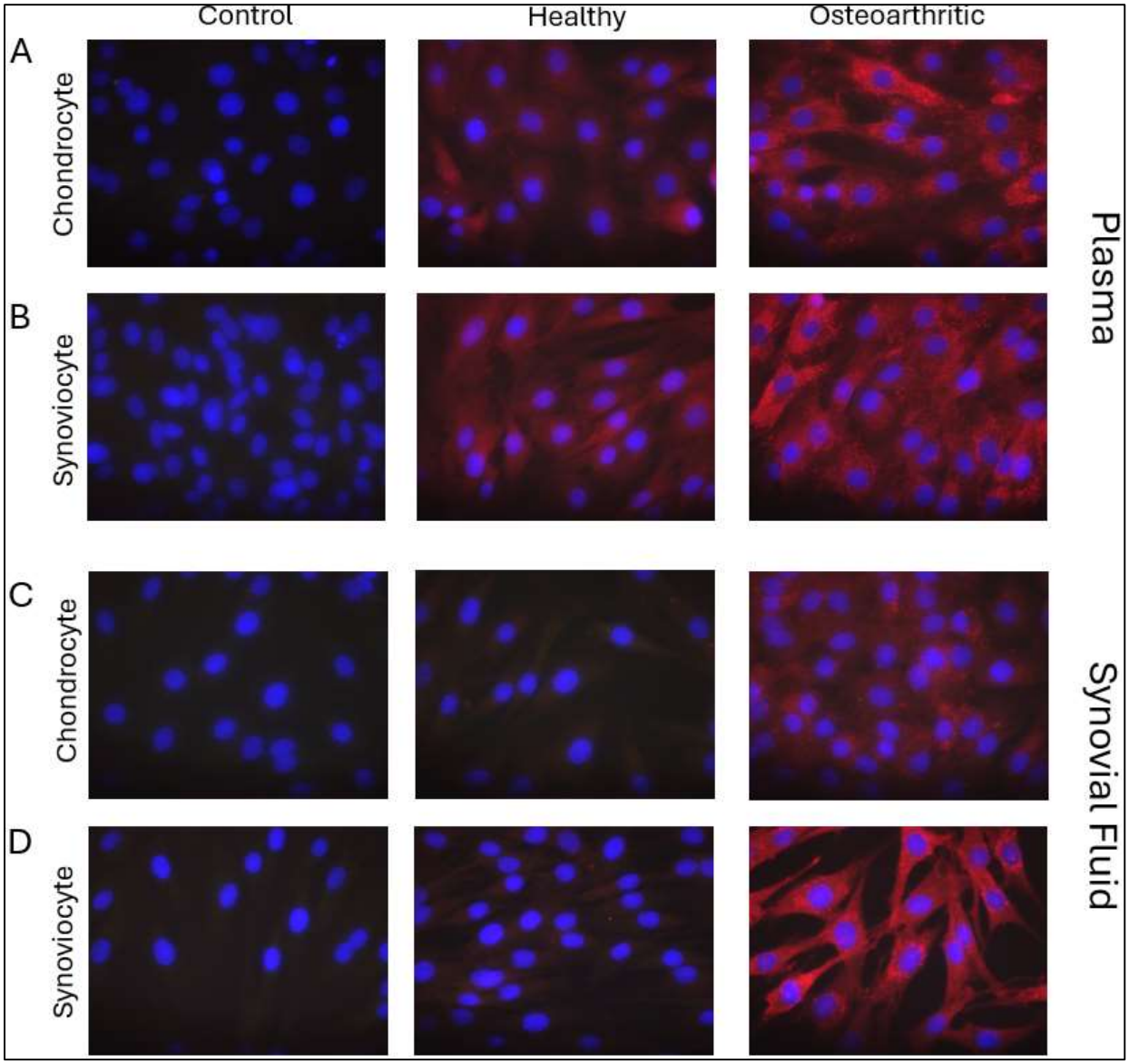


Figure 2.6 – Immunocytology images for the visualization of intracellular antigens in chondrocytes and synoviocytes. Healthy and osteoarthritic plasma autoantibody reactivity (diluted 1:100) towards chondrocyte (A) and synoviocyte (B) intracellular antigens were analyzed by fluorescent microscopy detected by Cy3 secondary antibodies anti-horse IgG (diluted 1:5000). Control samples consisted of fixed cells reactivity to secondary antibody only. Healthy and osteoarthritic synovial fluid reactivity (diluted 1:100) towards chondrocyte (C) and synoviocyte (D) intracellular antigens were also analyzed (secondary antibody diluted 1:5000). Imaging settings remained constant for all images, and image magnification = 40x, DAPI stain is seen as blue, and Cy3 anti-horse IgG is seen as red.

Immunoprecipitation reveals a prominent 60kDa protein recognized by antibodies in horses with OA - With the revelation of an intracellular antigen to OA antibodies, further characterization of the protein of interest is warranted. Dynabeads linked to protein G bind the Fc region of antibodies in OA plasma so they could recognize and bind chondrocyte antigens. After eluting this antigen and separating it through gel electrophoresis, we used a coommassie blue based stain and detected the presence of a prominent 60 kDa protein isolated from chondrocyte cell lysate (Figure 2.7A).

Antibodies recognizing several dominant proteins detected in both plasma and SF of horses with OA - The detection of a 60 kDa chondrocyte antigen found through immunoprecipitation of plasma justified further investigation into the presence of this antigen. Western blot was performed on chondrocyte and synoviocyte cell lysates comparing OA plasma and synovial fluid to healthy plasma and synovial fluid. Western blot membrane imaging shows slight autorecognition of chondrocyte and synoviocyte antigens in healthy plasma at numerous protein sizes (Figure 2.7C). OA plasma displays a similar amount of autorecognition to these numerous protein sizes but possesses an additional pronounced band recognition at 60 kDa (Figure 2.7B). There is also the presence of an additional but less substantial band seen at 45 kDa. Synovial fluid samples showed analogous blotting patterns in chondrocytes and synoviocytes at a decreased intensity, with slight auto-reactivity to numerous protein sizes in healthy and osteoarthritic samples, but osteoarthritic samples displaying a considerable band at 60kDa and secondary band at 45kDa (Figure 2.7D, E). Confirming the presence of a 60 kDa antigen in chondrocytes and synoviocytes as well as a secondary 45 kDa antigen shows the need for protein isolation and identification.

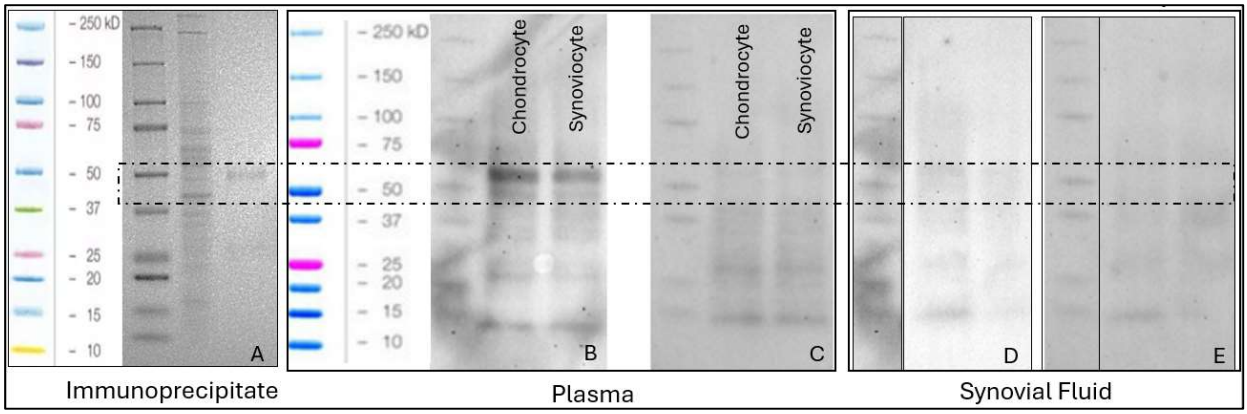


Figure 2.7 – Antigenic protein isolation and characterization by immunoprecipitation and western blot. A) Immunoprecipitation of our antigen was performed using Dynabeads linked to protein G. Coomassie blue based staining and imaging of gel-electrophoresed ladder (lane 1), chondrocyte cell lysate (lane 2) and eluted immunoprecipitate from osteoarthritic antibodies and chondrocyte cell lysate (lane 3). B) Western blot imaging of osteoarthritic plasma towards chondrocyte and synoviocyte cell lysate. C) Western blot imaging of healthy plasma towards cell lysates. D) Western blot imaging of osteoarthritic synovial fluid towards cell lysates. E) Western blot imaging of healthy synovial fluid towards cell lysates. All western blot images were taken under constant conditions. The cropped images of protein ladder in synovial fluid are due to OA plasma and synovial fluid running on the same gel and membrane, utilizing the same protein ladder. Healthy plasma and synovial fluid was also run on the same gel and membrane. Dashed lines were added post imaging to highlight region of interest.

Proteomic identification of potential proteins recognized by OA autoantibodies - In the final series of studies, we used proteomic analysis to identify proteins at the same molecular weight that are recognized by OA autoantibodies to identify potential candidates for the primary antigen. For this analysis, we focused on the 60 kDa protein band identified in western blots (Figure 2.7B) and cut this band out of a chondrocyte lysate gel for sequencing. After removing non-equine proteins and proteins smaller than 46 and larger than 67 kDa, the top 20 proteins represented in our chondrocyte lysate sample are listed according to intensity (Figure 2.8). Three of the top 20 proteins have been found to play substantial roles in the pathogenesis of rheumatoid arthritis: vimentin(15), calreticulin(16), and heat shock protein 60 kDa(17).

Protein ID	Gene	Protein Length	Organism	Description	Protein Probability	Combined Total Peptides	Combined Spectral Count	Combined Unique Spectral Count	Sample 1 Spectral Count	Sample 2 Spectral Count	Sample 1 Unique Spectral Count	Sample 2 Unique Spectral Count	Sample 1 Intensity	Sample 2 Intensity
B3IVM1	PKM	531	Equus caballus	Pyruvate kinase	1	84	900	0	336	562	0	0	1.36E+08	1.39E+08
F7B821	P4HB	510	Equus caballus	Protein disulfide-isomerase	1	55	756	0	362	394	0	0	1.02E+08	1.06E+08
A0A9L0SBW6	PDIA3	512	Equus caballus	Protein disulfide-isomerase	1	67	663	0	299	361	0	0	8.73E+07	8.37E+07
F7D854	CAT	563	Equus caballus	Catalase	1	49	613	97	250	321	27	28	5.10E+07	6.23E+07
F6VUR4	CKAP4	586	Equus caballus	Cytoskeleton associated protein 4	1	38	309	309	150	159	150	159	4.02E+07	3.81E+07
A0A3Q2HXD5	VIM	457	Equus caballus	Vimentin	1	72	983	94	50	44	50	44	2.18E+07	9.45E+06
A0A9L0RBQ7	CALR	412	Equus caballus	Calreticulin	1	26	282	45	117	158	21	17	2.03E+07	1.90E+07
F6Z8W0	WDR1	606	Equus caballus	WD repeat domain 1	1	42	301	26	116	185	12	14	1.67E+07	1.93E+07
P38029		421	Equus caballus	Alpha-1-antiproteinase 2	1	3	19	0	9	10	0	0	1.39E+07	6.69E+06
A0A9L0S8L8	HSPD1	587	Equus caballus	60 kDa heat shock protein, mitochondrial	1	39	252	0	97	155	0	0	1.30E+07	1.60E+07
F7CS30	CAVIN1	421	Equus caballus	Caveolae associated protein 1	1	19	173	91	89	84	46	45	1.13E+07	1.76E+07
A0A9L0TRC2	HNRNPK	463	Equus caballus	Heterogeneous nuclear ribonucleoprotein K	1	25	205	0	89	116	0	0	1.13E+07	1.52E+07
A0A3Q2GXS8	EHD2	543	Equus caballus	EH domain containing 2	1	39	273	0	102	171	0	0	9.33E+06	1.45E+07
A0A3Q2HLJ4	TUBB	492	Equus caballus	Tubulin beta chain	1	27	175	3	78	92	0	0	9.31E+06	6.22E+06
A0A3Q2IAD9	P4HA1	538	Equus caballus	Prolyl 4-hydroxylase subunit alpha-1	1	39	229	0	50	98	0	0	8.20E+06	1.05E+07
A0A9L0R368	WARS1	466	Equus caballus	Tryptophan--tRNA ligase, cytoplasmic	1	32	195	0	71	124	0	0	8.00E+06	7.03E+06
A0A3Q2HWP2	CALD1	537	Equus caballus	Caldesmon 1	1	40	201	0	81	120	0	0	7.55E+06	9.93E+06
A0A9L0R3M8	CCT8	593	Equus caballus	T-complex protein 1 subunit theta	1	30	121	0	58	63	0	0	7.20E+06	4.08E+06
A0A3Q2HGW8	PDLIM5	573	Equus caballus	PDZ and LIM domain 5	1	29	234	0	90	144	0	0	6.58E+06	1.25E+07
A0A9L0R5P4	CAP1	492	Equus caballus	Adenylyl cyclase-associated protein	1	22	161	0	74	87	0	0	6.55E+06	5.53E+06

Figure 2.8 – Proteomic results for proteins isolated with gel electrophoresis. Proteins isolated from the ~60kDa band in chondrocyte lysate were sorted according to intensity (takes into account total number of peptides in support of the protein identification, both unique peptides and commonly shared peptides) with MSFragger. Proteins were excluded if they were non-equine or below 46 and above 67 kDa. The top 20 ranked proteins in intensity are shown.

HSP60 detection bears resemblance to OA autoantibodies in cellular staining but not ELISA results. **–** After identification of possible antigens to these OA autoantibodies, we began studies to try to confirm potential protein candidates. HSP60, a recently confirmed stromal autoantigen detected in RA(17), was first to be examined. Intracellular immunocytology was performed to detect the presence and distribution of HSP60 in chondrocytes (Figure 2.9). Similarities to OA plasma staining in the occurrence and distribution of these antibodies are seen.

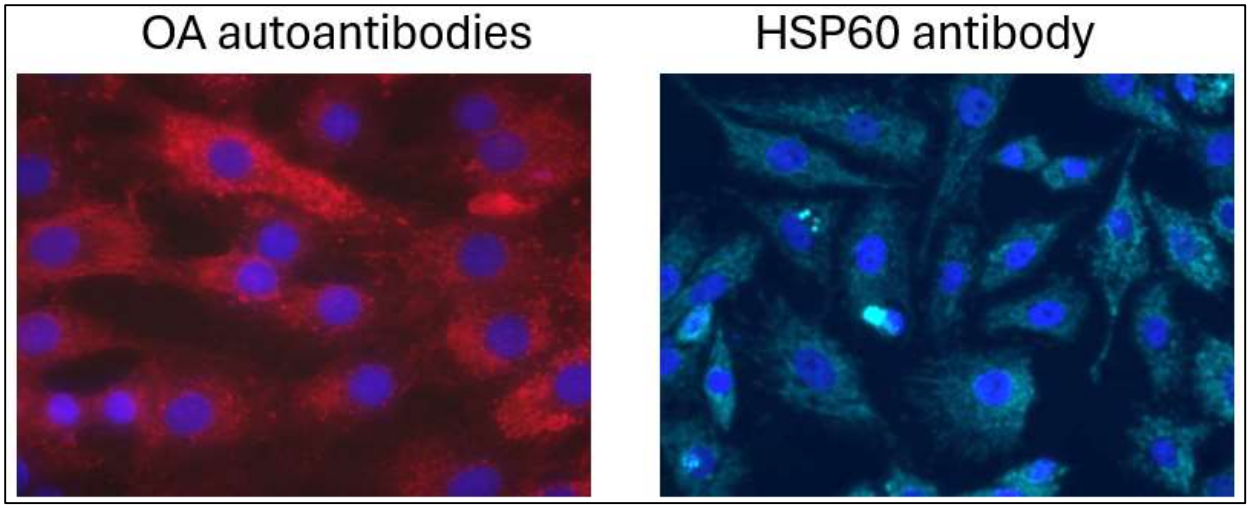


Figure 2.9 – Immunocytology comparison between antibodies in OA plasma and HSP60 antibody. Immunocytology of chondrocytes shows the presence of OA antibodies (left, Cy3) and anti-HSP60 antibodies (right, Cy5). Images taken at 40x magnification.

An ELISA plate was coated with hrHSP60 and 3 healthy and 3 OA plasma samples were used to detect antibody titers. Surprisingly, no differences were detected between the two groups (Figure 2.10). This disparity between intracellular imaging and ELISA warrants further investigation into experimental confirmation of HSP60 and moving to other possible antigens.

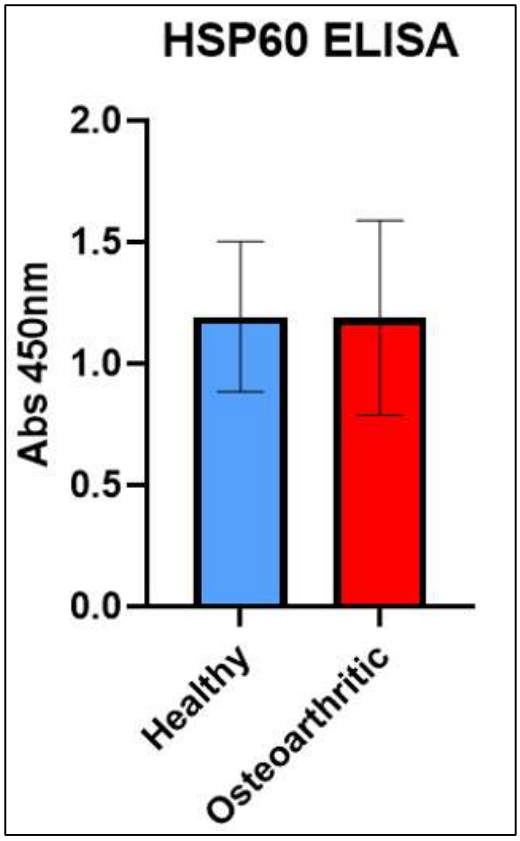


Figure 2.10 – ELISA to compare antibody titers to HSP60 in healthy and osteoarthritic horses. No differences were observed in an ELISA that was performed with healthy (n=3) and osteoarthritic (n=3) plasma to compare antibody titers to hrHSP60 protein.

