RECIPROCAL INTERACTIONS BETWEEN MESENCHYMAL STEM CELL REGENERATIVE MEDICINE MODALITIES AND THE INFLAMMATORY RECIPIENT ENVIRONMENT

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RECIPROCAL INTERACTIONS BETWEEN MESENCHYMAL STEM CELL REGENERATIVE MEDICINE MODALITIES AND THE INFLAMMATORY RECIPIENT ENVIRONMENT

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Advancement of regenerative medicine modalities is dependent on understanding the basic biology of both the cells being used as treatment and the recipient environment. This has become more apparent as clinical implementation of regenerative medicine has been pushed forward, and basic research has lagged behind, resulting in inconsistent response to therapy. The overall goal of this thesis was to further understand the bidirectional interactions between regenerative medicine modalities utilizing mesenchymal stem cells (MSCs) and the targeted inflammatory recipient environment.

Bone marrow concentrate (BMC) and platelet-rich plasma (PRP) are used extensively in regenerative medicine. The aim of Chapter 2 was to determine differences in the cellular composition and cytokine concentrations of BMC and PRP and to compare two commercial BMC systems in the same patient cohort. The most significant finding of this study was the previously undocumented high concentration of interleukin 1 receptor antagonist (IL-1ra) in bone marrow aspirate and bone marrow concentrate. IL-1ra is an antagonist of the pro-inflammatory cytokine IL-1β, which is an important mediator in early osteoarthritis. This highlighted that clinicians are currently using this regenerative medicine modality without a full understanding of the bioactive factors present, which could have differing clinical effects on musculoskeletal tissue. Future research could apply these results to design appropriately sized clinical studies to correlate clinical outcome with modality components.

In order to answer basic questions about the interactions between the recipient environment and MSCs, the horse was employed as a model. In Chapter 3, we sought to determine how different inflammatory cells and inflammatory stimuli impacted MSC immunomodulatory function. MSCs were exposed to polyinosinic:polycytidylic acid (poly I:C) to stimulate TLR3 receptors, lipopolysaccharide (LPS) to stimulate TLR4 receptors, or inflammatory macrophages. TLR priming or exposure of MSCs to inflammatory macrophages induced marked upregulation of many cytokines and chemokines in MSCs. All inflammatory stimuli treatments significantly enhanced MSC immunomodulatory function, demonstrated by a decrease in T-cell proliferation compared to unstimulated MSCs. While these results suggest MSC use in an inflammatory environment may not be

contraindicated, the recipient tissue bed in chronic injuries may not contain sufficient inflammatory signals to activate MSC immunomodulatory function. These results suggested that enhancement of MSCs immunomodulatory function through inflammatory priming prior to clinical application, known as "inflammatory licensing," might improve the therapeutic effect of MSC treatments.

In the Chapter 4 we sought to further understand how these inflammatory licensed MSCs would function in the recipient environment. MSCs were licensed with either poly I:C or IFN-γ and then exposed to inflammatory macrophages. Additionally, to investigate the significance of the inflammatory gene upregulation noted in MSCs following inflammatory licensing, we analyzed the effects of the inflammatory licensed MSCs secretome on macrophages and chondrocytes.
Inflammatory licensed MSCs maintained their superior ability to suppress T cell proliferation compared to naïve MSCs, even with subsequent exposure to inflammatory macrophages. There was little evidence that inflammatory licensing creates a pro-inflammatory MSC secretome, based on minimal changes to macrophage or chondrocyte gene expression. The secretome of inflammatory licensed MSCs continued to exert a beneficial effect on arthritic (IL-1β stimulated) chondrocytes, causing downregulation of pro-inflammatory genes. Compared to the licensing agents, inflammatory macrophages produced an inconsistent change in immunomodulatory function. This suggests that in vitro inflammatory licensing prior to clinical use could result in more consistent induction of immunomodulatory function, compared to in vivo inflammatory licensing by the recipient environment.

In Chapter 5 we evaluated if MSCs isolated from different tissue sources responded differently to inflammatory stimuli. If MSCs from one or more sources were resistant to inflammatory stimuli, then that source might be preferable for clinical application in regenerative medicine. We evaluated the immune-related gene expression profiles of equine MSCs from different tissue sources in response to IFN-γ stimulation. MSCs from all sources responded in a similar fashion to IFN-γ induced gene upregulation. Both before and after stimulation, peripheral blood derived-MSCs and adipose tissue derived-MSCs gene expression profiles clustered together and bone marrow derived-MSCs and umbilical cord blood derived-MSCs were most similar to each another. The similarity in response and in expression following IFN-γ stimulation, suggests that when selecting a source of MSCs for clinical use in an allogeneic or inflammatory environment, the MSCs from different sources will likely respond similarly to the inflammatory stimuli.