2.5 Discussion

The identification of antibodies in OA towards intracellular synovial joint cell antigens indicates a previously undetected humoral immune response in OA driven by antigens present intracellularly, and possibly secreted, in joint stromal cells (synoviocytes) and cartilage cells (chondrocytes). To investigate cellular antigens and their recognition by OA autoantibodies, we utilized various immunoassays for antigenic detection, characterization, and identification.

Here we first screened the cellular autoantibodies for their reactivity to chondrocyte and synoviocyte whole cell lysate in ELISA. We observed an upregulation in autorecognition of

cellular antigens in plasma and synovial fluid from osteoarthritic horses that correlated to their lameness grade and arthroscopic score. This reactivity was confirmed and further characterized by using multiple methods: (a) flow cytometry for cell surface antigens, (b) immunocytology using Cy3 conjugated secondary antibody to detect and visualize horse IgG intracellularly, (c) immunoprecipitation using protein g linked Dynabeads to bind antibody from plasma and antigen from chondrocytes, (d) western blot with OA plasma and synovial fluid in chondrocytes and synoviocytes, and (e) immunoprecipitate and western blot isolation of antigen for proteomics identification. The presence of autoantibodies towards 60 kDa and 45 kDa intracellular antigens in chondrocytes and synoviocytes in naturally occurring OA represents a new avenue to define the pathology and inform future treatments for osteoarthritis.

In the past, the detection of autoantibodies in OA was primarily focused on cartilage derived proteins. Autoantibodies against the cartilage derived osteopontin(18), YKL-39 (19), collagen(20), cartilage intermediate layer protein (21), aggrecan (22), and fibulin-4 (23) have all been characterized. Autoantibodies towards citrullinated proteins, a post translational modification, have also been detected in OA, however in a low percentage (~5%) of patients(24,25). The deposition(26) and cytotoxic effects(9) of these autoantibodies against cartilage derived proteins shows autoantibodies may play an important role in cartilage degeneration.

Research in rheumatoid arthritis may help inform us of potential antigens and treatment options. While rheumatoid factor is the main autoantibody in rheumatoid arthritis, there are other autoantibodies in rheumatoid arthritis comparable to OA, including especially antibodies to citrullinated(27) and carbamylated(28) proteins and cartilage derived proteins like collagen(29,30). A recent study also described the presence of autoantibodies directed against

joint stromal cell antigens in patients with RA(13,17) providing precedent for stromal cells as targets for autoimmune responses. The detection of synovial B cells that recognized stromal derived HSP60, and subsequent treatment with rituximab to deplete these autoantibody producing cells demonstrated a clinically relevant potential treatment path. Initial ELISA results for HSP60 do not confirm it as the antigen of interest. The examination of HSP60 antibodies in human osteoarthritic serum showed a non-significant elevation, due to extreme variability in measured titer levels, in serum(31). This extreme variability may be related to the correlation we observed between elevated arthritic indexes and autoantibody titers. Other pathogenic antibodies towards antigens observed in ~60kDa protein cellular isolate are observed in rheumatoid arthritis. Vimentin, a 54 kDa intermediate filament protein is immunomodulatory(32), induced by inflammatory cytokines(33), involved in wound healing(34), and is the target of antibodies in RA, ankylosing spondyloarthritis, and pulmonary fibrosis, among others(15). Calreticulin, also involved in RA pathology plays a role in the clearance of dying cells and interacts with dendritic cells to modulate immune regulation(35). Other proteins seen in our proteomics results were involved in inflammation and autoimmune disorders including pyruvate kinase(36), catalase(37), caveolae-associated protein(38), and caldesmon(39) also make them prospective candidates. With HSP60 results being ambiguous, further analysis, as well as analysis of other potential cellular antigen candidates is necessary. Ultimately, reductions in either expression of antigen or a decrease in antibody production in OA may exhibit reparative effects in its progression.

The intracellular nature of these antigens provides a new dimension to the already discovered extracellular autoantibodies against cartilage antigens in OA. Autoimmune diseases that involve autoantibodies to extracellular target proteins are ideal cases to deplete B lymphocytes to reduce levels of autoantibodies(40). With extracellular antigens, a significant

reduction in pathogenic autoantibodies typically results in clinical improvement. Autoantibody responses to intracellular antigens usually involve T-cell mediated degeneration rather than direct antibody associated damage. However, antibodies can also enter cells to create injury, suggesting a combination of antibody injury and cytotoxic T cell injury to these cells. Treatment of intracellular vs. extracellular autoantibodies may require somewhat different strategies, including suppressing both the development of autoantibodies to intracellular antigens and immune activated T cells and B cells, their signaling pathways, and regulating cytokine mediated immune activation(40).

The presence of autoantibody capable of binding to the cytosolic antigens suggests these autoantibodies may show increased cellular disruption and cytotoxicity compared to cartilage derived antigens. Chondrocytes, responsible for cartilage homeostasis, and synoviocytes, which secrete factors into synovial fluid including lubricating molecules and cytokines are highly connected to inflammation and degeneration in the joint. The disruption of cellular processes of these cells has been linked to an increase in cartilage degeneration and progression of OA(41–44). Other autoimmune diseases with intracellular targets exhibit specificity towards essential cellular proteins, including structural proteins, splicing machinery, RNA polymerases and enzymes(45). They have wide ranging effects depending on their target.

Fluorescent microscopy (Figure 2.4) showed dispersion of the antigen throughout the cytosol with concentrated points of antibody, no apparent evidence of reactivity to nuclear proteins or cell surface proteins, or cell membrane proteins was seen, which suggests these are not polyreactive autoantibodies. Likewise, the absence of obvious antibody binding to nuclear antigens suggests that these are not primary targets. The appearance of autoantibodies targeting these cytosolic antigens may be mechanistically explained by an increase in antigen release from

dying cells, breakdown in the regulation of B or T cell activation threshold, protein modification due to inflammation, or unsuccessful clearance of apoptotic cells and bodies(46).

Research into the immune mechanisms in OA shows an increase in inflammatory cytokines(47–49), cellular infiltrates(50–52), and subsequent synovial and cartilage tissue responses(10,53,54). Inflammatory cytokines present in OA, including IL-1β, TNF-α and IL-6, are upregulated and contribute to the pro-inflammatory environment seen in OA. These cytokines cause the upregulation of MMPs(49,53), induce apoptosis(55,56), cause generation of reactive oxygen species(57), cartilage degeneration(49), and synovial inflammation(58) that are responsible for releasing cellular antigens from catabolic tissue. Infiltrating mononuclear cells containing T cells have been observed in OA(59). Lymphocytic aggregates in synovium exhibited early, intermediate, and late activation markers suggesting the presence of an active adaptive immune response. Similarly, infiltrating cells have also been reported to contain activated B cells(60). The inflammatory nature of the synovial joint combined with antigenic release and the presence of adaptive immune cells suggests the initiation and production for these autoantibodies happens locally.

Moving forward, identification and confirmation of the antigen of interest is a primary objective, which will inform on the function of the antigen and the role that this autoantibody plays in cell function in the context of OA. Modulating the expression of this antigen could play an important role in reducing the adaptive immune effects related to its expression. Likewise, reducing the production/concentration of the autoantibody may result in beneficial effects if they exhibit cytotoxic effects or the antigen is being hindered from producing cellular effects that would be favorable for OA repair. Examining the expression levels of this antigen in synovial joint tissue could provide contextual evidence for which cells are involved and may be affected

the greatest. Alternatively, illuminating the mechanisms for the expression and upregulation of this antibody in OA would provide knowledge towards the pathogenesis of the humoral immune system in OA. Finally, the role of the innate immune system needs to be analyzed for its effects on this adaptive immune response.

Caveats to study design include a small donor horse sample size, the nature of in vitro cultures, the non-uniform state of OA severity in study horses, and the lack of a study population matched for age, but without OA. Different donor horses were used to isolate chondrocytes and synoviocytes than ones that donated plasma and synovial fluid for immunoassays. This in vitro model is not fully representative of the multitude of conditions encompassing OA and does not capture the chronic inflammatory response exhibited by a degenerated joint. The inflammatory milieu present in OA most likely modulates the antigenic expression of the cell, and certainly has an effect on T cells and B cells. Without measuring the cellular response to these autoantibodies in vitro or in vivo we are unable to determine their influence on cell function or the role they play in degeneration, though there is ample precedent in RA for the damage induced by autoantibodies. For example, antibody binding to RA antigens in the joint has been associated with complement activation and recruitment of inflammatory neutrophils and monocytes, which leads to cartilage injury(61–67). Further studies to elucidate these effects should be performed with an emphasis on their role in the pathogenesis and treatment of OA.

2.6 Conclusions

The discovery of autoantibodies that recognize intracellular antigens in animals with naturally occurring OA represents an important finding that has not been previously reported in OA. Examining their effects on cell function and tissue degeneration would be important to further define their role in progressive OA Elucidating the pathogenesis for these autoantibodies

can inform potential future treatments for OA, and possibly other arthritic diseases. Targeting the immune cells that initiate or drive this autoantibody response could be an effective alternative to traditional and ineffective OA treatments.

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CHAPTER 3 - INNATE IMMUNE PATHWAY ACTIVATION TO MODULATE MESENCHYMAL STROMAL CELL INTERACTION WITH SYNOVIUM AND CARTILAGE

3.1 Summary

Mesenchymal stromal cells have emerged as a potential therapeutic option to treat osteoarthritis (OA). Priming MSCs with immune stimulants may increase immunomodulatory properties and homogeneity of cell products and therefore treatment efficacy. We have shown pre-activation of MSC with Toll-like-receptor (TLR)-3 polyinosinic:polycytidylic acid (pIC) and STimulator of Interferon Genes (STING) ligands improves outcomes in a murine model of OA and investigate that further mechanistically here using an equine in vitro culture system. The objective of Chapter 3 was to use relevant in vitro bioassays to compare the anti-inflammatory and immunomodulatory properties of TLR3- or STING- immune activated MSC supernatants on synoviocytes, chondrocytes and macrophages.

3.2 Introduction

Osteoarthritis (OA) is a common degenerative joint disease in companion animal species and humans, resulting in pain and socio-economic burden. Estimated to affect up to 80% of horses over 15 years old, and one third of people over 65, OA is the second most costly health condition treated in the US(1). The comparable disease prevalence and rate of progression, articular cartilage loading forces, cartilage thickness and joint volume between horses and humans, and greater ease with which to obtain equine synovial tissues for modeling makes studying spontaneously occurring OA using horse tissues in vitro a valuable translational model for both species(2–5). Despite the high frequency and economic burden of OA across species, there are no approved interventions or treatments to mitigate or reverse joint degeneration.

Regenerative therapies, including mesenchymal stromal cells (MSCs), have gained increasing recognition for their therapeutic and immunomodulatory potential in OA but have reportedly variable efficacy which has been attributed potentially in part to heterogeneity between donors and within culture populations. Priming MSCs with immune stimulants that activate pattern recognition receptors (PRRs) on their surface such as TLR-3 agonist polyinosinic:polycytidylic acid (pIC) may increase homogeneity of cell products and therefore treatment efficacy(6–11). We have recently shown pre-activation of MSC with two PRRs, Toll-like-receptor (TLR)-3 polyinosinic:polycytidylic acid (pIC) and STimulator of Interferon Genes (STING) ligands improves histologic and functional gait outcomes in a murine model of OA(12). Agonism of STING receptors has been previously described to induce production of Type I interferons in immune and sensory cells following tissue injury, with demonstrated potential to be both inflammatory and antinociceptive depending on the context in which it is injected(13,14). While STING has been evaluated in the context of nociception and regulation of

neuropathic pain and injected locally in other animal models, our recent work represents the first use of STING to stimulate MSC to induce an immunomodulatory response in the context of OA(13,15–17). Current understanding of OA supports a key role to innate immune effector cells to regulate and perpetuate low-grade inflammation in disease, which may be modulated by treatment with stromal cell therapy. Myeloid cell lines (*e.g.,* macrophages), which represent the most common cell type in synovium, are thought in particular to propagate inflammation through sustained release of inflammatory mediators in response to damage-associated molecular patterns released in the joint(18–20). We hypothesized that the observed beneficial effect of STING could be through induction of differential gene expression and cytokine secretion by activated MSC secreted cargo on key joint effect cells (*e.g.,* macrophages, synoviocytes, and chondrocytes). We sought to investigate that observed effect further mechanistically here using an in vitro co-culture system designed to simulate the in vivo joint environment.

Therefore, the objective of this study was to use relevant in vitro bioassays to compare the anti-inflammatory and immunomodulatory properties of TLR3- or STING- on equine MSC themselves and that of immune activated MSC supernatants on synoviocytes, chondrocytes and macrophages using cytokine and transcriptomic readouts. Our analysis revealed induction of interferon related pathways and suppression of pro-inflammatory cytokines relevant to OA progression. These findings indicate potential benefit to 'immune licensing' of MSC prior to in vivo injection in OA although mechanism varied between agonists and readout cell lines in vitro. Overall, this work provides key insights to further our understanding of the interaction between licensed MSC products with joint cell lines relevant to their application in orthopedic disease.

3.3 Methods

Study overview – The Institutional Animal Care and Use Committee at Colorado State University (No. CSU IACUC #5672) approved tissue collection for this study. All methods were conducted according to the national guidelines under which the institution operates and NIH Guidelines for the Care and Use of Laboratory Animals (8th edition). Horses (n=3 total) were donors of bone marrow aspirate for mesenchymal stromal cell culture, which were activated with either poly I:C (TLR3) or 2'3'cGAMP (STING), conditioned media were generated, and cells were submitted for transcriptomic analyses to determine gene expression following activation, as described below. Activated or non-activated conditioned media were then applied to key joint cells (synoviocytes, chondrocytes, macrophages) from one healthy donor horse and cells were assessed for relative cytokine secretion and transcriptomic expression (Figure 3.1).

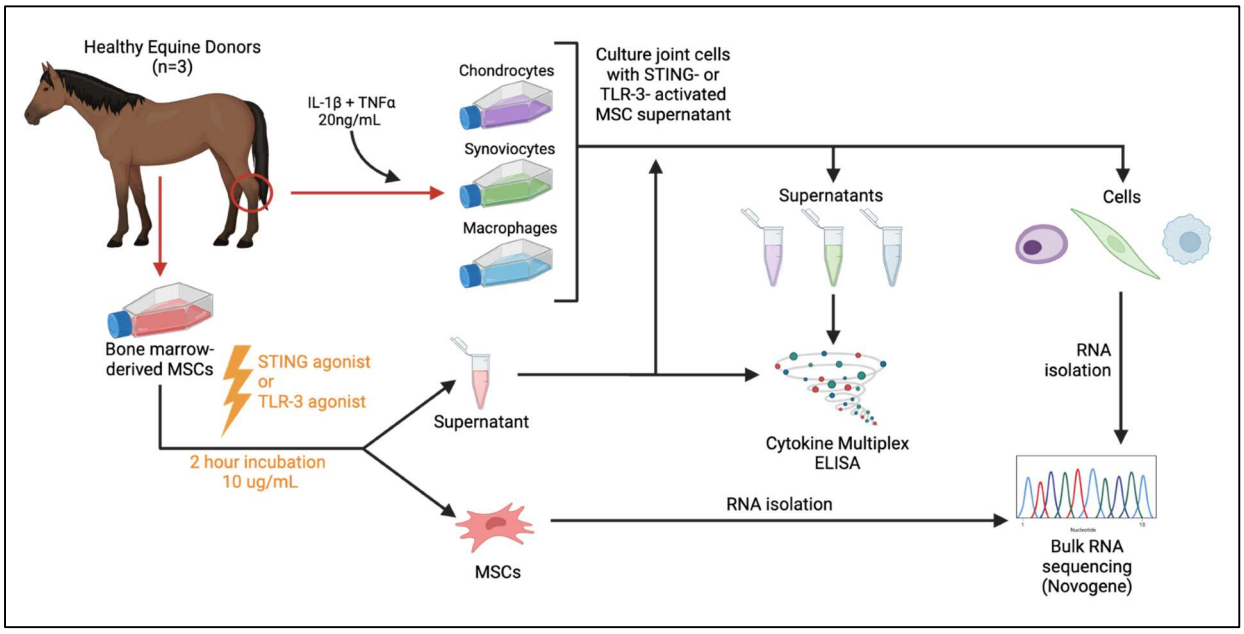


Figure 3.1 – Study overview. Horses (n=3 total) were donors of bone marrow aspirate for mesenchymal stromal cell culture (MSC), which were activated with either poly I:C (TLR3) or 2'3'cGAMP (STING) and conditioned media were generated. Activated or non-activated conditioned media were then applied to key joint cells (synoviocytes, chondrocytes, macrophages) and cells were assessed for relative cytokine secretion and transcriptomic expression following application.

Mesenchymal stromal cell culture – MSCs were isolated from bone marrow aspirates collected from the sternum of donor horses (n=3). Briefly, approximately 5-10 mL of bone marrow aspirate was obtained from the sternebrae using a Jamshidi needle (BD) into a syringe containing 1ml heparin (5,000 U/mL). Mononuclear cells were isolated by density separation (Ficoll-Paque Plus; GE Healthcare BioSciences) at 400 g for 18 minutes (Radcliffe, Schnabel), plated at 10,000 cells/cm^2, and expanded in culture media to 80% confluency (Dulbecco's Modified Eagle Medium with 1g/L glucose, 10% fetal bovine serum, penicillin (100U/ml), streptomycin (100ug/ml), 1M HEPES). Cells were detached via trypsinization and cryopreserved in liquid nitrogen at 5x10^6 cells/mL freeze media (90% FBS, 10% DMSO) for future use. To generate MSC conditioned supernatant, cells were thawed in a 37°C water bath, plated and cultured 24 hours in complete growth media, activated with poly I:C (10 μg/mL) or 2'3'cGAMP (10 μg/mL) for 2 hours, washed with PBS, then replated on 24 well-plates at 100,000 cells/well. After 24 hours, conditioned supernatants were collected and frozen in –20°C, and cells were lysed and frozen in RLT lysis buffer (QIAGEN) for RNA analysis.