BIOGRAPHICAL SKETCH

Jennifer Michelle Cassano was born in Manhasset, New York on December 9, 1987 to Steve and Valerie Cassano. She graduated Salutatorian from Valley Stream Central High School in 2006. She attended Cornell University for her undergraduate degree and completed an honors thesis in the laboratory of Dr. Doug Antczak on the interactions between horse genotype and BPV in equine sarcoid. She graduated Magna Cum Laude with Distinction in Research from Cornell University in 2009 and she received her Bachelor's degree in Animal Science. Jennifer was accepted into the Cornell University College of Veterinary Medicine's Combined D.V.M/Ph.D. Program in 2009 and received her D.V.M. degree in 2013. During veterinary school, she received the American College of Internal Medicine Clinical Excellence Award. Jennifer first rotated in Dr. Fortier's laboratory in 2009 where she began investigating the interactions between the environment and regenerative therapies. Following her veterinary degree, Dr. Cassano began pursuing a Ph.D. degree in the laboratory of Dr. Fortier in the Graduate Field of Comparative Biomedical Sciences, Department of Clinical Sciences, funded for the first two years by a Cornell University Graduate Research Assistantship (2013-2015). In 2015, she was awarded a Morris Animal Health Foundation Fellowship Training Grant (2015-2016). During her Ph.D. training, Dr. Cassano assisted in and taught numerous veterinary laboratories in order to further her professional development and prepare herself for an academic position consisting of research, clinical work, and teaching.

This dissertation is dedicated to all the angels that walk above me and with me, your love and support gives me the strength to strive to be better.

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Chapter 2: Bone marrow concentrate and platelet-rich plasma differ in cell distribution and interleukin 1 receptor antagonist protein concentration.

Authors: Jennifer M. Cassano, John G. Kennedy, Keir A. Ross, Ethan J. Fraser, Margaret B. Goodale, Lisa A. Fortier

Study design was proposed by JMC, KAR, and LAF. JGK performed all surgical procedures. Samples were shipped by EJF and received by JMC for analysis. Tissue culture was performed by JMC, EJK, and Lynn Pezzanite. Cell counts and differentials were performed by the clinical pathology department. Samples were processed for multiplex by JMC and the multiplex was performed by Heather Freer. Data analysis and statistical analysis was performed by JMC and MBG. The manuscript was written by JMC and reviewed and edited by all authors.

Chapter 3: Priming through an inflammatory microenvironment and TLR ligands enhance MSC immunomodulatory function.

Authors: Jennifer M. Cassano, Lauren V. Schnabel, Margaret B. Goodale, Lisa A. Fortier

Study design was proposed by JMC, LAF, and LVS. Samples (lymphocytes, macrophages, and MSCs) were isolated by JMC and MBG. Tissue culture was performed by JMC and MBG. Flow cytometry was performed by JMC. RNA was extracted and made into cDNA by Andrew Bullis. qRT-PCR was performed by JMC. Data analysis and statistical analysis was performed by JMC with consultation from Lynn Johnson. The manuscript was written by JMC and reviewed and edited by all authors.

Chapter 4: Inflammatory licensing equine MSCs improves immunomodulatory function and does not induce a pro-inflammatory MSC secretome

Authors: Jennifer M. Cassano, Lauren V. Schnabel, Margaret B. Goodale, Lisa A. Fortier

Study design was proposed by JMC, LVS, and LAF. Samples (lymphocytes, macrophages, and MSCs) were isolated by JMC and MBG. Tissue culture was performed by JMC and MBG. Flow cytometry was performed by JMC. RNA was extracted and made into cDNA by Kerry Schneider and JMC. qRT-PCR was performed by JMC. Data analysis and statistical analysis was performed by JMC with consultation from Lynn Johnson. The manuscript was written by JMC and reviewed and edited by all authors.

Chapter 5: Equine mesenchymal stem cells (MSCs) from different tissue sources display similar immune-related gene expression profiles in response to IFN-γ stimulation.

Authors: Jennifer M. Cassano, Lisa A. Fortier, Rebecca B. Hicks, Rebecca M. Harman, Gerlinde R. Van de Walle

Study design was proposed by JMC, GVdW, and LAF. Samples (MSCs) were isolated by JMC, MBG, and RMH. IFN-γ stimulation tissue culture was performed by RBH. RNA was extracted and made into cDNA by Kerry Schneider, JMC, and RBH. qRT-PCR was performed by JMC and RBH. Data analysis and statistical analysis was performed by JMC with consultation from Lynn Johnson. The manuscript was written by JMC and reviewed and edited by all authors.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

GENERAL INTRODUCTION

Overall goal of dissertation research

The overall goal of this dissertation research was to further understand the bidirectional interactions between regenerative medicine modalities and the recipient environment. Regenerative medicine seeks to restore or establish to the normal functional state of tissues by utilizing knowledge of molecular biology and tissue engineering. Adult and embryonic stem cells are studied in regenerative medicine for their ability to serve as progenitor cells, as well as their capacity to change the environment into which they are placed. Signals in the microenvironment of injured or chronically inflamed tissue will shape the fate of that tissue and determine if a return to normal function is possible. Regenerative medicine seeks to manipulate the tissue environment to achieve the best clinical outcome. While there is a drive for increasing the use of regenerative medicine modalities in the clinic, the basic research to understand the components and effects of these modalities has lagged behind the clinical implementation.