Synovial cell isolation and treatment with conditioned media – Synoviocytes were obtained from synovium of one horse; briefly, synovium was excised from the femoropatellar joint, minced and digested for 4 hours in complete media (Dulbecco's Modified Eagle Medium, 10% FBS, penicillin (100units/mL), streptomycin (100 μg/mL), 1M HEPES) with type II collagenase (0.75 mg/mL), as previously described(21,22). Digested synovium was filtered through 70 μM and 40μM cell strainers where cells were counted, plated and expanded to second passage, then cryopreserved in freeze media (90% fetal bovine serum (FBS), 10% DMSO). For treatment, synoviocytes were thawed in 37°C water bath and recovered overnight in complete media. Cells were plated at 100,000 per well in a 24 well plate.

Synoviocytes were stimulated with IL-1β (20 ng/mL) and TNF-α (20 ng/mL) and treated with activated MSC conditioned culture media (MSC CM) at a ratio of 1:3 MSC CM:complete media(21). The stimulated and treated synoviocytes were cultured for 24 hours, then washed with PBS, and cultured for an additional 24 hours in complete media. At that time, synoviocyte supernatant was collected and frozen at –20°C for cytokine analysis (multiplex bead assay and ELISA immunoassay), and synoviocytes were collected in RLT lysis buffer and frozen at –20°C until RNA isolation was performed.

Chondrocytes were isolated from cartilage from the femur and caudal surface of the patella of the femoropatellar joint, as previously described(21). Cartilage tissue samples were digested as described for synoviocytes overnight, passed through cell strainers, and expanded in culture to second passage where they were cryopreserved. Chondrocytes were stimulated and treated as synoviocytes above, and supernatant and chondrocytes in RLT lysis buffer were collected as above.

Macrophages were isolated from whole blood of one horse, peripheral blood mononuclear cells were isolated by density gradient centrifugation (Ficoll-Paque TM plus, GE Healthcare BioSciences) and cultured in macrophage media (Dulbecco's Modified Eagle Medium with 1g/L glucose, 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100μg/ml), 1M HEPES) supplemented with human M-CSF (PeproTech) at 30 ng/ml for 5 days(23).

Monocyte-derived macrophages were stimulated with IL-1β (20 ng/mL) and TNF-α (20 ng/mL), and treated with activated MSC conditioned culture media at a ratio of 1:3 MSC CM:macrophage media. Controls included non-stimulated macrophages, and macrophages stimulated with IL-1β/TNF-α with no MSC treatment. The stimulated and treated macrophages

were cultured for 24 hours, then washed with PBS, and cultured for an additional 24 hours in macrophage media. At that time, macrophage supernatant was collected and frozen at -20°C for cytokine analysis (multiplex bead assay and ELISA immunoassay), and macrophages were collected in RLT lysis buffer and frozen at –20°C until RNA isolation was performed.

Cytokine and PGE_2 concentration determination - MSC and treated cell supernatants were analyzed for cytokine concentrations using a bead-based multiplex assay (Milliplex MAP Equine Cytokine/Chemokine Magnetic Beads Multiplex Assay, Millipore Sigma). The multiplex assay was used to quantify the concentrations of 23 analytes (Eotaxin/CCL11, FGF-2, Fractalkine/CS3CL1, G-CSF, GM-CSF, GRO, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8/CXCL8, IL-10, IL-12, IL-13, IL-17a, IL-18, IP-10, MCP-1, RANTES/CCL5 and TNF-α). ELISA immunoassay (PGE_2 high sensitivity ELISA kit, Enzo Life Sciences, TGF-β quantikine ELISA, R&D Systems, Inc.) was used to measure TGF-β and PGE_2 concentrations.

Transcriptomic analyses - RNA was sent to Novogene Corporation Inc. for bulk RNA sequencing. Briefly, RNA was extracted from frozen samples in RLT lysis buffer using the RNeasy kit (Qiagen) according to manufacturer's instructions. Total RNA sample quality and quantity was verified with Agilent 5400 Fragment Analyzer system (Agilent). RNA integrity number ranged from 7.3~ 9.7. Library was constructed with Abclonal Fast RNA-seq Lib Prep Kit V2 (ABclonal Technology). Samples were sequenced on NovaSeq X Plus, 150 bp paired end sequencing.

Data analysis - Cytokine data was assessed for normality via Shapiro-Wilk tests, and visual assessment of diagnostic plots. The effect of activation of MSC supernatant treatment on cytokine production was evaluated by one-way ANOVA with post-hoc Tukey's adjustment for multiple comparisons (normal data) or Kruskal-Wallis test (non-normal data). Statistical

analyses, graph analyses and graphical representations were performed using Prism Software v9.1.1. Statistical significance was established as $p<0.05$.

RNAseq data was analyzed on Partek Flow v10 (Illumina, Inc. San Diego, California). Median read for 33 samples was 47709688 pre alignment, sequences were trimmed for min Pred value of 20, adapters were trimmed with CUTADAPT v1.12(24). Trimmed reads were aligned with STAR - 2.7.3a(25) with reference genome Equab3.0. Aligned reads were counted with HTSeq v0.11.0 (26) with Ensembl gene annotation 108. Features were filtered for "protein coding" and lowest total coverage of 10 counts, DESeq2 was used for differential analysis(27). Median ratio normalized counts were used for pathway analysis using GSEA v4.3.2. Gene sets used include Hallmarks, Reactome, WikiPathways, KEGG and Biocarta (UC San Diego).

3.4 Results

MSCs respond in related but un-equivalent ways to activation by TLR3 or STING agonists with changes in cytokine secretion and transcriptome favoring interferon responses. Out of 23 equine-cross reactive cytokines, TLR-3 did not cause any significant changes, while STING increased IP-10 (interferon γ-induced protein 10kDa) secretion ($p=0.0068$) (Figure 3.2); other detectable cytokines included IL-1B, IL-6, IL-8, GRO and RANTES which were not significantly different between activated and resting MSCs (Figure3.2). The remaining cytokine levels were below detectable limits.

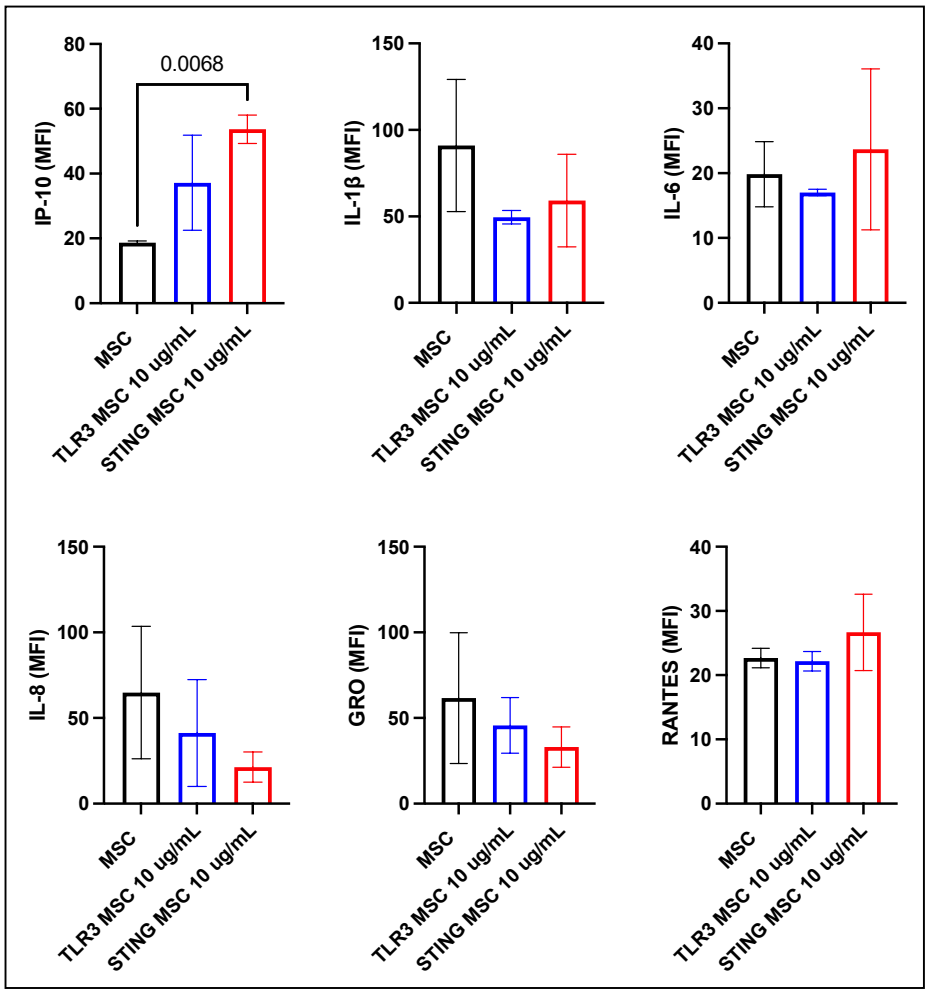


Figure 3.2: Cytokine secretion in activated MSCs. IP-10 cytokine secretion results of untreated MSC compared to either TLR3- or STING-MSC. Statistical analysis using one way ANOVA with Tukey Post test. P value < 0.05 considered significant. Non-significant measurements of detected IL-1β, IL-6, IL-8, GRO, RANTES also shown. Data is represented as mean fluorescent intensity.

Principle component analysis for RNA transcriptomic analysis shows no clear clustering either by treatment or individual horse. (Figure 3.3). Activation of bone marrow MSCs showed significant upregulation of 76 protein coding genes (TLR3 agonist, Figure 3.4A) and 99 genes (STING agonist, Figure 3.4C). The top upregulated genes are similar in the TLR3- and STING-MSCs. Of the top 10 genes, 9 are shared between the groups (IFIT3, RTP4, CXCL10, DDX60L, DHX58, ISG15, HERC6, BST2, MX1). Only STING showed significantly downregulated genes,

C1H15, ITGAL, and CCL20 (Figure 3.4B, D). GSEA shows the TLR3 activation upregulated genes that are mapped to pathways mostly involving interferon signaling, as well as cell cycle and DNA response (Figure 3.4E). Modulation of interferon signaling pathways was also the main effect of STING activation, but in addition, STING activation also downregulated pathways related to protein translation and synthesis (Figure 3.4F).

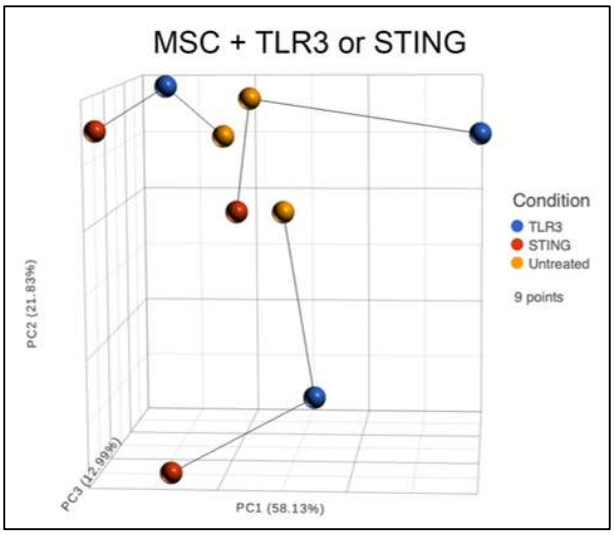
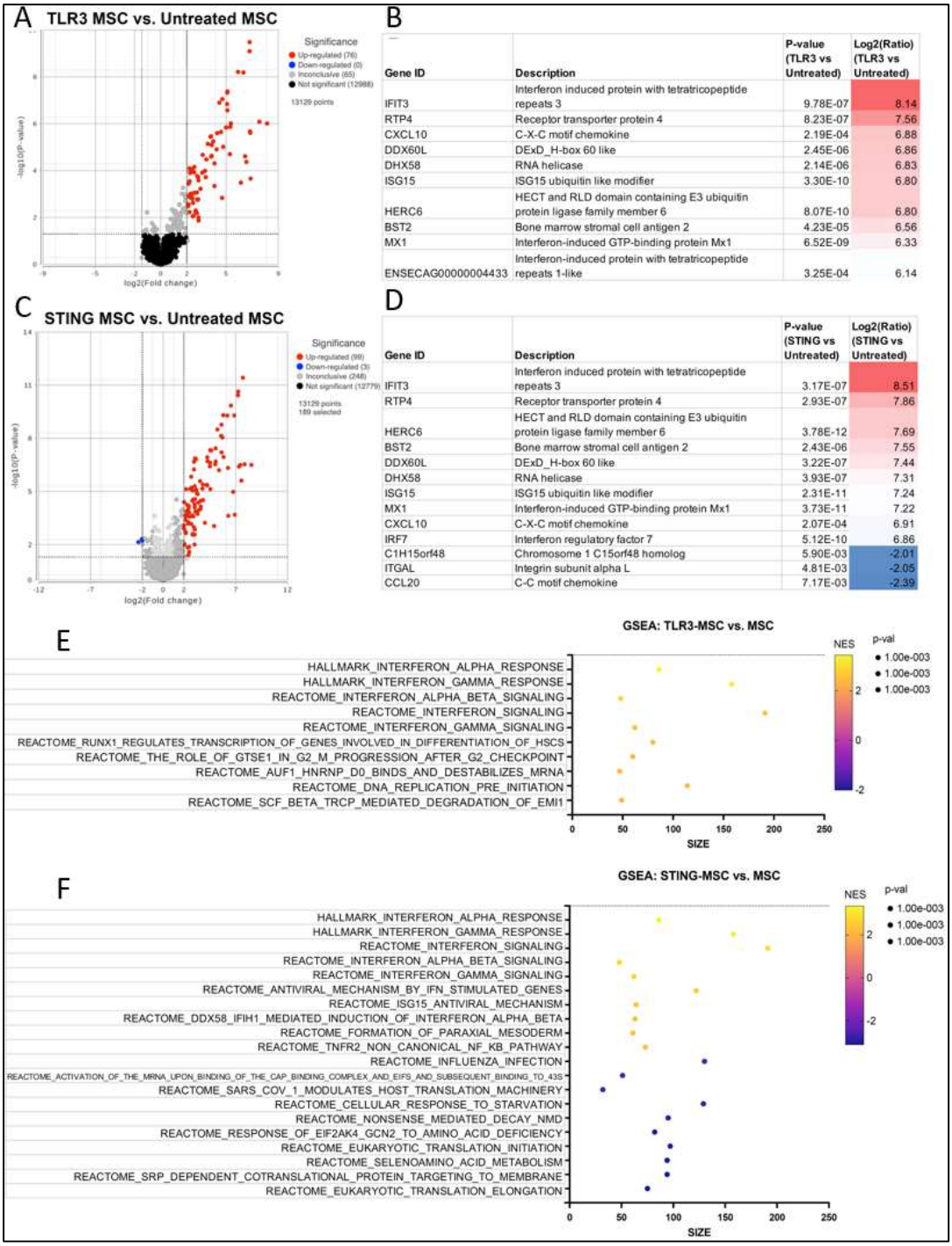


Figure 3.3: Transcriptome of MSC treated with innate immune stimulants. Principle component analysis plot (PCA) of dimensionality reduced samples of n=3 biological replicates of MSC activated with either TLR3 agonist (blue), STING agonist (red) and untreated (yellow). Lines show connecting biological replicates



B

Gene ID	Description	P-value (TLR3 vs Untreated)	Log2(Ratio) (TLR3 vs Untreated)
IFIT3	Interferon induced protein with tetratricopeptide repeats 3	9.78E-07	8.14
RTP4	Receptor transporter protein 4	8.23E-07	7.56
CXCL10	C-X-C motif chemokine	2.19E-04	6.88
DDX60L	DExD_H-box 60 like	2.45E-06	6.86
DHX58	RNA helicase	2.14E-06	6.83
ISG15	ISG15 ubiquitin like modifier	3.30E-10	6.80
HERC6	HECT and RLD domain containing E3 ubiquitin protein ligase family member 6	8.07E-10	6.80
BST2	Bone marrow stromal cell antigen 2	4.23E-05	6.56
MX1	Interferon-induced GTP-binding protein Mx1	6.52E-09	6.33
ENSECAG00000004433	Interferon-induced protein with tetratricopeptide repeats 1-like	3.25E-04	6.14

D

Gene ID	Description	P-value (STING vs Untreated)	Log2(Ratio) (STING vs Untreated)
IFIT3	Interferon induced protein with tetratricopeptide repeats 3	3.17E-07	8.51
RTP4	Receptor transporter protein 4	2.93E-07	7.86
HERC6	HECT and RLD domain containing E3 ubiquitin protein ligase family member 6	3.78E-12	7.69
BST2	Bone marrow stromal cell antigen 2	2.43E-06	7.55
DDX60L	DExD_H-box 60 like	3.22E-07	7.44
DHX58	RNA helicase	3.93E-07	7.31
ISG15	ISG15 ubiquitin like modifier	2.31E-11	7.24
MX1	Interferon-induced GTP-binding protein Mx1	3.73E-11	7.22
CXCL10	C-X-C motif chemokine	2.07E-04	6.91
IRF7	Interferon regulatory factor 7	5.12E-10	6.86
C1H15orf48	Chromosome 1 C15orf48 homolog	5.90E-03	-2.01
ITGAL	Integrin subunit alpha L	4.81E-03	-2.05
CCL20	C-C motif chemokine	7.17E-03	-2.39

Figure 2: Transcriptome of MSC treated with innate immune stimulants. A) Volcano plot of differential gene expression analysis comparing protein coding genes in the TLR treated MSC to resting MSC. Significance defined as unadjusted pvalue <= 0.05. Fold Change (FC) >= $2Log_2$ or <= -2 Log_2. Significantly upregulated genes shown in red, significantly downregulated genes shown in blue. B) List of top 10 significantly upregulated genes in panel A comparison. C) Volcano plot of differential gene expression comparing STING activated MSC to unactivated MSC. D) list of significant genes from panel E, top 10 upregulated and all 3 of the significantly downregulated genes. E) Gene set enrichment analysis (GSEA). Top 10 highest enrichment score (NES) upregulated pathways comparing TLR treated MSC to resting MSC. X-axis denotes the number of genes found in corresponding pathways (size). Enrichment score colored from purple (-2 downregulated) to yellow +2 enrichment score, upregulated. Size of dots show p-value for significance. All pathways shown filtered for unadjusted p-value of <=0.05 and FDR adjusted p-value of 0.25. F) GSEA of top 10 up and downregulated pathways comparing STING treated MSC to resting MSC.

Activated MSC supernatant reduces secretion of inflammatory cytokines associated with cartilage degradation in synoviocytes and chondrocytes – Synoviocytes exhibited a trend towards reduction of IL-8 when treated with STING-MSC supernatants ($p=0.09$), as well as a trend towards reducing pro-inflammatory IL-6 levels ($p=0.09$). Non-activated MSC treatment ($p=0.003$) and TLR3-MSCs ($p=0.02$) showed a significant reduction in IL-6 secretion in synoviocytes. TLR3-MSCs also reduced levels of IL-1β ($p=0.02$), which has catabolic effect in chondrocytes (Figure 3.5A). Other detected cytokine levels were not-significantly different between treatment groups (FGF-2, eotaxin, G-CSF, IL-1α, GM-CSF, fractalkine, IL-13, IL-5, IL-18, IL-17A, IL-2, IL-4, GRO, TGF-β, PGE_2) or were below detectable limits of the assay (IFNγ, IL-10, IL-12, IP-10, MCP-1, RANTES/CCL5 and TNF-α).

Chondrocytes showed a similar but significant reduction in the pro-inflammatory IL-18 when treated with non-activated MSC ($p<0.001$) and TLR3-MSC supernatants ($p=0.002$) compared to IL-1β/TNF-α stimulated controls (Figure 3.5B). The inflammatory chemokine IL-8, increased in patients with OA, was trending towards a reduction when chondrocytes were treated with STING-activated MSC supernatants ($p=0.06$). Other detected cytokine levels were not-significantly different between treatment groups (FGF-2, eotaxin, G-CSF, IL-1α, GM-CSF, fractalkine, IL-13, IL-5, IL-1β, IL-6, IL-17A, IL-2, IL-4, IL-12, IFNγ, IP-10, GRO, MCP1, IL-10, TNF-α, RANTES, TGF-β, PGE_2).

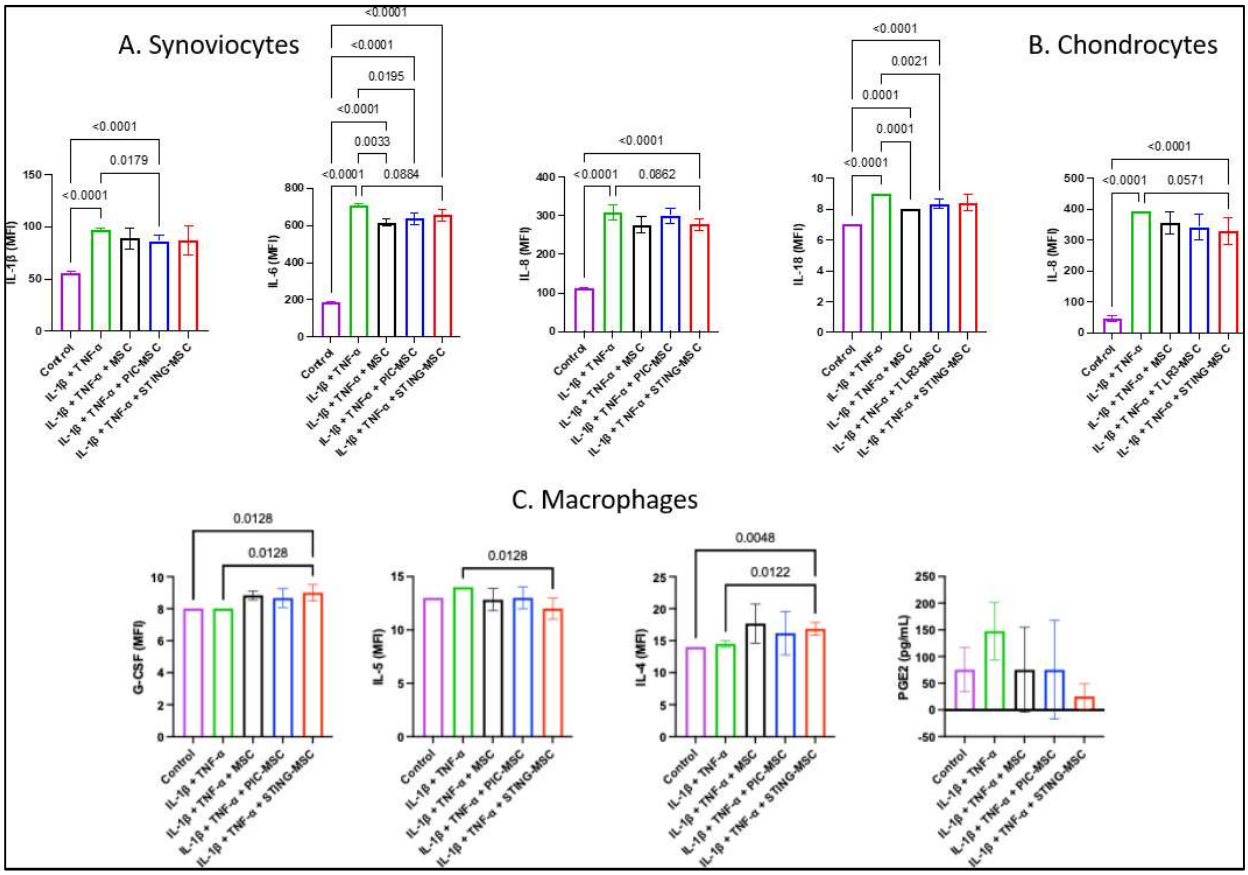


Figure 3.5 – Cytokine and PGE_2 expression in cell culture supernatants of joint cells treated with activated MSC supernatants. Fluorescent bead-based multiplex assay (Milliplex MAP Equine Cytokine/Chemokine Magnetic Beads Multiplex Assay, Millipore Sigma, Burlington, MA, USA) and ELISA ELISA PGE_2 high sensitivity ELISA kit, Enzo Life Sciences, TGF-β quantikine ELISA, R&D Systems, Inc.**)** were used to quantify concentrations of 23 analytes in cell culture supernatants of synoviocytes, chondrocytes, and macrophages treated with activated MSC conditioned media. Significant differences were noted in IL-1β, IL-6, and IL-8 levels for synoviocytes, IL-18 and IL-8 for chondrocytes, and G-CSF, IL-5 and IL-4 for macrophages. Results reported in MFI; * significance was assessed at $P<0.05$.

STING-MSCs modulate macrophage inflammatory cytokines – Macrophage cytokine concentrations were measured after activated MSC treatment, as with chondrocytes and synoviocytes. Significant differences were seen with STING-MSC treatment, where significant increases in markers commonly associated with M2 (reparative) macrophages, G-CSF (p=0.01), and IL-4 (p=0.01), were seen (Figure 3.5C). IL-5 was also significantly downregulated by STING activated MSC treatment (p=0.01). Trends towards reduction in pro-inflammatory PGE_2

were seen with all MSC treatments, with STING-MSC treatment showing a trend to below control cells unstimulated with IL-1β and TNF-α. Other measured cytokines were not significantly different between treatment groups (Eotaxin/CCL11, FGF-2, Fractalkine/CS3CL1, GM-CSF, GRO, IFN-γ, IL-1α, IL-1β, IL-2, IL-6, IL-8/CXCL8, IL-10, IL-12, IL-13, IL-17a, IL-18, IP-10, MCP-1, RANTES/CCL5, TNF-α, TGF-β).

Synoviocytes treated with activated MSC supernatant show increased interferon signaling and DNA replication – Synoviocytes, chondrocytes or macrophages were treated with activated and unactivated MSC supernatant. For each comparison, n=3 horses were used for the donor cells. Synoviocytes treated with TLR3-MSC supernatant showed significant differential expression of a total of 40 protein coding genes. Interestingly, similarly to the chondrocytes presented next, upregulated pathways in synoviocytes included mostly interferon signaling; while downregulated pathways included those related to cell cycle, DNA replication, and chromosome dynamics, translation, ribosome function, and protein targeting as well as a few metabolic pathways (Figure 3.6A, D, G).

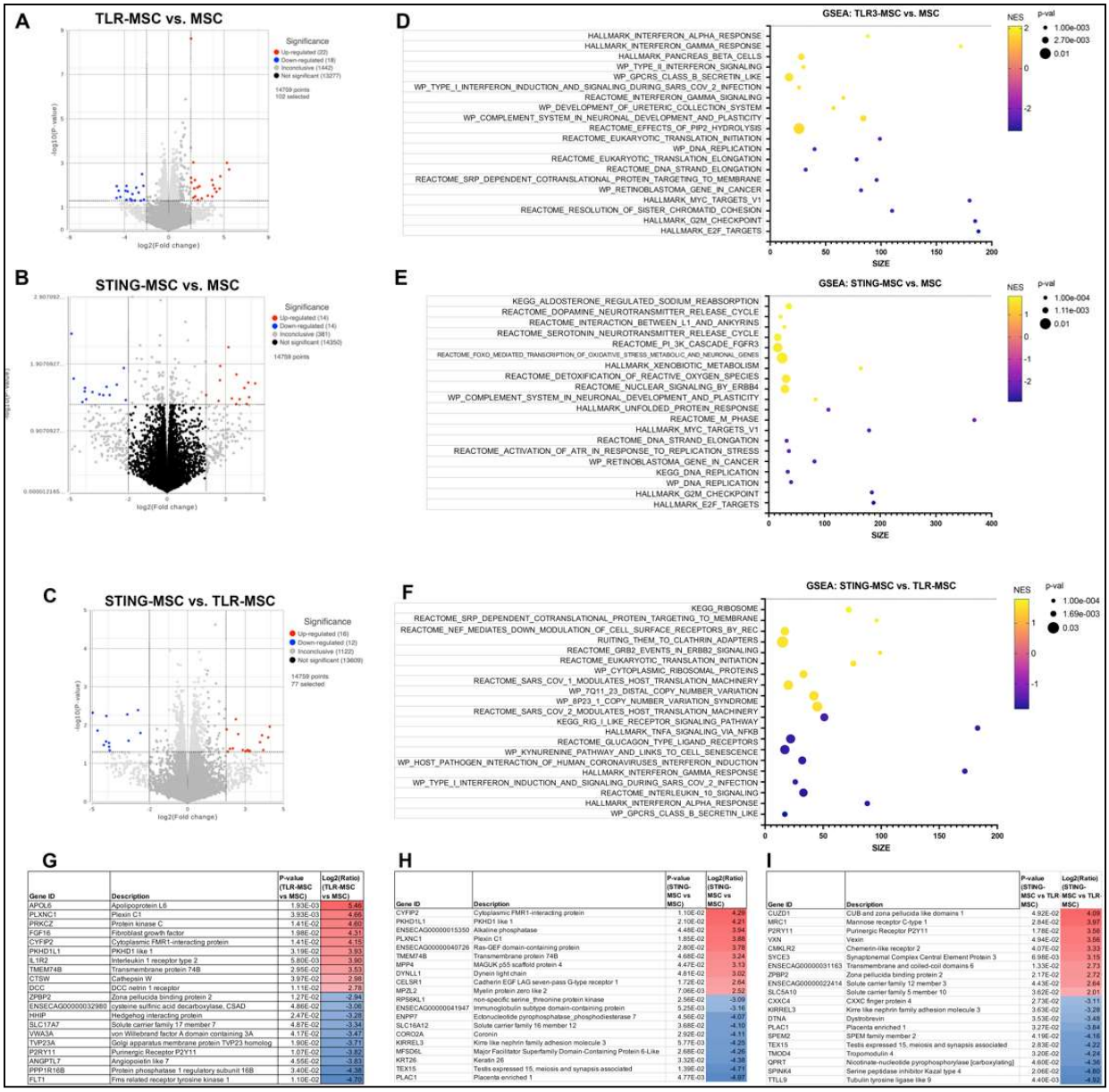


Figure 3.6 – Transcriptome of synoviocytes treated with activated MSC supernatants. A) RNAseq differential gene expression analysis results (protein coding genes) comparing n=3 biological replicates of synoviocytes treated with TLR activated MSC supernatant compared to matched biological replicates of synoviocytes treated with resting MSC supernatant. Figure shows volcano plot with significantly upregulated genes ($p <= 0.05$, FC $>= 2Log_2$) in red and significantly downregulated genes ($p <= 0.05$, FC $<= -2Log_2$) in blue. B) Volcano plot of differential gene expression analysis (DESeq) comparing synoviocytes treated with STING-MSC supernatant vs. synoviocytes treated with resting MSC supernatant. C) Volcano plot of differential gene expression analysis comparing synoviocytes treated with STING-MSC supernatant vs. synoviocytes treated with TLR3-MSC supernatant. D-F) top significant GSEA pathways of the above listed comparisons, using hallmarks, reactome, wiki pathways and KEGG. X-axis denotes the number of genes found in corresponding pathways (size). Normalized Enrichment score (NES) colored from purple (-2 downregulated) to yellow +2 enrichment score, upregulated. Size of dots show p-value for significance. All pathways shown filtered for unadjusted p-value of <=0.05 and FDR adjusted p-value of 0.25. G-I) List of top 10 significantly upregulated and top 10 significantly downregulated genes in the above comparison.

Synoviocytes treated with STING-MSC supernatant have less DEGs compared to the TLR3- supernatant, with a total of 14 significantly upregulated and 14 significantly downregulated protein coding genes. Downregulated genes include some related to cytoskeletal components (KRT26, CORO2A) cell signaling SLC16A12, upregulated genes also include some related to cellular structure (CYFIP2, PLXNC1, MPP4) as well as signaling proteins. According to pathway analysis, significantly upregulated pathways include reactomes to PI3K, FOX0 oxidative stress, detoxification of ROS, and ErbB4 signaling, while significant downregulated pathways include 2 broad categories cell cycle, DNA replication, and chromosome dynamics; as well as Protein Response, Translation, and Stress Response (Figure 3.6B, E, H).

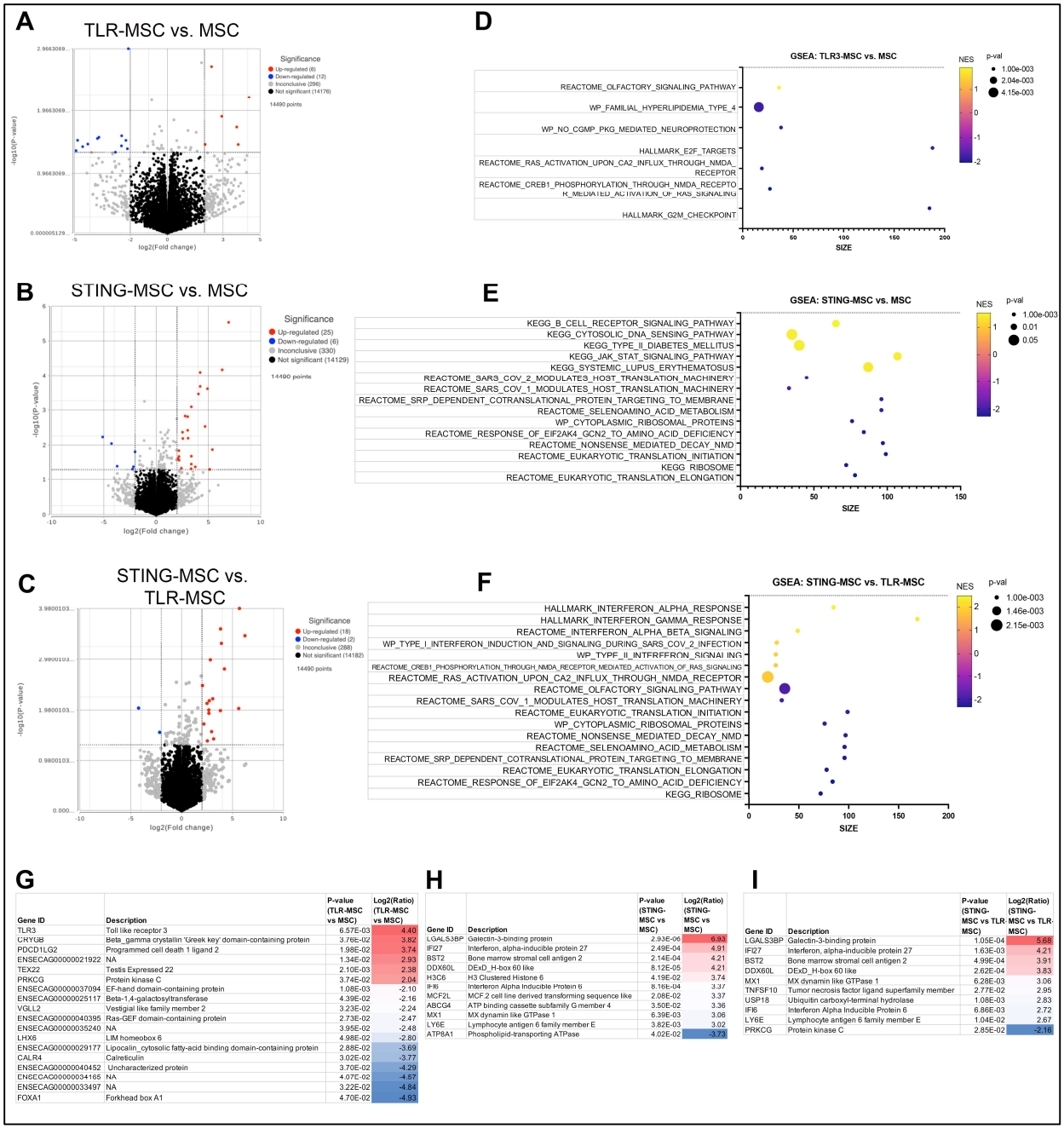
Direct comparison of STING- to TLR3-MSC supernatant treated synoviocytes reveal a total of 28 significant DEGs. Pathway analysis reflects an increased translational environment and decreased TNF-α, IFN-γ, and IL-10 reactomes in STING-MSC treated synoviocytes. (Figure 3.6 C, F, I).