In the first study presented, a detailed analysis was performed on various biologics produced in the same human patient cohort that are presently in clinical use (Chapter 2). This study highlighted that although these biologics are allowed by the Food and Drug Administration, not all of their components are known. Thus, biologics are being utilized without a full understanding of their potential for positive or negative clinical implications. While implementation of biologics in regenerative medicine is expanding, many basic science gaps remain that require an animal model to address these questions.

In the studies presented in Chapters 3, 4, and 5 the horse was utilized as a model, to further understand the impact of the environment on mesenchymal stem cells (MSCs). The horse is an ideal animal model to study how the environment affects MSCs and can be superior to the mouse and other

models for several reasons. The horse allows for non-invasive access to large quantities of blood and bone marrow needed to generate and test MSCs. The horse is a valuable model for studying the human immune response because the horse and human genome share high synteny, and over 90 hereditary diseases in horses have similarities to human conditions.[1–4] Stem cell therapy has been studied for its benefits in many analogous conditions in the horse and human, including musculoskeletal injury, amyotrophic lateral sclerosis, and acute neural injury. Moreover, studying the immunomodulatory function of MSCs using the mouse as a model has been called into question, since murine MSCs act via upregulation of intracellular inducible nitric oxide synthase (iNOs) and human MSCs act via indoleamine 2,3 deoxygenase (IDO) expression.[5] Phylogenetic analysis predicts that equine MSCs utilize the IDO pathway like humans.[5] Our expertise and developed reagents facilitate utilizing the equine model, and we propose that the horse is not only a clinically relevant model, but the optimal model to replicate immunomodulation of human MSCs. Additionally, horses and humans are outbred species, unlike many laboratory animals. Lastly, proof of efficacy and safety in large animal models (sheep, goat, or horse) is commonly required by regulatory agencies prior to the approved use of regenerative therapies in humans because they more closely approximate the situation and response to treatment of human patients when compared to laboratory species.[6]

Biologics are investigated in Chapter 2 as they are the current FDA approved clinical implementation of regenerative medicine in humans. Basic questions about the interactions between the recipient environment and MSCs are studied in Chapters 3-5. MSCs have great promise in regenerative medicine, however it is not well understood how the recipient environment can alter their function. We sought to determine how different inflammatory cells and inflammatory stimuli impact MSC immunomodulatory function (Chapter 3). To further understand the changes documented in MSC function induced by inflammatory signals, the effects of inflammatory stimuli priming with inflammatory cell exposure and on the MSC secretome was determined (Chapter 4). Finally, the effects of an

inflammatory cytokine on MSCs derived from different tissue sources was studied to guide the selection of stem cell source for clinical application (Chapter 5).

Biologics

Regenerative medicine seeks to utilize various biologics, or therapeutic products made from biologics sources, typically autologously prepared. Currently, in vitro expansion of MSCs prior to transplantation into human patients is not allowed by the FDA. Culture expansion of MSCs also takes a variable amount of time, from 2-6 weeks depending on the tissue source of MSCs,[7] resulting in a delay in clinical application. As a result, both human and veterinary medicine have turned to biologics such as bone marrow concentrate (BMC) to administer MSCs. BMC is generated by a centrifugation process that concentrates the MSCs and the other cellular components in bone marrow aspirate. As a result of the BMC preparation process, numerous leukocytes at various stages of differentiation comprise the majority of the cell population in the BMC product, which also contains various growth factors and cytokines secreted by the MSCs and leukocytes. Secretion of paracrine factors and modulation of the environment is thought to be the main mechanism of action of MSCs, so BMC has the potential to deliver many of the same benefits as expanded MSCs, despite a lower number of MSCs.[8,9]

Platelet rich plasma (PRP) is another popular biologic for patient side administration and is produced through a centrifugation process of whole blood. PRP is mainly thought to be beneficial due to the concentrated platelets containing growth factors in their granules.[10]

Because biologics are prepared from individuals for autologous use, there is tremendous variability in the end therapeutic product,[11] making it difficult to compare them to each other or between patients. There are numerous commercial machines available for clinical use, and there are known differences in how these cells concentrate the various components of blood or bone marrow aspirate.[8] In order to select the most appropriate biologics for a clinical application, further understanding of the

component differences is needed. The objectives of the study presented in Chapter 2 were to utilize samples of BMC and PRP derived from the same patient cohort to determine how these biologics differ, and to compare BMCs generated from two different commercial systems.