Chondrocytes treated with activated MSC supernatant exhibit increased interferon signaling and extracellular matrix protein gene expression - Replicates for treated chondrocytes cluster by donor horse rather than treatment condition. Differential gene expression of TLR3- MSC supernatant treated chondrocytes compared to resting MSC supernatant treated chondrocytes revealed a small number (18 total) of significant DEGs (differentially expressed genes) with p-value <= 0.05 and Log2 fold change <=2 or <= -2. Top upregulated gene is TLR3, the target for poly I:C, followed by crystalin gene which is likely part of the chondrocyte extracellular matrix(29) then programmed death ligand gene PDCD1LG2. Most downregulated genes include the FOXA1 associated with chondrocyte differentiation(30) calcium signaling gene CALR4 and VGLL2 gene which has been found in muscle. Upregulated pathways include

signaling pathways for RAS reactome, G2M, and PKG, while there was only one significantly downregulated pathway (FDR p value <0.25) (Figure 3.7A, D, G).

Treatment of chondrocytes with STING-MSC supernatant resulted in additional upregulated genes and pathways. A total of 25 significant DEGs including galectin 3 binding protein, and several interferon inducible genes were upregulated. Upregulated pathways in GSEA involve B cell receptor signaling, cytosolic DNA sensing and JAK-STAT signaling. Downregulated pathways broadly revolve around translation. (Figure 3.7B, E, H).

Direct comparison of STING-MSC supernatant treatment vs. TLR3-MSC supernatant showed 20 significant DEGs, with STING-MSC upregulating genes that are mostly part of interferon responses. STING-MSC treated chondrocytes exhibit reduced translational signaling as well. (Figure 3.7C, F, I)



G

Gene ID	Description	P-value (TLR-MSC vs MSC)	Log2(Ratio) (TLR-MSC vs MSC)
TLR3	Toll like receptor 3	6.57E-03	4.40
CRYGB	Beta_gamma crystallin 'Greek key' domain-containing protein	3.76E-02	3.82
PDCD1LG2	Programmed cell death 1 ligand 2	1.98E-02	3.74
ENSECAG00000021922	NA	1.34E-02	2.93
TEX22	Testis Expressed 22	2.10E-03	2.38
PRKCG	Protein kinase C	3.74E-02	2.04
ENSECAG00000037094	EF-hand domain-containing protein	1.08E-03	-2.10
ENSECAG00000025117	Beta-1,4-galactosyltransferase	4.39E-02	-2.16
VGLL2	Vestigial like family member 2	3.23E-02	-2.24
ENSECAG00000040395	Ras-GEF domain-containing protein	2.73E-02	-2.47
ENSECAG00000035240	NA	3.95E-02	-2.48
LHX6	LIM homeobox 6	4.98E-02	-2.80
ENSECAG00000029177	Lipocalin_cytosolic fatty-acid binding domain-containing protein	2.88E-02	-3.69
CALR4	Calreticulin	3.02E-02	-3.77
ENSECAG00000040452	Uncharacterized protein	3.70E-02	-4.29
ENSECAG00000034165	NA	4.97E-02	-4.67
ENSECAG00000033497	NA	3.22E-02	-4.84
FOXA1	Forkhead box A1	4.70E-02	-4.93

H

Gene ID	Description	P-value (STING-MSC vs MSC)	Log2(Ratio) (STING-MSC vs MSC)
LGALS3BP	Galectin-3-binding protein	2.93E-06	6.93
IFI27	Interferon, alpha-inducible protein 27	2.49E-04	4.91
BST2	Bone marrow stromal cell antigen 2	2.14E-04	4.21
DDX60L	DExD_H-box 60 like	8.12E-05	4.21
H3C6	H3 Clustered Histone 6	4.19E-02	3.74
IFI6	Interferon Alpha Inducible Protein 6	8.16E-04	3.37
MCF2L	MCF.2 cell line derived transforming sequence like	2.08E-02	3.37
ABCG4	ATP binding cassette subfamily G member 4	3.50E-02	3.36
MX1	MX dynamin like GTPase 1	6.39E-03	3.06
LY6E	Lymphocyte antigen 6 family member E	3.82E-03	3.02
ATP8A1	Phospholipid-transporting ATPase	4.02E-02	-3.73

I

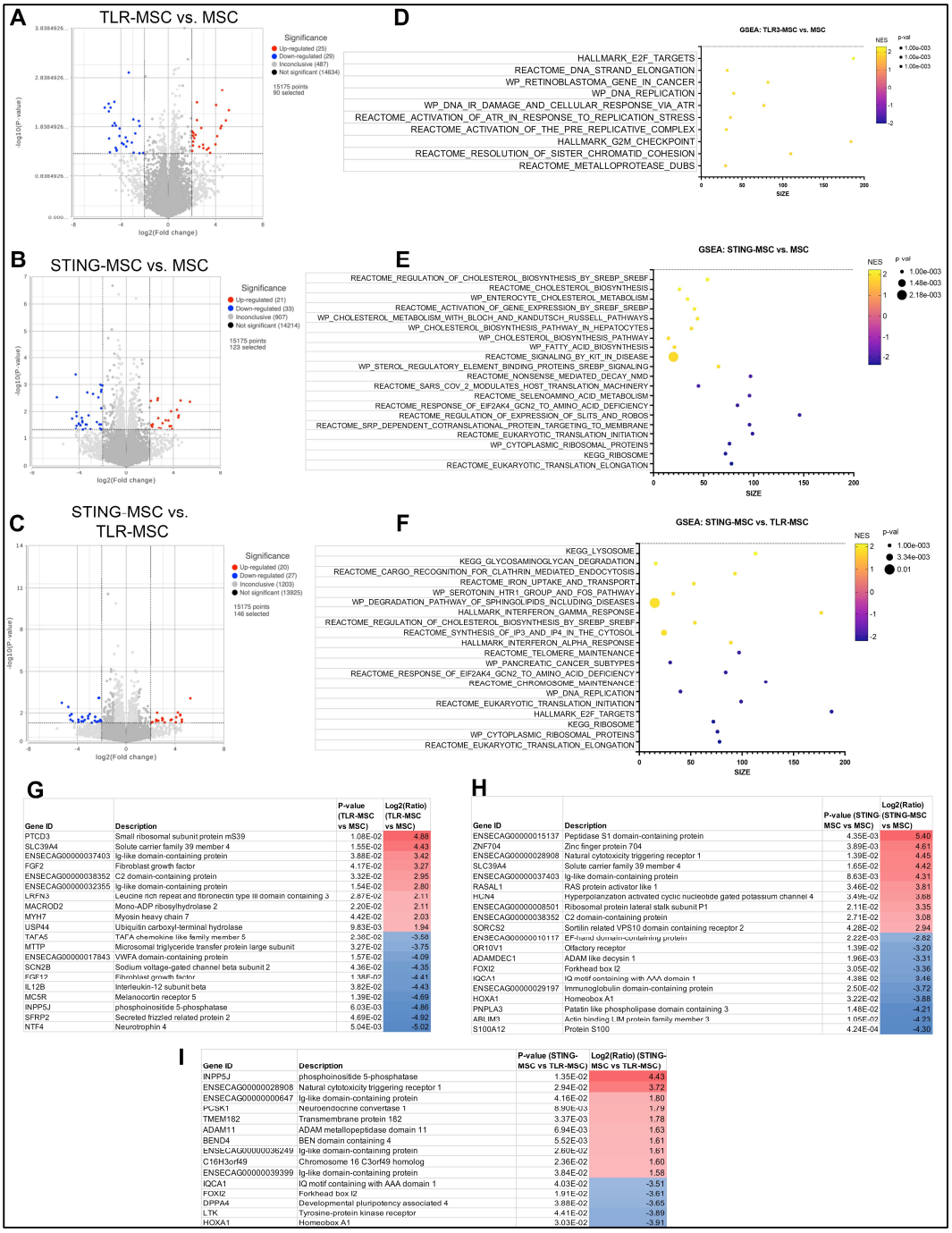
Gene ID	Description	P-value (STING-MSC vs TLR	Log2(Ratio) (STING-MSC vs TLR
LGALS3BP	Galectin-3-binding protein	1.05E-04	5.68
IFI27	Interferon, alpha-inducible protein 27	1.63E-03	4.21
BST2	Bone marrow stromal cell antigen 2	4.99E-04	3.91
DDX60L	DExD_H-box 60 like	2.62E-04	3.83
MX1	MX dynamin like GTPase 1	6.28E-03	3.06
TNFSF10	Tumor necrosis factor ligand superfamily member	2.77E-02	2.95
USP18	Ubiquitin carboxyl-terminal hydrolase	1.08E-03	2.83
IFI6	Interferon Alpha Inducible Protein 6	6.86E-03	2.72
LY6E	Lymphocyte antigen 6 family member E	1.04E-02	2.67
PRKCG	Protein kinase C	2.85E-02	-2.16

Figure 3.7 – Transcriptome of chondrocytes treated with activated MSC supernatants. A) RNAseq differential gene expression analysis results (protein coding genes) comparing n=3 biological replicates of synoviocytes treated with TLR3-MSC supernatant compared to matched biological replicates of synoviocytes treated with resting MSC supernatant. Figure shows volcano plot with significantly upregulated genes (p<= 0.05, FC >=2Log$_2$) in red and significantly downregulated genes (p<= 0.05, FC <= -2Log$_2$) in blue. B) Volcano plot of differential gene expression analysis (DESeq) comparing chondrocytes treated with STING-MSC supernatant vs. chondrocytes treated with resting MSC supernatant. C) Volcano plot of differential gene expression analysis comparing chondrocytes treated with STING-MSC supernatant vs. chondrocytes treated with TLR3-MSC supernatant. D-F) top significant GSEA pathways of the above listed comparisons, using hallmarks, reactome, wiki pathways and KEGG. X-axis denotes the number of genes found in corresponding pathways (size). Normalized Enrichment score (NES) colored from purple (-2 downregulated) to yellow +2 enrichment score, upregulated. Size of dots show p-value for significance. All pathways shown filtered for unadjusted p-value of <=0.05 and FDR adjusted p-value of 0.25. G-I) List of top 10 significantly upregulated and top 10 significantly downregulated genes in the above comparison.

Macrophages treated with stimulated MSC supernatant show increased metabolic gene expression - Macrophages treated with TLR3-MSC supernatant have significant upregulated expression of 25 genes as well as 29 genes that were downregulated. These genes map to a diverse category of upregulated pathways such as those related to DNA Replication, Repair, and Cell Cycle Control or signaling, with no significantly downregulated pathways (Figure 3.8A, D, G).

Macrophages treated with STING-MSC supernatant showed significant upregulated expression of 21 genes as well as 33 genes that were downregulated. Pathway analysis shows that STING-MSC supernatant treated macrophages upregulate cholesterol metabolism, biosynthesis, and signaling, along with some other metabolic and disease-related pathways. Downregulated pathways are related to Translation and Ribosome Function, Disease and Pathological Conditions or Cellular Responses and Metabolic Pathways (Figure 3.8B, E, H).

Although the direct comparison of STING-MSC supernatant treated vs. TLR3-MSC supernatant treated macrophages only showed a moderate number of significant DEGs (47 total), there were many pathways that were up or downregulated that were not seen in the above comparisons. STING-MSC treatment upregulated pathways involved in interferon response and metabolic pathways, while downregulating DNA replication and translational pathways. (Figure 3.8C, F, I).



G

Gene ID	Description	P-value (TLR-MSC vs MSC)	Log2(Ratio) (TLR-MSC vs MSC)
PTCD3	Small ribosomal subunit protein mS39	1.08E-02	4.88
SLC39A4	Solute carrier family 39 member 4	1.55E-02	4.43
ENSECAG00000037403	Ig-like domain-containing protein	3.88E-02	3.42
FGF2	Fibroblast growth factor	4.17E-02	3.27
ENSECAG00000038352	C2 domain-containing protein	3.32E-02	2.95
ENSECAG00000032355	Ig-like domain-containing protein	1.54E-02	2.80
LRFN3	Leucine rich repeat and fibronectin type III domain containing 3	2.87E-02	2.11
MACROD2	Mono-ADP ribosylhydrolase 2	2.20E-02	2.11
MYH7	Myosin heavy chain 7	4.42E-02	2.03
USP44	Ubiquitin carboxyl-terminal hydrolase	9.83E-03	1.94
TAFA5	TAFA chemokine like family member 5	2.38E-02	-3.58
MTTP	Microsomal triglyceride transfer protein large subunit	3.27E-02	-3.75
ENSECAG00000017843	VWFA domain-containing protein	1.57E-02	-4.09
SCN2B	Sodium voltage-gated channel beta subunit 2	4.36E-02	-4.35
FGF12	Fibroblast growth factor	1.38E-02	-4.41
IL12B	Interleukin-12 subunit beta	3.82E-02	-4.43
MC5R	Melanocortin receptor 5	1.39E-02	-4.89
INPP5J	phosphoinositide 5-phosphatase	6.03E-03	-4.86
SFRP2	Secreted frizzled related protein 2	4.69E-02	-4.92
NTF4	Neurotrophin 4	5.04E-03	-5.02

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Gene ID	Description	P-value (STING-MSC vs MSC)	Log2(Ratio) (STING-MSC vs MSC)
ENSECAG00000015137	Peptidase S1 domain-containing protein	4.35E-03	5.40
ZNF704	Zinc finger protein 704	3.89E-03	4.61
ENSECAG00000028908	Natural cytotoxicity triggering receptor 1	1.39E-02	4.45
SLC39A4	Solute carrier family 39 member 4	1.65E-02	4.42
ENSECAG00000037403	Ig-like domain-containing protein	8.63E-03	4.31
RASAL1	RAS protein activator like 1	3.46E-02	3.81
HCN4	Hyperpolarization activated cyclic nucleotide gated potassium channel 4	3.49E-02	3.68
ENSECAG00000008501	Ribosomal protein lateral stalk subunit P1	2.11E-02	3.35
ENSECAG00000038352	C2 domain-containing protein	2.71E-02	3.08
SORCS2	Sortilin related VPS10 domain containing receptor 2	4.28E-02	2.94
ENSECAG00000010117	EF-hand domain-containing protein	2.22E-03	-2.82
OR10V1	Olfactory receptor	1.39E-02	-3.20
ADAMDEC1	ADAM like decysin 1	1.96E-03	-3.31
FOXI2	Forkhead box I2	3.05E-02	-3.36
IQCA1	IQ motif containing with AAA domain 1	4.38E-02	-3.46
ENSECAG00000029197	Immunoglobulin domain-containing protein	2.50E-02	-3.72
HOXA1	Homeobox A1	3.22E-02	-3.88
PNPLA3	Patatin like phospholipase domain containing 3	1.48E-02	-4.21
ARL14	Actin binding LIM protein family member 3	1.05E-02	-4.29
S100A12	Protein S100	4.24E-04	-4.30

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Gene ID	Description	P-value (STING-MSC vs TLR-MSC)	Log2(Ratio) (STING-MSC vs TLR-MSC)
INPP5J	phosphoinositide 5-phosphatase	1.35E-02	4.43
ENSECAG00000028908	Natural cytotoxicity triggering receptor 1	2.94E-02	3.72
ENSECAG00000000647	Ig-like domain-containing protein	4.16E-02	1.80
NCSK1	Neuroendocrine convertase 1	8.90E-03	1.79
TMEM182	Transmembrane protein 182	3.37E-03	1.78
ADAM11	ADAM metallopeptidase domain 11	6.94E-03	1.63
BEND4	BEN domain containing 4	5.52E-03	1.61
ENSECAG00000036249	Ig-like domain-containing protein	2.60E-02	1.61
C16H3orf49	Chromosome 16 C3orf49 homolog	2.36E-02	1.60
ENSECAG00000039399	Ig-like domain-containing protein	3.84E-02	1.58
IQCA1	IQ motif containing with AAA domain 1	4.03E-02	-3.51
FOXI2	Forkhead box I2	1.91E-02	-3.61
DPPA4	Developmental pluripotency associated 4	3.88E-02	-3.65
LTK	Tyrosine-protein kinase receptor	4.41E-02	-3.89
HOXA1	Homeobox A1	3.03E-02	-3.91

Figure 3.8 – Transcriptome of macrophages treated with activated MSC supernatants. A) RNAseq differential gene expression analysis results (protein coding genes) comparing n=3 biological replicates of macrophages treated with TLR3-MSC supernatant compared to matched biological replicates of macrophages treated with resting MSC supernatant. Figure shows volcano plot with significantly upregulated genes ($p <= 0.05$, FC $>= 2Log_2$) in red and significantly downregulated genes ($p <= 0.05$, FC $<= -2Log_2$) in blue. B) Volcano plot of differential gene expression analysis (DESeq) comparing macrophages treated with STING-MSC supernatant vs. macrophages treated with resting MSC supernatant. C) Volcano plot of differential gene expression comparing macrophages treated with STING-MSC supernatant vs. macrophages treated with TLR3-MSC supernatant. D-F) top significant GSEA pathways of the above listed comparisons, using hallmarks, reactome, wiki pathways and KEGG. X-axis denotes the number of genes found in corresponding pathways (size). Normalized Enrichment score (NES) colored from purple (-2 downregulated) to yellow +2 enrichment score. Size of dots show p-value. All pathways shown filtered for unadjusted p-value of <=0.05 and FDR adjusted p-value of 0.25. G-I) List of top 10 significantly up and downregulated genes in the above comparison.

Bioinformatics reveals potential immune and metabolic treatment pathways between activated MSCs and treated cells. Combining the transcriptome data from activated MSC and their effects on target cells, a prediction can be made on the potential mechanisms of how activated MSCs differ for OA treatment. First, gene expression results from non-activated and activated MSCs were matched to the human protein atlas (https://www.proteinatlas.org/humanproteome/tissue/secretome) to predict secreted proteins. TLR3 activation shows a predicted 12 upregulated secreted proteins, STING activation upregulates those same 12 proteins and includes an additional 23 secreted proteins (Figure 3.9). The heat map in figure 8 shows all 23 of the upregulated proteins along with normalized expression value for all biological replicates in all treatment groups. Transcriptomic responses to activated MSC treatment from chondrocytes, synoviocytes and macrophages were then matched to these predicted secreted proteins. Among these proteins, both STING- and TLR3-MSCs secreted TNFSF10, CXCL10, ISG15 and C2 were found upstream of several affected immune response and infection pathways in all 3 cell types. STING activation additionally increased proteins ITGBL1, NOTUM, SRGN, IL4L1, SERPING1, which influence downstream metabolic signaling and transcription pathways in the 3 joint cell types.