The following review article details the current status of MSCs in regenerative medicine and highlights the current knowledge gaps in the field. The article has been modified from the printed version to include the MSC inflammatory licensing theory. Following the article is information regarding in vitro measurement of immunomodulation, macrophage polarization, and MSC tissue sources, all of which are important for understanding the rationale of the studies presented in Chapters 3, 4, and 5.

Mesenchymal Stem Cell Therapy: Clinical Progress and Opportunities for Advancement

Modified from: Mesenchymal Stem Cell Therapy: Clinical Progress and Opportunities for Advancement Published in Current Pathobiology Reports 2015;3(1):1–7

The goal of regenerative medicine research is to improve tissue healing and return the patient to function after injury. MSC therapies deliver a multitude of growth factors and cytokines to the injured site. As important are the bidirectional interactions between transplanted MSCs with the host environment which can dictate the difference between function and non-functional tissue regeneration.

Mesenchymal stem cells (or multipotent stromal cells, MSCs) can differentiate into chondrocytes, adipocytes, and osteoblasts. MSCs can be derived from numerous adult tissue sites, such as bone marrow, adipose, peripheral blood, umbilical cord blood, umbilical cord tissue, and muscle. They have been identified as having great therapeutic potential to treat a wide variety of disease processes. However, there is still much to understand about how stem cells elicit a healing response and how the recipient environment impacts MSC phenotype and function.

The use of stem cells for cartilage repair began in the 1980's with several in vivo animal studies, but it was the work of Wakitani[12] in 1994 that incited international attention by suggesting that bone marrow-derived MSCs injected into the knees of rabbits with cartilage defects lead to formation of

hyaline-like repair tissue. It was believed at that time that MSCs functioned to facilitate cartilage repair by differentiating into functional chondrocytes that would replenish the extracellular matrix. In the past several years, however, there has been a paradigm shift toward the concept that the benefit of MSCs in regenerative applications is due to modulation of the local environment, mainly through paracrine signaling, and to decreased immune response, rather than the MSCs acting as a cell source for new tissue.[13–15]

MSC-driven immunomodulation

MSCs have numerous effects on cells of the immune system, and secrete paracrine factors culminating in multiple levels of enhanced tissue regeneration (Figure 1). MSCs induce regulatory T cells (Tregs) in animal model studies of rheumatoid arthritis[5] and uveoretinitis[16,17] resulting in suppressed induction and proliferation of effector T cells. In vitro, MSCs have been shown to decrease T cell proliferation, decrease IFN gamma secretion, and induce a helper T cell response (Th2) response necessary for humoral immunity.[18] Differentiation of B cells into plasmablasts is inhibited by adipose-derived MSCs in a T cell independent manner, suggesting that MSCs can be a tool for the modulation of the B cell response in immune disease.[19] Natural killer cells have decreased proliferation, cytotoxicity, and secrete less IFN gamma when exposed to MSCs in vitro.[20] MSCs also have a significant impact on monocytes leading to decreased differentiation and promotion of polarization towards an M2 regenerative phenotype essential for functional regeneration.[21,22]

Paracrine signaling from MSCs

The secretome of MSCs contains numerous paracrine factors thought to produce a therapeutic effect through modulation of the injury reparative process and immunomodulation of the local environment.[23,24] Several studies have investigated how MSCs affect the transplantation recipient environment via paracrine mediators.[21,25–28] MSCs secrete various cytokines such as prostaglandinE_2 (PGE_2), transforming growth factor β (TGFβ), interleukin 1 receptor antagonist (IL-1ra), IL-6, and IL-

10.[20] MSCs also secrete indoleamine 2,3 deoxygenase (IDO) which is involved in tryptophan catabolism and is one mechanism by which MSCs inhibit T cell proliferation (Figure 1).[29] Collectively, these cytokines act to provide an environment conducive to tissue repair.

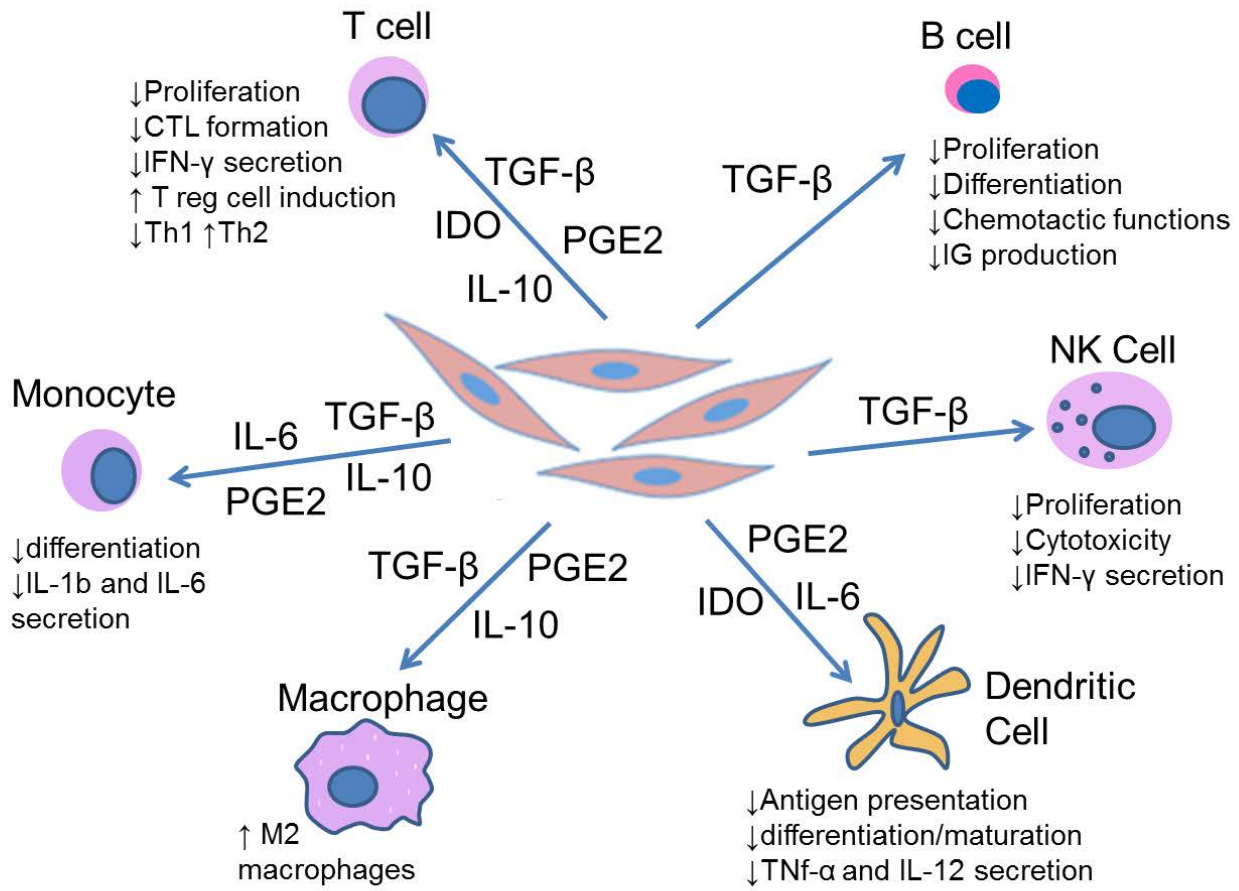
MSCs in transplantation

The immunomodulatory potential of MSCs has led to their use in numerous studies to improve survival of allografts. Survival of corneal allografts in a mouse model was improved when allogeneic MSCs were injected intravenously, compared to syngeneic MSCs or no MSC treatment.[30] A recent study found that co-administration of an immunosuppressive drug along with MSCs promoted long-term acceptance of allogeneic heart grafts, compared to treatment with the drug or MSCs alone, via induction of Treg cells.[31] If MSCs are able to be used as an adjunct to promote modulation of the local transplant environment, complete immune suppression would not be necessary for transplant survival. Additionally, numerous preclinical studies have explored models using MSCs to treat acute graft versus host disease (GVHD).[32,33] In Germany, 25 patients were treated at least 2 times, with bone marrow-derived, allogeneic MSCs for steroid non-responsive acute GVHD, and 17 (68%) of the patients had a positive response with no significant adverse events.[34] These studies suggest that the main benefit of utilizing MSCs to alter the local immunomodulatory environment is a reduced need for suppression of the entire immune system in a particularly vulnerable patient population.

MSCs for treating cardiovascular disease

One of the most clinically advanced indications for MSC therapy is in the treatment of cardiac dysfunction. Pilot studies are in progress to evaluate safety and efficacy of MSCs primarily for acute cardiac ischemic events. Intra-coronal artery infusion of either bone marrow or adipose-derived MSCs in a mouse experimental model of acute myocardial infarction resulted in enhanced cardiac function and a switch from infiltration of pro-inflammatory to anti-inflammatory (M2) macrophages at the infarction

Fig. 1.1 Mesenchymal stem cells have multimodal effects on the immune system. MSCs secrete numerous inflammatory mediators that alter the differentiation, proliferation, and cytokine profiles of various immune system cells



site. This macrophage polarization switch was presumptively due to secretion of PGE_2 from MSCs leading to IL-10 secretion from macrophages.[35,36] A recent study in a porcine model of acute myocardial infarction showed that MSC infusion of bone marrow-derived MSCs into the coronary artery improved cardiac function to a greater degree when treatment was delayed for 2-4 weeks after myocardial infarction, compared to MSC treatment at 3 hours, 1 day, or 3 days after the ischemic event.[37] These studies document safety and efficacy in animal models. Currently in Spain, a placebo controlled, double blind, phase II study is underway to investigate the benefits of intravenous allogeneic adipose-derived MSCs following an acute ischemic event.[38] Patients will be followed for 2 years after treatment to document safety and efficacy.