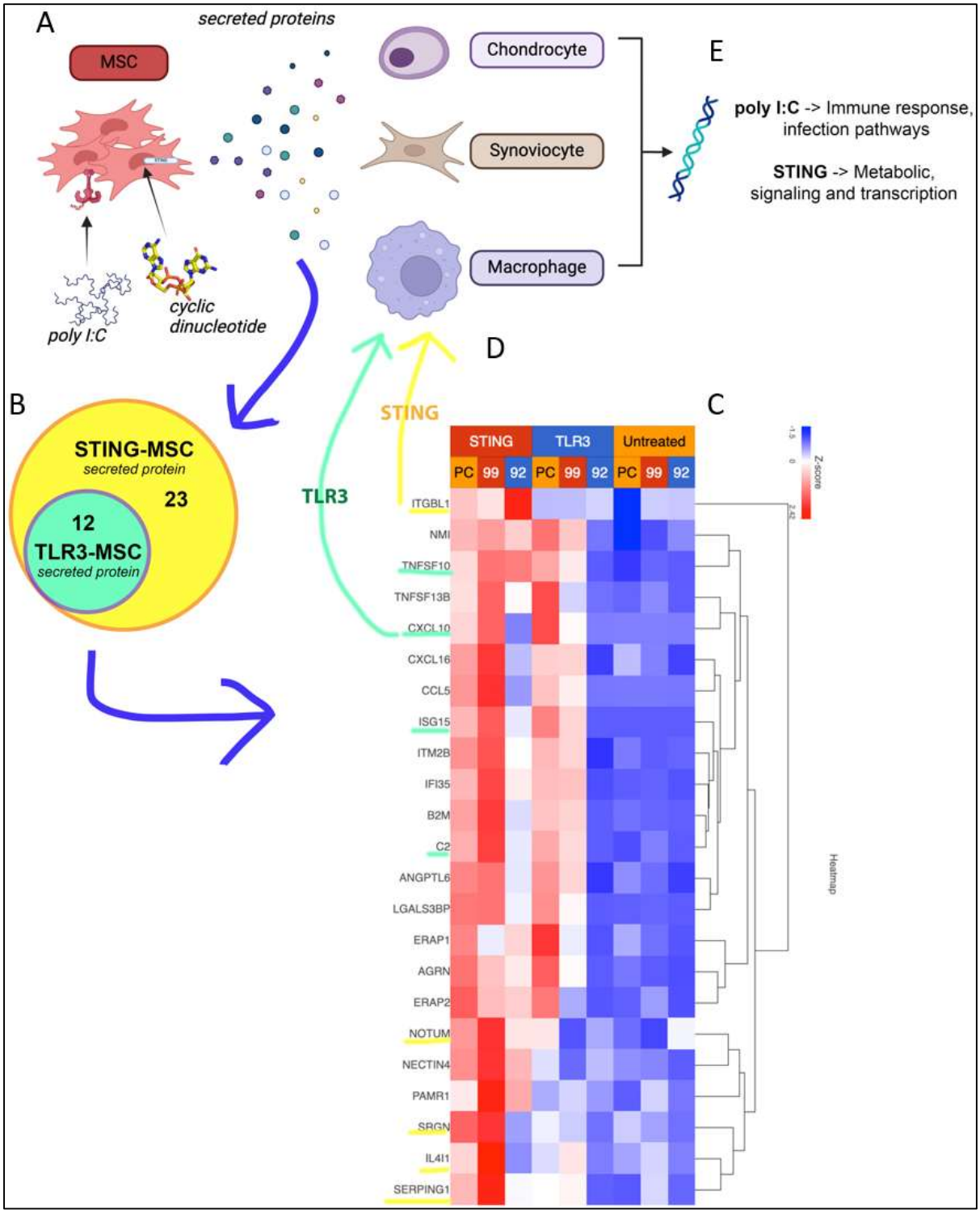

Figure 3.9. Proposed mechanism of increased treatment efficacy using stimulated MSCs based on joint cell type interaction with MSC secreted proteins. Figure shows diagram depicting experimental work flow. A) shows MSCs treated with either TLR3 or STING agonist, then secreting different proteins. B) upregulated protein coding genes (Figure 2) were matched to human protein atlas for prediction of secreted protein, which included 12 in the TLR treated MSCs and an additional 23 in the STING MSCs shown in a Venn diagram. C) heat map of normalized expression values of 35 predicted secreted proteins (data from Figure 2) including 3 biological replicates (PC, 99, 92) as well as all treatment groups. Untreated in yellow, TLR3 in blue (top) and STING in red (top). D) Yellow and green highlighted proteins were found to effect downstream pathways in 3 joint cell types (chondrocytes, synoviocytes and macrophages). E) Key take aways from pathway matching.

3.5 Discussion

Priming MSCs with immune stimulants may increase homogeneity of cell products and therefore treatment efficacy in the context of OA. We have recently shown pre-activation of MSC with Toll-like-receptor (TLR)-3 polyinosinic:polycytidylic acid (pIC) and STimulator of Interferon Genes (STING) ligands improves histologic and functional gait outcomes in a murine model of OA and sought to investigate that further mechanistically here using an in vitro co-culture system. The objective was to use relevant in vitro bioassays to compare the anti-inflammatory and immunomodulatory properties of TLR3- or STING immune activated MSC supernatants on synoviocytes, chondrocytes and macrophages. Key findings included induction of interferon related pathways and suppression of pro-inflammatory cytokines relevant to OA progression. These findings indicate potential benefit to 'immune licensing' of MSC prior to in vivo injection in OA although mechanism varied between agonists and readout cell lines in vitro. Below we examine this effect more closely by cell line.

When examining transcriptomic responses of MSC themselves following activation, stimulation with TLR3 and STING largely induced interferon related responses, as well as transcription and DNA replication in MSCs. Production of type I interferons by immune cells and sensory neurons has been described to elicit antinociceptive effects in murine models of neuropathic pain(13,15–17), with potential implications for pain mitigation in OA. Following TLR3 activation of MSC, 76 genes were upregulated, with the top 10 representing interferon induced or related genes. When this transcriptomic response was matched to previously secreted proteins, we provided prediction analyses for secreted proteins that may be active in the clinical application of these cell products; 12 potential secreted proteins were identified, notably interferon stimulated gene 15 (ISG15) and agrin (AGRN) have shown beneficial effects in

chondrogenesis(31) and cartilage formation(32). Mechanistically, ISG15 has been shown to increase USP18(33), an IL-6 and IL-18 inhibitor(34), and AGRN works to repair cartilage by attracting tissue progenitor cells and differentiation of articular chondrocytes through simultaneous activation of CREB and suppression of canonical Wnt signaling(32). STING activation augmented MSC response compared to TLR3 activation.

When examining the transcriptomic and cytokine response of MSC to STING activation, the same 76 genes were upregulated in STING compared to TLR3 activated MSC and an additional 23 genes were upregulated while 3 were downregulated. Of the top 10 upregulated genes, 9 were the same as in TLR3 activated MSC with IRF7, the "master regulator of type-1 interferon dependent-immune responses(35)," rising into the top 10. GSEA shows a similar increase in mostly interferon-based pathways, but also shows a significant upregulation in ISG15 signaling and reactome to the non-canonical NFκB pathway. STING activation also induced downregulation of three genes as well as significant reduction in protein translation and metabolism pathways. Predicted secreted proteins included the 12 same increased proteins in TLR3 activated MSCs, along with 11 additional proteins predicted. Multiple exclusive proteins show efficacy in reducing osteoarthritis or enhancing cartilage formation (ITGBL1, Notum, serpinG1. Integrin beta-like 1 (ITGBL1) inhibits integrin-ECM interactions and promotes chondrogenic differentiation(36), and inhibits cartilage inflammation decreasing TNF-α, IL-1β, and IL-6(37). Notum stimulates repair(38) of cartilage as a negative regulator of the Wnt pathway(39). SerpinG1, a serine protease inhibitor may also inhibit catabolism and inflammation of cartilage but has not been extensively studied. Finally, STING activation induced increased IP-10 secretion from MSC, a chemokine known to be secreted in response to IFN-γ that further plays a role in activation and regulation of immune responses. These data provide further

mechanistic insight to the effect of MSC priming with pattern recognition receptors, implicating induction of interferon responses as a primary pathway with relevance to OA.

When examining transcriptomic and cytokine responses of synoviocytes to treatment with activated MSC media, TLR3-MSC treatment induced an increase in interferon response and downregulation of translation related pathways. Differentially expressed genes with known relevance to OA and cartilage homeostasis include protein kinase C (PKC), FGF-16, and IL-1r2. PKC has been implicated to have dual roles in OA, both inhibiting inflammation through TNF-α induced ICAM-1 expression(40) while increasing MMP-1 -3 and -13 production in cartilage(41). FGF signaling has been associated with several functions, including cell differentiation, proliferation and tissue repair. While some FGFs have demonstrated an effect in OA and cartilage (both regenerative and degenerative), literature supporting a role for FGF-16 specifically in the context of OA is limited. IL-1r2 suppresses the immune response to IL-1 reducing arthritis in mice(42). STING-MSC induced downregulation of similar pathways, while the upregulated pathways include increases in reactome to PI3K cascade, detoxification of ROS and nuclear signaling by ERB84. The increased plexin C1 is notable, as it ameliorates injury, inflammation, apoptosis and ECM degradation of chondrocytes exposed to IL-1β(43). Finally, non-activated MSC treatment reduced secretion of IL-6 from synoviocytes, an immunomodulatory cytokine with potential dual roles in OA. In OA, IL-6 is released from synovial fibroblasts and may contribute to OA through production of inflammatory mediators and MMP production through the JAK/STAT pathway and MAPK/PI3K cascade(44–47). TLR3-MSCs also reduced IL-18 in chondrocytes and IL-6 in synoviocytes, and reduced IL-1β, a key cytokine in OA that amplifies synovitis and cartilage degradation, secretion in synoviocytes. STING activated MSC treatment further reduced expression of IL-8 from synovicoytes,

indicating a potential anti-inflammatory role as IL-8 is known to attract neutrophils to regions of inflammation.

When assessing transcriptomic and cytokine responses of chondrocytes to treatment with activated MSC media, TLR3-MSC treatment of chondrocytes induced upregulation of 6 genes and downregulation of 14 genes. Pathway analysis shows a significant reduction in pathways involved with molecules targeted by E2F and molecules involved with NMDA receptor. Upregulation of TLR3 and Protein Kinase C genes suggest an inflammatory response by the cells that is not reflected in their secretory profile. STING-MSC treatment induced increased expression of 25 genes and decreased expression 6 genes in chondrocytes, showing an increase in the JAK-STAT, B cell receptor, and cytosolic DNA sensing pathways, and reduced expression of translation related pathways. Notable increased genes include IFI6, a negative regulator of innate immune responses(48), and MCF2L which is correlated to cartilage maintenance(49,50). Unactivated MSC treatment resulted in decreased chondrocyte production of IL-18, described to contribute to cartilage degradation by inhibiting TGF-β induced proliferation as well as increasing nitric oxide production(51). STING-MSC also reduced expression of IL-8, which has been correlated to severity of OA through upregulation of MMP levels and chondrocyte hypertrophy.

When assessing transcriptomic and cytokine responses of macrophages to treatment with activated MSC media, TLR3-MSC treatment did not induce significant changes in cytokine secretion but did induce upregulation of DNA replication pathways and cell cycle pathways. Significant changes in individual genes show an upregulation of FGF2, an ambivalent mediator in OA depending on conditions in the joint(52). STING-MSC treatment exerted significant effects in secretory changes, and changes in genetic expression with an upregulation in mainly

cholesterol and fatty acid synthesis pathways. A downregulation in translational pathways also occurred. Significant changes in secretory cytokines of macrophages happened only when MSCs were activated with STING agonist, causing an increase in G-CSF and IL-4 while reducing secretion of IL-5. Macrophages, a key regulator in joint inflammation, in the synovial joint include synovial macrophages and infiltrating macrophages are classified as either M1, an inflammatory cytokine producing or destructive macrophage, or M2, an anti-inflammatory or reparative macrophage. Polarization states of these macrophages are essential for modulating the inflammatory state of the synovial joint. Granulocyte stimulating factor (G-CSF), shown to repair osteochondral defects(53) and damaged cartilage(54), decreases the M1/M2 ratio of macrophages(55). IL-4 is associated with M2 macrophages and inducing M2 polarization(56) and is also chondroprotective in osteoarthritis(57). IL-5 promotes eosinophilic inflammation(58), the implications of which are not fully characterized in the context of OA. Taken together these alterations in transcriptomic and cytokine responses with STING-MSC treatment of macrophages in particular support an immunomodulatory and overall anti-inflammatory role for STING induction in OA.

Limitations of this study include the in vitro nature of study design, short time course in culture, and small donor horse sample size with some inter-individual variability noted between primary cells lines in differential gene expression and cytokine secretion. Given the in vitro nature of study design, there is potential for lack of translation of findings to the in vivo osteoarthritic environment, and it is acknowledged that the culture conditions described here could not replicate the spectrum of disease processes encompassed by OA. These findings, interpreted in light of future application in OA, corroborate our recent findings supported a

beneficial effect of activated MSC therapy in murine OA models and support further investigation of these therapeutic strategies in large animal models in vivo.

3.6 Conclusions

These findings indicate potential benefit to ‘activation’ or ‘immune licensing’ of MSC prior to in vivo injection in OA although mechanism of action observed varied between agonists and readout cell lines in vitro. These findings indicate that innate immune pathway activation modulates MSC interaction with joint cell lines which warrants further investigation to improve OA treatment.

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CHAPTER 4: ADULT OVINE CONNECTIVE TISSUE CELLS RESEMBLE MESENCHYMAL STROMAL CELLS IN THEIR PROPENSITY FOR EXTENSIVE EX VIVO EXPANSION[1]

4.1 Summary

In expansion culture connective tissue cells can adopt mesenchymal stromal cell properties that are favorable for applications in regenerative medicine. Given that cell therapies are anticipated to require a large number of cells, this study characterized growth and selected MSC immunophenotypical properties of five connective tissue cells through at least 20 population doublings (PDs). Culture expansion of cells can cause dedifferentiation, and the adoption of properties associated with mesenchymal stromal cells. Likewise, the presence of tissue progenitor cells highlights the potential for tissue specific MSC treatments. Therefore, the objective for chapter 4 was to evaluate selected MSC properties of different connective tissue cells with expansion through greater than 20 population doublings (PDs). A phenotypical drift towards an MSC phenotype may be advantageous for these tissue derived cells in the treatment of degenerative diseases.

1: A version of this manuscript has been submitted to *Connective Tissue Research*: Adult ovine connective tissue cells resemble mesenchymal stromal cells in their propensity for extensive ex vivo expansion: Linde P, Puttlitz C, and Kisiday J.

4.2 Introduction

Expansion in monolayer culture is a routine laboratory technique for amplifying the number of cells isolated from connective tissues. While cells can be induced to grow in an exponential manner, it is well recognized that culture expansion can alter the phenotype of connective tissue cells. For example, chondrocytes begin to dedifferentiate to a fibroblastic phenotype in primary expansion culture, with progressive loss of the native phenotype with additional growth(1). Similar changes in the phenotype of tenocytes(2) and intervertebral disc cells(3) with expansion have been described. Concomitant with loss of the native phenotype, expansion of connective tissue cells can result in the adoption of properties that are associated with mesenchymal stromal cells (MSCs). For example, culture-expanded stromal cells from fat(4,5), chondrocytes(6–11), and different strains of fibroblasts(12) resemble bone marrow MSCs in their expression of cluster of differentiation (CD) molecules, propensity for immunomodulation, and/or multilineage differentiation. Expression of MSC-like CD molecules and/or multilineage differentiation has been reported for cells from the meniscus (13,14) and annulus fibrosus(15,16). Taken together, these data indicate a general propensity of connective tissue cells to acquire an MSC-like phenotype with culture expansion.

While loss of the native phenotype with expansion has been considered a challenge for tissue engineering, enthusiasm for the use of MSCs in regenerative medicine suggests a benefit of implanting expanded connective tissue cells. However, it is important that MSC-like connective tissue cells are capable of extensive expansion for treatments that are anticipated to consist of millions of cells. For example, for autologous therapies, only a small number of cells can be obtained from biopsied (17) or debrided(18) tissues. For allogeneic treatments, cell expansion is anticipated to focus on the creation of many cellular treatments from each donor

lot(19), with suggested amplification of cells up to 20 population doublings(20). Therefore, the objective of this study was to evaluate selected MSC properties of different connective tissue cells with expansion through greater than 20 population doublings (PDs).

In this study adult ovine cells from cartilage, tendon, meniscus, annulus fibrosus, and nucleus pulposus were compared to bone marrow MSCs. Cultures were established with unfractionated cell populations from tissue digests. Expansion was conducted using culture medium that has been shown to support rapid growth of bone marrow MSCs (21) and different connective tissue cells (22). Cultures were analyzed for expression of CD molecules that are associated with MSCs, using a small panel based on the availability of an ovine CD44 antibody, and previously established cross-reactivity of human CD29 and CD166 antibodies with sheep cells. MSCs are also defined by the propensity of individual cells to proliferate in a colony-forming manner with time in expansion culture. Therefore, we used time-lapse imaging to evaluate early colony formation after seeding into expansion culture. From these data, we conclude that unfractionated populations of connective tissue cells resemble MSCs in their immunophenotype and strong propensity for colony-formation and expansion.