MSCs for treating osteoarthritis

Intra-articular injections of MSCs have been proposed as a treatment for osteoarthritis (OA) based on their potential to resolve chronic inflammation via immunomodulation.[39] There are several early-stage clinical trials testing intra-articular delivery of isolated stem cells into the knee, but the type of stem cell, optimal dose, and treatment schedule remain to be established. In 2013, the first clinical results were published. Two level 4 studies[40] (observational studies, without controls) showed improved clinical signs of OA, the first at 1-year post-injection using autologous, adipose-derived stromal vascular fracture containing stem cells in 56 patients[41] and the other at 2-years follow-up after injection of autologous bone marrow-derived MSCs in 12 patients.[42] A level 2 randomized controlled trial studied the effects of autologous, bone marrow-derived MSCs in combination with microfracture and medial opening-wedge high tibial osteotomy in 56 patients with unicompartmental OA knees and genu varum with 2-years follow-up. This group also reported sustained significant improvement in clinical signs of pain and function associated with OA in patients treated with MSCs. Finally, very encouraging results were published by Vangsness et. al in the first level 1 randomized, double-blind, prospective study of MSC therapy for joint disease.[43] Patients were randomized to three groups and received either a single dose of 50 or 100 million stem cells in 20 mg of sodium hyaluronate, or 20 mg of sodium hyaluronate seven to

ten days after meniscectomy. Follow-up data was obtained at 6 months, 1 year, and 2 years post-injection. Follow-up data included patient-reporting systems of visual analog scale (VAS) of 0 to 100 and the Lysholm knee scale self-assessment, and MRI to assess meniscal volume, cartilage degeneration, and formation of ectopic tissue. MSC treatment resulted in significantly increased meniscal volume and significant reduction in pain compared with those who received the control. Continued pain relief from OA following a single injection of MSCs represents a major advancement in the symptomatic treatment of OA. One injection of MSCs, rather than repeated injections of steroids or sodium hyaluronate and/or frequent consumption of NSAIDs, should decrease patient morbidity and decrease the economic burden of OA to society.

MSC environmental interaction theories

At the onset of this dissertation research, a MSC polarization paradigm was proposed as the most likely explanation for how the environment affects MSC function. Throughout the work described in this thesis and in recent publications, MSC licensing has emerged as another framework to view the effects of the environment on MSCs. These two theories are explored in our research in Chapters 3 and 4.

MSC polarization

Similar to macrophages, MSCs polarize into pro-inflammatory MSC1, and immunomodulatory MSC2 phenotypes.[44,45] Much more is known about polarization of macrophages[46–48] compared to MSCs,[44,45] but there are similarities in triggers and resultant signaling pathways.[44,45] For example, lipopolysaccharide (LPS) results in a shift to pro-inflammatory MSC1 and M1 phenotypes. Similarly, MSC2 and M2 cells are desired for regenerative therapy to achieve immunomodulatory and functional tissue regeneration.[22,49–52] In vivo, paracrine signals from the environment regulate macrophage polarization, the ratio of M1:M2 cells, and the resultant reparative response.[52] MSCs can effect changes in macrophage phenotype both in vitro and in vivo and promote development of M2 macrophages.[21,28,35,53,54] Taken together, the MSC → environment ↔ macrophages ← MSC parts of the equation are known to

some extent, but the environment → MSCs interaction is largely uninvestigated. In disease states such as osteoarthritis where pro-inflammatory M1 macrophages predominate in the synovial membrane[22], it is possible that paracrine signals secreted from those M1 macrophages could polarize transplanted MSCs to a pro-inflammatory MSC1 phenotype which would elicit a further inflammatory reaction rather than an immunomodulatory response. To harness the full capacity of MSCs for regenerative medicine approaches, further understanding of how the transplantation/recipient environment affects MSC function is critical.

Priming MSCs to induce a uniform MSC1 or MSC2 population has promising potential to direct the actions of MSCs in modifying the inflammatory status of the local environment. In an in vivo model of painful diabetic neuropathy, MSC2 primed MSCs had a superior ability to ameliorate pain compared to unprimed MSCs.[55] Additionally, MSC1 primed MSCs, which are desired for their pro-inflammatory ability to overcome immune tolerance in tumor environments lead to a reduction in tumor size in vitro and in vivo compared to unprimed and MSC2 primed MSCs.[56] Collectively, these studies support MSC priming and polarization as a way to direct MSC immunomodulation to facilitate tissue regeneration.

Inflammatory licensing

The inflammatory licensing theory suggests that exposure of MSCs to inflammatory cytokines activates their immunomodulatory capabilities. [57–61] This idea was first proposed following the clinical observation that while MSCs appeared beneficial in the treatment of established graft versus host disease (GVHD),[62,63] when MSCs were co-administered with a bone marrow transplant at the time of initial treatment, they had no effect on the development of graft versus host disease.[64] It was proposed that there may be something present in the established disease environment that was stimulating the MSCs and thus eliciting an immunomodulatory function of the MSCs. Numerous studies have found that the timing of administration of MSCs to be critical, with delayed infusion of MSCs causing reduced mortality in experimental models of GVHD.[59,65] The timeline of clinical efficacy for MSCs is consistent with the delay required for IFN-γ secretion by T cells in the early adaptive immune response, as T cells secrete

IFN-γ in response to antigen presentation. IFN-γ treatment of MSCs prior to application improved their ability to treat graft versus host disease (GVHD).[59] While IFN-γ has been shown to be necessary in induction of MSC immunomodulatory capabilities, it is not sufficient for activation and requires the presence of TNF-α, IL-1α, or IL-1β [57]. Interestingly, a study found exposure of MSCs to IFN-γ and TNF-α abolishes the innate immunomodulatory heterogeneity of MSC clones in vitro and in vivo.[61] These results indicate that placing MSCs in an inflammatory recipient environment would not be contraindicated and instead may maximize their immunomodulatory potential.

Allogeneic MSC transplantation

The concept that MSCs are "immunoprivileged" is based on laboratory animal experiments and is frequently called into question.[66] MSCs express major histocompatibility (MHC) class I, a cell surface protein that the immune system uses to distinguish self from non-self in allorecognition. MSCs were initially reported to have low to absent MHC class II expression, which is utilized in self recognition and immune activation. When explored more extensively, it became clear that MSCs have variable MHC class II expression, in numerous species.[67–69] Our laboratory found that MHC class II expression in bone marrow-derived MSCs was variable between individuals and could change with passage in culture.[67] MSCs can upregulate MHC II expression upon exposure to IFN-γ. [67] Furthermore, only MHC class II negative MSCs are immunomodulatory in vitro as indicated by decreased lymphocyte proliferation in a modified mixed lymphocyte assay. This switch in the immunomodulatory properties of MSCs with changes in MHC class II expression is consistent with previous studies where MSC2 MSCs were capable of immunomodulation, while MSC1 MSCs stimulated lymphocyte proliferation.[44]

To study the consequences of using allogeneic MHC class II negative MSCs in vivo, our laboratory measured antibody responses in horses injected with MHC haplotype mismatched MSCs.[70] All horses injected with mismatched MSCs developed antibodies against the injected MHC haplotype, regardless of the MHC II status of the MSCs used for injection.

Investigation for subtle, subclinical evidence of an immune response to allogeneic MSCs, while essential for evaluation of MSC treatment for safety and efficacy, is rarely performed even in pre-clinical animal model studies. In the Vangsness study, allogeneic stem cells were used and no cross-matching was performed, which is presently typical for MSC studies. The MSCs were cultured in good manufacturing practice (GMP) fashion, but the donor human leukocyte antigen (HLA) status was not known and there was a real potential for immunological reaction to the transplanted, allogeneic MSCs. To test for a cell-mediated or humoral immune response, expression of immune cell markers for T cells, natural killer cells, and B cells including CD3, CD4, CD8, CD20 and CD56 were measured in peripheral blood. The authors report that there were no shifts in immunological parameters measured, suggesting that there was no immune reaction to the allogeneic stem cells. However, donors will vary between studies, so it is important for investigators to continue to be vigilant and look for immune reactions at a cellular level and not just based their conclusions on external findings at physical examination such as heat or swelling of the joint. Although a single MSC treatment by direct injection in the knee joint appears safe, it is important to recognize that the potential effect of immune priming would need to be evaluated if a repeated dosing regimen were to be studied.

Conclusion

Despite challenges and limitations to allogeneic MSC use, the need for allogeneic cells in many disease indications, some of which are described above, drives further investigation. Acute circumstances such as myocardial infarction, genetic factors affecting autologous stem cell quality, and costs associated with isolation and expansion of autologous MSCs all make the use of allogeneic MSC appealing and clinically important. Continued investigation into the optimal type of stem cell and therapeutic dosing of stem cells with respect to cell number and timing of injection(s) should lead to further clinical advances in stem cell therapy.

In vitro measurement of immunomodulation

To assess MSC immunomodulatory function in vitro, researchers primarily rely on measuring MSCs ability to suppress lymphocyte proliferation.[5,26,61,67] MSCs can suppress mitogen activated lymphocyte proliferation or suppress responder lymphocyte proliferation in a mixed leukocyte reaction (MLR). In an MLR there are two populations of lymphocytes derived from different animals and in a lymphocyte proliferation assay there is a single lymphocyte population. The lymphocyte proliferation assay utilizes a mitogen such as concavalinA (conA) to induce proliferation. ConA acts specifically on T cells, whereas other mitogens such as pokeweed induce proliferation in both T and B cells.[71] Prior to incubation with the mitogen, the lymphocytes are labeled with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE). As cells divide the CFSE marker is split between daughter cells resulting in a decrease in fluorescence on flow cytometry, which represents cell proliferation.[71] The mitogen stimulated T cell proliferation assay more closely approximates the clinically relevant situation when MSCs are injected into a patient, where there is a single lymphocyte responder population, compared to a true MLR with two MHC mismatched lymphocyte populations. The studies presented in Chapters 3 and 4 extensively utilize lymphocyte proliferation assays.