4.3 Methods

Isolation and expansion of annulus fibrosus cells, nucleus pulposus cells, chondrocytes, tenocytes, and meniscus cells. Samples of each tissue was collected from three (n=3) adult female Columbia-Rambouillet sheep that were euthanized for reasons unrelated to this study, harvested under blanket Institutional Animal Care and Use Committee (IACUC #18-8031A) approval for tissue shaving. Tendon was harvested from the midsubstance of the core of the patellar tendon. Meniscus tissue was harvested from the inner 2/3rd of the meniscus, or approximately the avascular white zone. Annulus fibrosus and nucleus pulposus cells were

harvested from the lumbar spine intervertebral discs. Each tissue was minced, and then transferred to alphaMEM medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO), 10 mM HEPES (Thermo Fisher Scientific), 0.5% antibiotics/antimycotics solution (Thermo Fisher Scientific), and 0.5% pronase (Worthington Biochemical Corp., Lakewood, NJ) for 1 hour. The tissue was then transferred to alphaMEM medium supplemented with 10% fetal bovine serum, 10 mM HEPES, 0.5% antibiotics/antimycotics solution, and 0.1% collagenase (Worthington Biochemical Corp., Lakewood, NJ). After incubation overnight, fresh collagenase was added, and the digestion was continued for an additional 3-4 hours to fully digest the pericellular matrix. All cells were cryopreserved at a concentration of 10 million per milliliter (ml) in 95% FBS and 5% dimethyl sulfoxide. For each experiment, cells were recovered from cryopreservation by thawing and then seeding at ~$20x10^3$ cells/cm^2 in expansion medium overnight, and then seeded into expansion culture in alphaMEM supplemented with 10% FBS,10 mM HEPES, 2 ng/ml fibroblast growth factor 2 (FGF2, Peprotech, Rocky Hill, NJ), and 0.5% antibiotics/antimycotics solution. Primary studies were conducted by seeding at 500 cells/cm^2.

Isolation and expansion of bone marrow MSCs. Approximately 2 ml of bone marrow was harvested from the iliac crest of three (n=3) adult female Columbia-Rambouillet sheep unrelated to the sheep used for harvesting other cell types. The aspirates were centrifuged at 1000g for 10 minutes to pellet both red and nucleated cells. To lyse the majority of the red cells, the pellet was resuspended in 1 ml of sterile tissue culture water for ~10 seconds, after which the water was quickly diluted with 12 ml of phosphate buffered saline (PBS). The nucleated cells were collected by centrifugation, washed with PBS, resuspended in low glucose DMEM plus 10% FBS, 10 mM HEPES and 0.5% antibiotics/antimycotics solution, and seeded at $200x10^3$

cells/cm^2 in tissue culture flasks. The medium was changed after 24 hours, and colony-forming cells were collected after an additional 4-6 days. The MSCs were cryopreserved and expanded as described for annulus fibrosus cells, nucleus pulposus cells, tenocytes, and meniscus cells.

Time-lapse image analysis of cell proliferation. MSCs, annulus fibrosus cells, nucleus pulposus cells, tenocytes, chondrocytes and meniscus cells were seeded into wells of a 96 well plate at 50 cells/cm^2 after recovery from cryopreservation or after primary cultures were passaged. Proliferation was tracked using an Incucyte live cell imaging system (Essen BioScience, Inc., Ann Arbor, MI), with light microscope images taken every three hours for up to 51 hours. Four views per well were recorded. The low seeding density allowed for tracking of individual cells in the days after seeding. Colony-forming behavior was characterized by manually counting cells in regions that contained one cell at the first recorded image, three hours after seeding.

Flow cytometry for analysis of CD molecule expression. MSCs, annulus fibrosus cells, nucleus pulposus cells, tenocytes, chondrocytes and meniscus cells were harvested from expansion culture and washed in FACS buffer (phosphate buffered saline with 2% FBS and 0.1% sodium azide). Next, the cells were blocked by incubating for 20 mins at 4^{o}C with 5% FBS, and then incubated with fluorochrome-conjugated antibodies (supplemental figure) in FACS buffer for 30 minutes in the dark at room temperature. Additional cell aliquots were stained with an isotype-matched antibody. Flow cytometric analysis was performed using an Attune NxT flow cytometer (Invitrogen, Carlsbad, CA), at least 5000 events were recorded for each cell type, and data were analyzed using FlowJo software (FlowJo LLC, Ashland, OR). Antibodies used for analysis of CD molecule expression include CD29 (BioLegend, cat. no.

303003), CD44 (Invitrogen, cat. no. MA5-28372), and CD166 (BD Biosciences, cat. no. 560903).

***Statistical analysis*:** Data were analyzed for normal distribution using the Shapiro-Wilk test. Normal data were analyzed by one-way ANOVA with *post-hoc* Tukey tests. Non-normal data were analyzed using Wilcoxon matched pairs signed rank test. Statistical tests were performed using R (v3.5.2). P-values less than 0.05 were considered statistically significant. Data are presented as mean ± standard error.

4.4 Results

Expansion through 20 population doublings: Expansion cultures were maintained for 20 days. Preliminary studies indicated that seeding at 500/cm^2 resulted in greater than 50% confluence for all cell types after 5 days (data not shown). Therefore, cultures were trypsinized and reseeded on days 5, 10, and 15. For bone marrow MSCs, the cumulative number of PDs increased in a near-linear fashion, reaching a mean of 27.0 +/- 0.9 on day 20 (Fig. 1A). All connective tissue cells proliferated in a similar pattern. On day 20, cumulative PDs for tenocytes (mean: 26.3 +/- 1.5, Fig. 1B), meniscus cells (25.1 +/- 1.2, Fig. 1C), annulus fibrosus cells (26.9 +/- 0.2, Fig. 1D), and nucleus pulposus cells (27.0 +/- 0.2, Fig. 1E) were not significantly different than MSCs (P=0.95, 0.27, 1.00, 0.99 respectively). Mean cumulative PDs for chondrocytes on day 20 was 22.0 +/- 0.6 (Fig. 1F), which was at least 12% less than all other cell types (P<0.05).

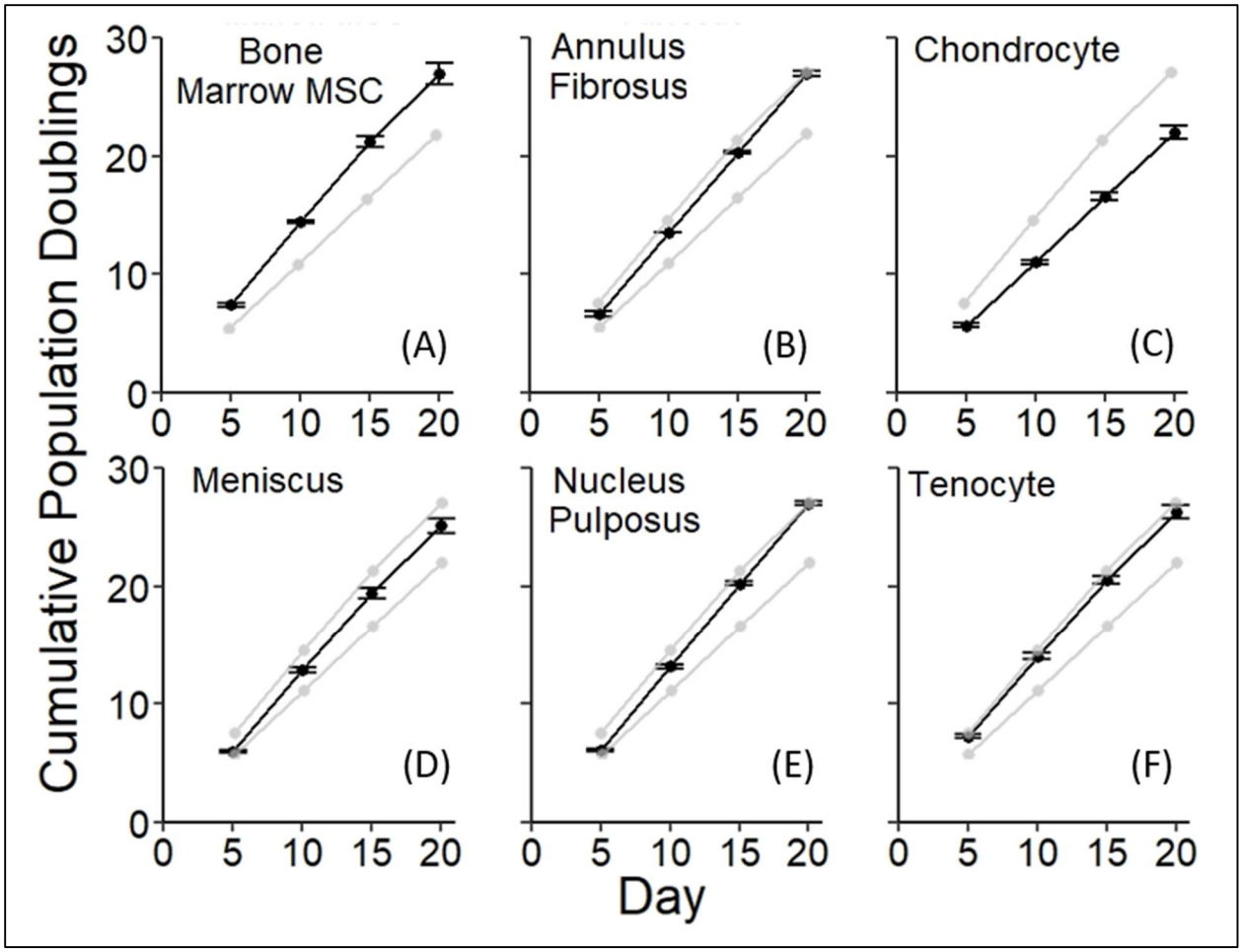

Figure 1: Cumulative population doublings over 20 days of culture. The cell yield on day 20 was not significantly different among tissue sources except for chondrocytes (**C**), which proliferated through at least 3.1 fewer population doublings than the other cell types. ($P<0.05$). N=3 donor animals, data are mean +/- s.e.m. Maximum (MSC) and minimum (chondrocyte) values are shown in gray.

CD molecule expression: CD expression was evaluated at the first subculture on day 5 and at the end of the experiment (day 20). Figure 2A is a representative histogram with isotype and uniform expression of CD166 for a MSC sample on day 20. For CD29 and CD44, all cell types were 99-100% positive at both timepoints (Figure 2C and 2D show representative histograms for CD29 and CD44, respectively). CD166 expression was 98-99% positive for bone marrow MSCs, and moderately variable for connective tissue cells (Fig. 2B). CD166 expression was highest for annulus fibrosus and nucleus pulposus cells (greater than 88%), while expression

ranged between 64-75% for chondrocytes, meniscus cells, and tenocytes. Between days 5 and 20, expression of CD166 modestly increased for annulus fibrosus cells (88% to 97%, P<0.002). Otherwise, expression was not significantly different between timepoints for chondrocytes, meniscus cells, nucleus pulposus cells, or tenocytes (P=0.24-0.87).

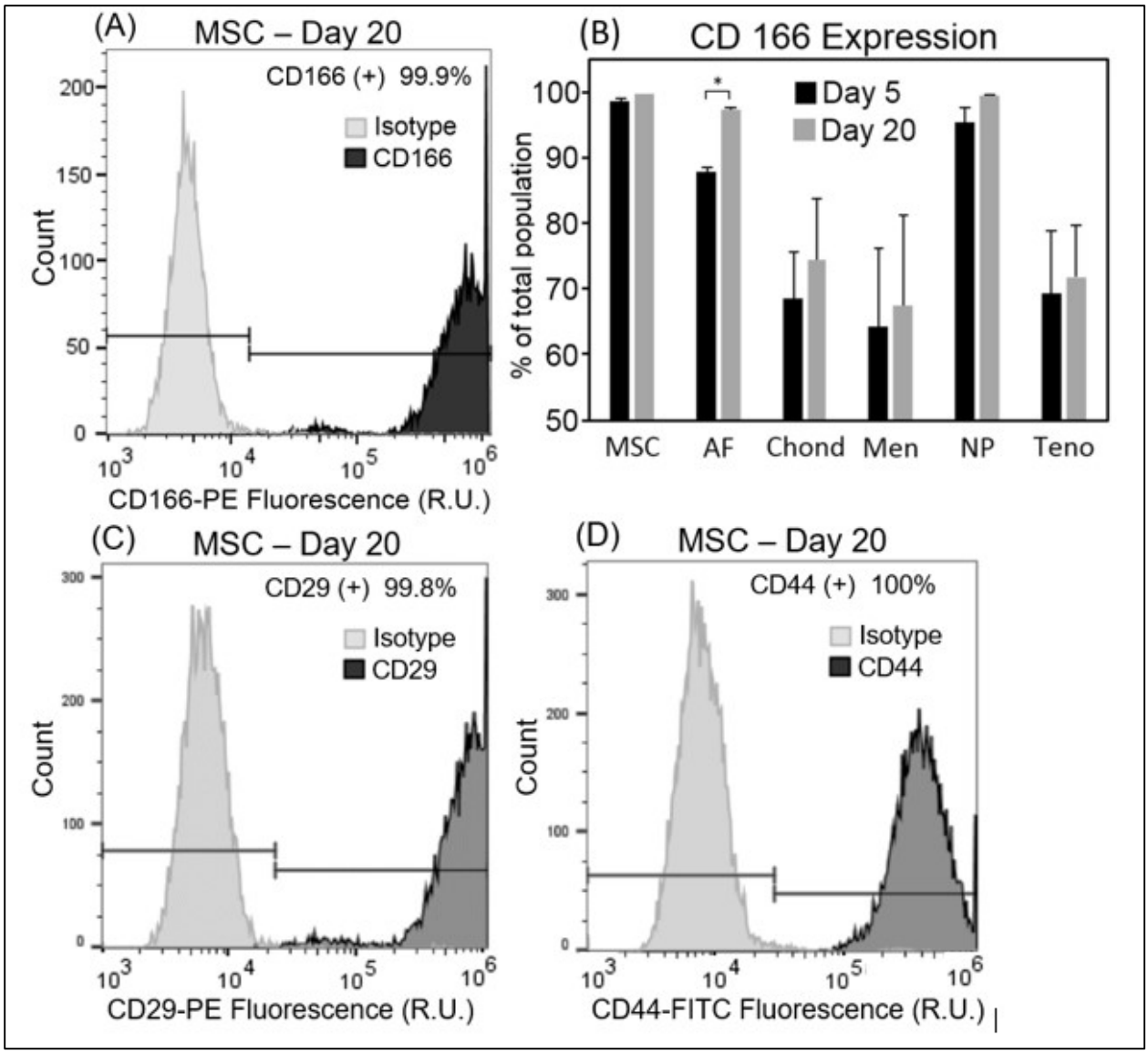

Figure 2. (**A**) Representative histogram of uniform expression of CD166 by MSCs. (**B**) CD166 expression after 5 and 20 days of culture expansion. Annulus fibrosus cells were the only cells to significantly increase expression of CD166. P<0.05 denoted by *. N=3 donor animals, data are mean +/- s.e.m. (**C**) Representative histogram of uniform expression of CD29 by MSCs. (**D**) Representative histogram of uniform expression of CD44 by MSCs.

Early colony formation with low density seeding: Colony-formation was evaluated at the start of the experiment or after the third subculture on day 15. Seeding at 50 cells/cm^2 generally resulted in cells that were at least 500 microns away from neighboring cells (Fig. 3A, B), which allowed for evaluation of colony-formation without encroaching on neighboring colonies for up to 52 hours. Cells were categorized as having undergone at least 3 PDs, 2 PDs, 1 PD, or did not proliferate. For example, Fig. 3B consists of time lapse images of an annulus fibrosus cell undergoing at least 3 PDs. For the same timepoints, images of an annulus fibrosus cell that did not proliferate are presented in Fig. 3C. Figure 3A summarizes the percentage of cells that fell into each category. **Day 0** – For all cell types the most frequent outcome was proliferation through at least 3 PDs, which ranged between 94% (annulus fibrosus, nucleus pulposus) and 58% (chondrocytes). The frequency of non-dividing cells was at most 21% (bone marrow MSC, tenocytes), approximately 10% for chondrocytes and meniscal cells, and less than 5% for annulus fibrosus and nucleus pulposus cells. For 2 PDs, the highest frequency was observed for chondrocytes (22%), followed by bone marrow MSCs and meniscal cells (~10%). Only chondrocytes were observed to undergo 1 PD (9%). **Day 15** – Similar to Day 0, the most frequent outcome was proliferation through at least 3 PDs, with the lowest percentage for bone marrow MSCs (78%). The frequency of non-dividing cells was at most 20% (bone marrow MSCs), and less than 10% for all other cell types, except for tenocytes (13%). For all cell types, less than 3% underwent 1 or 2 PDs.

(A) Cumulative Population Doublings After 52 Hours

Day 0				
Tissue	0 PD	1 PD	2 PD	3 PD
Bone Marrow MSC	22%	0%	10%	68%
Annulus Fibrosus	4%	0%	2%	94%
Chondrocyte	13%	9%	22%	58%
Meniscus	9%	0%	11%	81%
Nucleus Pulposus	3%	0%	1%	94%
Tenocyte	21%	0%	5%	73%

Day 15				
Tissue	0 PD	1 PD	2 PD	3 PD
Bone Marrow MSC	19%	1%	2%	78%
Annulus Fibrosus	4%	0%	2%	94%
Chondrocyte	9%	0%	2%	90%
Meniscus	5%	1%	1%	94%
Nucleus Pulposus	13%	3%	3%	82%
Tenocyte	13%	3%	3%	82%

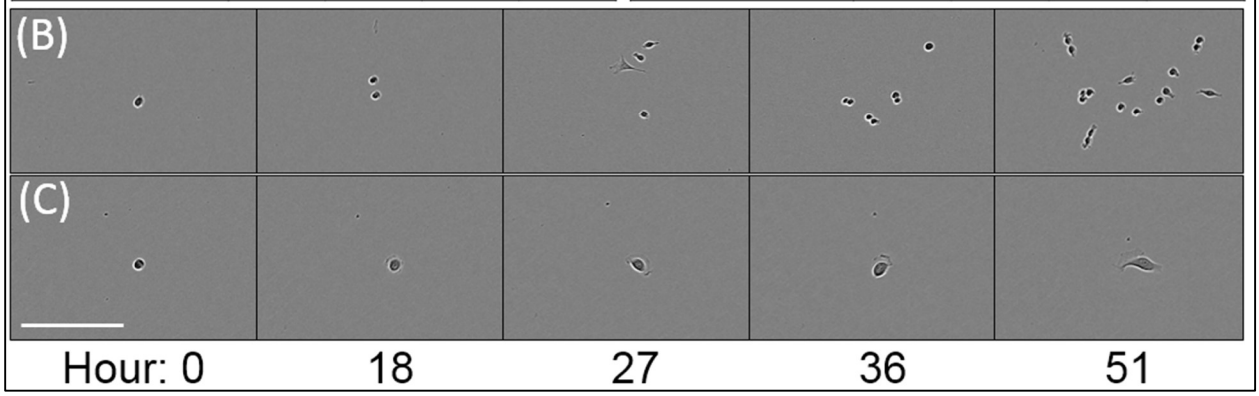

Figure 3. (**A**) Percentage of cells that had undergone 0, 1, 2, or 3 PDs 52 hours after seeding. Cultures were established at the start of the experiment (day 0) and at the third subculture (day 15). (**B**) Time-lapse images of dividing annulus fibrosus cells, with cell division occurring at 18, 27, 36, and 51 hours. (**C**) Time-lapse imaging of a non-dividing annulus fibrosus cell. Scale bar is 200µm.

Growth surface distribution of chondrocytes and tenocytes during expansion: Time-lapse imaging was conducted for chondrocytes and tenocytes to investigate the propensity of each cell type to distribute across the growth surface during expansion. Cultures were seeded at 1,000 cells/cm^2 such that the cells would approach confluence by day 4. Images from days 0, 2, and 4 are presented in Fig. 4. On day 0, an area of the plate containing cells adjacent to open growth surfaces was identified, which in Fig. 4 is the area left of the dotted line. This area was tracked through day 4. For chondrocytes, few cells had migrated to the vacated region by day 2. On day 4, the initially open area was moderately populated with cells, while the adjacent area that contained cells on day 0 was approaching confluence. For tenocytes, by day 2 the cells had

distributed onto the open surface, and on day 4 all areas were approaching confluence. Time-lapse video files of the cultures from which the images were taken are available as supplemental data.

Chondrocyte and tenocyte expansion following seeding at 50 cells/cm^2: Chondrocytes or tenocytes were expanded to near confluence, which was determined to be 9 days for chondrocytes and 8 days for tenocytes by visual inspection with a microscope. Chondrocytes expanded through 9.2 +/- 0.1 PD, for an average growth rate of 1.0 PD/day, and an average yield of 30,600 cells/cm^2 (data not shown). Tenocytes proliferated through 10.5 +/- 0.1 PDs for an average growth rate of 1.3 PDs/day, and an average yield of 81,400 cells/cm^2 (data not shown).

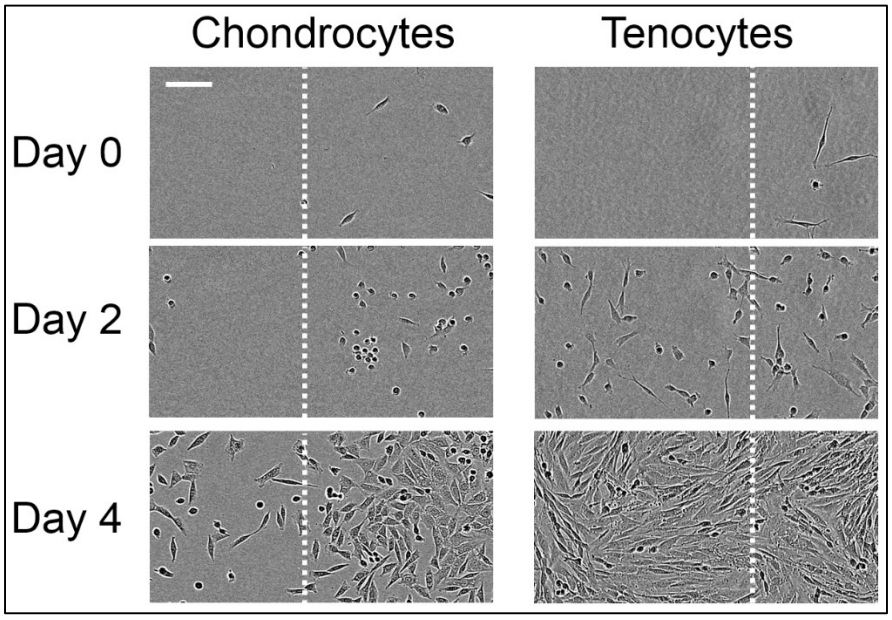

Figure 4. Time-lapse imaging of the cell distribution on growth surfaces over 4 days. Views were selected to capture an area that did not contain cells immediately after seeding, which is to the left of the dotted lines. Over time tenocytes were more efficient in filling open surface. Scale bar is 100 µm.

4.5 Discussion

This study demonstrated that whole populations of cells from selected connective tissues that are needed for regenerative therapies are capable of rapid and extensive *ex vivo* expansion.

At more than a PD per day, the growth rate of ovine connective tissue cells and MSCs was faster than typical growth rates reported for human MSCs(23,24), The near-linear relationship between cumulative PDs and time is not consistent with the onset of senescence, which is typically identified by a severe and sudden decrease in proliferation. Further, similarities in the frequency of cells that demonstrated colony-forming growth between the initiation of expansion and after ~17-21 PDs indicates sustained, homogeneous growth. The propensity of all cells to proliferate through at least 22 PDs suggests a strong tolerance for biomanufacturing of cell therapies.

When measured using flow cytometry, expression of CD29 and 44 has been reported to be uniform, or nearly so, for human (25) and ovine bone marrow MSCs(26,27). Also, high levels of CD29 and/or CD44 have been reported for MSC-like cells from cartilage (10), meniscus (13,28), tendon (29), and intervertebral disc (16,30). The current study is consistent with these data, as CD29 and 44 expression was 99-100% for all cell types at both timepoints. For CD166, expression for human MSCs has been moderately variable among studies, ranging from partial (31,32) to uniform (25,33). For ovine MSCs, CD166 expression has been reported to be between 40 and 90% (26,27,34). Expression of CD166 by chondrocytes has ranged from partial to uniform (7,9,10,13), and in a comparative study was similar to expanded cells from the avascular region of the meniscus (13). Uniform expression of CD166 was reported for intervertebral disc cells (16,30). Taken together, these data are generally consistent with expression of CD166 for ovine MSCs (98-99%) and connective tissue cells (64-99%). To the authors' knowledge, the propensity of populations of MSCs or MSC-like connective tissue cells to partially express CD166 is not well understood. Several studies have reported that expression of CD166 can increase with time in expansion culture (4,7,13,35), and partial expression may reflect a timepoint before full expression is reached. However, in the current study, stable levels of

CD166 expression between the first passage and the end of the time course strongly suggest a steady state of partial expression had been reached.

For connective tissue cells, previous reports of an increase in MSC-like CD molecules with isolation and expansion suggest two possibilities. First, a small number of resident MSC-like cells may proliferate more rapidly than committed cells, resulting in an increasing percentage of an MSC-like cells with growth. This possibility is supported by the practice of isolating colony-forming cells by seeding unfractionated populations at a density (less than ~100/cm^2) that allows for the identification of distinct colonies that emerge over time (28,29). Alternatively, connective tissue cells as a whole may transition to an MSC-like phenotype with expansion, which has been proposed for chondrocytes(7,35). We postulate that data from this study suggest the latter. Time lapse imaging of cultures seeded at 50 cells/cm^2 demonstrated that ~60-90% of primary cells underwent 3 PDs in the first 52 hours, which indicates that the overall increase in cell numbers was not due to a small subset of cells. Further, the calculated growth rate at the first passage was comparable to the next three passages, while a slower overall growth rate would be expected at the first passage if a small number of MSC-like cells was anticipated to dilute the population of slower growing, committed cells.

Among the cell types tested, chondrocytes proliferated at the slowest rate, which is consistent with a previous study in which growth of adult ovine chondrocytes, tenocytes, and meniscus cells were compared through 10 days of expansion(22). Visual inspection of the cultures throughout the study indicated that chondrocytes proliferated in the closest proximity, which could have led to the modestly lower rate of growth from contact inhibition. Time-lapse imaging supported this possibility as chondrocytes accumulated to near-confluence adjacent to

areas that contained fewer cells. This growth pattern was in contrast to tenocytes that more evenly distributed across the expansion surface as they proliferated.

Monolayer expansion can be optimized for efficiency by seeding at the lowest possible density that promotes rapid growth and results in almost complete coverage of growth surface. While MSC cultures are typically seeded with 1,000s of cells/cm (2,23) reducing the seeding density to 10s (36) or 100s (37) of cells/cm^2 can promote rapid growth. However, coverage of the growth surface is not necessarily achieved. For example, for human bone marrow MSCs decreasing the seeding density from 1,000 cells/cm^2 to 100 or 10 cells/cm^2 resulted in a 50% and 80% decrease in cell yield, respectively(36). Similarly, equine MSCs seeded at 25 cells/cm^2 proliferated through 11 PDs in 8 days but resulted in dense colonies of cells that occupied approximately half of the growth surface (21). In the current study, when seeded at 50 cells/cm^2, the growth rate for tenocytes (1.3 PDs/day) and chondrocytes (1.0 PDs/day) was comparable to 500 cells/cm^2. Further, the average yield for tenocytes after 8 days (81,400/cm^2) and chondrocytes after 9 days (30,600/cm^2) indicate efficient coverage of the growth surface, which was grossly confirmed by visual inspection with light microscopy. Taken together, these data indicate the potential for highly efficient expansion of connective tissue cells using seeding densities of 10s of cells/cm^2.

There are several limitations of this study. CD markers do not correlate with a specific regenerative phenotype, nor are they an indicator of potency. Instead, regenerative potential should be defined using assays that are specific to diseases or injuries that are to be treated(38). We evaluated ovine cells given the frequent use of sheep in orthopedic preclinical animal models. However, to ensure translation, it will be important to define the degree to which the properties of ovine cells resemble human. We evaluated unfractionated populations of cells as

enzymatic digestion followed by seeding into expansion culture is the simplest approach for generating MSC-like cells. However, other techniques for establishing cultures of MSC-like cells include selection of colony-forming cells (28) or cellular outgrowth from partially digested tissue (30); the degree to which cell properties are affected by culture techniques is not known. Lastly, more thorough investigations are needed to determine whether different connective tissue cells are more or less favorable for a given disease or injury. While these limitations can be addressed with additional testing, this study indicates that cell biomanufacturing should not be considered a barrier for the use of connective tissue cells in regenerative therapy strategies.

4.6 Conclusions

This study demonstrated that sheep are a favorable species for reliably generating adult MSC-like connective tissue cells. Importantly, cell proliferation and CD expression was consistent among donor animals for a given tissue, and animal-to-animal variability is not expected to be a major confounding factor for laboratory or preclinical animal testing.

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CHAPTER 5 - CONCLUDING REMARKS AND FUTURE DIRECTIONS

Osteoarthritis is an exceedingly common degenerative disease that affects up to 45% of individuals in their lifetime. This disabling disease is characterized by pain, reduced mobility and a diminished quality of life. The economic burden is substantial, costing the US $480 billion in 2018 alone. OA is a chronic disease resulting from articular damage and synovial inflammation induced by a multifaceted relationship of mechanical, genetic, metabolic, biological and inflammatory factors. OA has long been viewed as a cartilage based degenerative disease, but recently focus has shifted to the significant role that inflammation plays in pathogenesis. Localized inflammation, characterized by infiltration of inflammatory cells and the production of inflammatory mediators can alter chondrocyte and synoviocyte function and survival, as well as induce expression of degenerative molecules in the joint. The innate immune system recognizes pathogens and tissue damage through pattern recognition receptors. The damage associated molecular patterns are produced during tissue damage and activate inflammatory mediators and have been implicated in the chronic inflammation exhibited OA. Evidence of the contribution of the adaptive immune system to OA has been observed with autoantibodies produced to mainly cartilage derived proteins.

This dissertation has contributed to OA research by recognizing a previously unidentified autoantigen present in the heterogenous inflammatory environment of OA, as well as provided a preliminary experimental treatment for immunomodulation in synovial joint cells. The novel discovery of an autoantibody in osteoarthritis recognizing a primary 60kDa and secondary 45 kDa protein in the cytoplasm of chondrocytes and synoviocytes improves the understanding of the adaptive immune response seen in naturally occurring OA that is not exhibited in a short term

PTOA model. For the treatment of OA, immune activation of MSCs with interferon inducing TLR3 and STING provides pathway specific benefits in OA immunomodulation. MSCs have shown inconsistent previous benefit in OA(1), with variability due to multiple factors including potential heterogeneity within MSC populations. In addition to these pathway and cell specific benefits seen with activation, the activation of MSCs with these receptor ligands prior to in vivo application may further improve treatment efficacy through the increase in treatment homogeneity in the context of OA. Additionally, large quantities of connective tissue cells can be cultured as an alternative source for regenerative therapies with limited phenotypical drift. Alternate sources of MSCs were evaluated for their ability to expand to high passages under the hypothesis that tissue sources of MSCs and MSC like cells may be more optimally matched to recipient site to improve therapeutic outcomes associated with treatment. These findings demonstrate the involvement of the adaptive immune system towards cellular antigens not previously seen in the inflammatory environment of OA. Progression of inflammation in osteoarthritis may be mitigated in part by an activated stromal cell regenerative therapy that could be enhanced utilizing stromal cells tailored to specific tissue sources.

In chapter two, we demonstrated that autoantibodies detected in OA are specific to intracellular proteins expressed by chondrocytes and synoviocytes. Pending additional proteomic identification of the antigen of interest, future studies will evaluate the effects of antibodies has on joint tissue homeostasis. Functional assays measuring the effect on cell growth and metabolism in vitro and the effect on healthy or degenerated joint tissue in vivo would determine the effects that autoantibodies have in the propagation of OA. Antibodies may have other tissue or systemic effects if specific to a common protein expressed in various cell types. Treatments aimed at neutralizing these antibodies may mitigate OA progression. Current treatments for OA,

such as nonsteroidal anti-inflammatories (NSAIDs), corticosteroid and regenerative therapy injections, or arthroscopic debridement, could be analyzed for their effects on the production of autoantibodies. Similarly, determining the regulatory mechanisms of the targeted antigen(s) and how to modulate their expression could lead to future therapies to stop or slow this adaptive response in the joint. Transgenic overexpression, or alternatively knocking out the gene for this antigen in a spontaneous osteoarthritic model in mice could inform the role of this autoantibody response in OA. The higher relative antibody levels in synovial fluid compared to plasma suggest that these antibodies are produced in the joint. The identification and reduction or elimination of the peripheral lymphoid structures responsible for antibody production could reduce adaptive inflammation in the joint, which could be investigated further. Exploration into a longer term PTOA model that exhibits increased autoantibody production, perhaps examining mice at 26 weeks after destabilization of the medial meniscus(2), would allow us to study this adaptive immune response more in depth in induced models of OA. Further investigation of whether autoantibodies are produced in induced animal models is warranted and would facilitate further investigation of the adaptive immune system in OA disease processes. Our data indicate that in the equine osteochondral fragment model of post traumatic osteoarthritis, autoantibodies are not produced at 18 weeks postoperatively, which may reflect disease duration and/or lack of severity in this model. Introduction of the targeted antigen, once identified, to elicit an adaptive response could shed further light on its role in disease progression, using naturally occurring or induced OA models.

In chapter three, we assessed further mechanistically the effect of immune activation of MSCs in the context of OA, using transcriptomic and immunoassay readouts. Activating MSCs in the context of OA showed that immunostimulants provide MSCs with an enhanced capability

to modulate inflammation. Although both poly I:C and STING induce interferon production, differences exist between the two activators in functional secretion of cytokines and differential gene expression in activated MSC and subsequently treated synovial joint cells. Heterogeneity in MSC populations has been proposed to be responsible for differential responses to therapy in the past, which may be potentially overcome through immune activation prior to clinical application. Future studies may further evaluate this concept through single cell RNA sequencing of MSCs before and after activation culture protocols to determine if the observed treatment effect with licensed MSC populations is, in fact, the result of increased homogeneity within stromal cell populations. Bulk RNA sequencing performed here provided a glimpse into molecular pathways involved between the two activators. To expand on the effects of certain pathways/molecules in OA progression, silencing mRNA through small interfering RNAs (siRNAs) could facilitate the understanding of these pathways and also inform which pathways are imperative in immunomodulatory differences seen between the activators. Our diluted aMSC treatment elicited relatively modest changes in target cell function in vitro. Further optimization of culture conditions in vitro may yield additional information. Higher concentrations of activator, longer exposure, multiple activator combinations, and increased ratio of MSC supernatant:treated cells could all potentially enhance target cell response. Similarly, co-culture of aMSCs with immune cells like macrophages could show improved or altered responses through cell-cell interactions. In addition, as the complexity of synovial cell interactions are not fully reflected in the *in-vitro* culture conditions simulated here, further evaluation in vitro using synovial or articular tissue explants could provide a more representative cellular landscape for OA. An organ-on-a-chip model could provide further insight into the complex intra-articular environment seen in OA, involving multiple cell types, fluid movement and the interplay between differing cell types.

Finally, as initial studies in mice support a beneficial effect to activated MSC therapy in osteoarthritis progression (Cody Plaisance, MS thesis, unpublished data), further investigation of aMSC treatment in large animal models of OA is warranted. Furthermore, as naturally occurring OA frequently may involve multiple synovial structures, further examination of the described therapies via multiple routes of administration (e.g., intravenous vs intra-articular) is warranted.

In chapter four, we investigated alternate connective tissue cell sources for MSC cell culture, with the idea that stromal cells cultured from the target tissue of interest may be beneficial when treating that specific tissue target. Large quantities of cultured cells were obtained from five connective tissues (annulus fibrosus, nucleus pulposus, meniscus, tendon and articular cartilage), which exhibited rapid and constant growth characteristics with limited phenotypical drift. Given the few sheep specific reagents and cluster of differentiation (CD) markers limited the characterization of the cell lines assessed using this relevant large animal model. The use of a different translational animal model with more available reagents could allow for more extensive characterization. As CD markers have been questioned in their correlation to functional outcomes, future studies could include further characterization of these cell lines through the use of functional assays, such as tri-lineage differentiation and immunoassays including cytokine secretion would inform studies on expanded cell capabilities in the context they will be applied. The phenotypical drift towards an MSC phenotype seen in annulus fibrosus cells with increased CD166, a glycoprotein of the immunoglobulin superfamily of proteins, expression at higher passages warrants further investigation of whether continued culture may in fact be advantageous if applying this cell line.

In summary, the studies described here contribute to the field's understanding of the role of the adaptive immune system in osteoarthritis, and further introduces evidence for the use of

TLR3- and STING-activated cellular immunotherapies to modulate progressive inflammation in osteoarthritis. These findings may be broadly applicable to future studies investigating intra-articular treatments for osteoarthritis.

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