Measuring the ability of MSCs to suppress T cell proliferation is focusing on only one aspect of MSC immunomodulation; however, this in vitro assay has been predictive of MSCs ability to reduce a delayed hypersensitivity response in vivo.[61] Additionally, the primary mechanism by which MSCs suppress T cell proliferation is through prostaglandin E_2 (PGE_2) secretion,[18] which can impact numerous other immune cells.[72] Suppression of T cell proliferation indicates MSCs are secreting PGE_2, in vitro T cell suppression could indicate MSCs potential for other immunosuppressive effects such as inhibition of differentiation of monocytes and dendritic cells. T cell proliferation suppression is an informative assay to begin to measure MSC immunomodulatory capabilities in vitro; however additional in vitro assays as well as in vivo studies are needed to further characterize changes in MSC immunomodulatory function.

Macrophage polarization

Macrophages can be polarized in vitro to take on different characteristics along a spectrum, from the classically activated pro-inflammatory M1 phenotype, to the alternatively activated regenerative M2 phenotype.[46–48] Stimulation of naïve macrophages with lipopolysaccharide (LPS) and IFN-γ produces the M1 phenotype characterized by increased inflammatory cytokine and iNOS production and increased phagocytic activity. Alternatively activated macrophages can be produced through stimulation with a combination of interleukin-4 (IL-4), interleukin-13 (IL-13), immune complexes, interleukin-10 (IL-10), glucocorticoids, and transforming growth-factor-β (TGF-β).[46,73] The resultant M2 macrophages phenotypes are slightly different depending on the individual combination of stimulants, but they have relatively lower expression of inflammatory cytokines compared to M1 macrophages, secrete IL-10, and have high levels of intracellular arginase activity.[48] M2 macrophages are desired to achieve functional tissue regeneration.[22,49–52] The M1 and M2 phenotypes described are in vitro extremes of the spectrum of what exists in vivo, and polarization states have been shown to be somewhat plastic.[48,73,74] In vivo, paracrine signals in the microenvironment regulate macrophage polarization, the ratio of M1:M2 cells, and the resultant reparative response.[52] An M1 macrophage placed in an environment with M2 stimulants will downregulate some of its M1 attributes and adopt a more M2 phenotype. This plasticity makes sense in the context of injury and repair, where in the acute phase an inflammatory environment is necessary to recruit cells to the site, and then a switch needs to occur to a non-inflammatory environment for repair. However, in many disease states, this switch does not occur and there is chronic inflammation. MSCs can affect changes in macrophage phenotype both in vitro and in vivo and promote development of M2 macrophages.[21,28,35,53,54]

The objectives of the study in Chapter 3 were to determine how inflammatory macrophages, similar to what would be present in an inflamed recipient environment, impacted MSC

immunomodulatory function and to test the polarization paradigm in the equine model. Our hypothesis was that exposure to inflammatory macrophages or TLR4 stimulation would impair the immunomodulatory function of MSCs, whereas TLR3 stimulation with poly I:C would promote their immunosuppressive properties. Contrary to our hypothesis, we found TLR3 stimulation, TLR4 stimulation, and exposure to inflammatory macrophages increased the immunomodulatory function of equine MSCs. These results were consistent with the inflammatory licensing paradigm, and led us to further explore inflammatory licensing in equine MSCs by studying the effect of poly I:C and IFN-γ stimulation followed by inflammatory macrophage exposure, and the secretome of equine MSCs following inflammatory priming in Chapter 4.

MSC tissue sources

MSCs isolated from various sources share the minimum defining characteristics of cell surface markers, trilineage differentiation, and plastic adherence, but they differ in many other properties. MSCs immunomodulatory capabilities vary based on the tissue source.[75,76] The levels of expression of different cell surface markers such as the major histocompatibility complex (MHC) and other markers such as CD44 are also dependent on the source of cells.[77] MSCs from different sources vary in the extent to which they undergo trilineage differentiation, and MSCs from some sources are able to differentiate into additional lineages such as hepatocytes.[78,79] From a practical standpoint, isolation ease and availability of MSCs from different sources is also distinct. While the fact that these cells do not behave identically is not surprising, it is important to carefully consider the source of cells for regenerative therapies and not treat all MSCs as equal. MSCs have previously been regarded as uniformly MHC class II negative, however our laboratory has shown this is not the case for all bone marrow derived equine MSCs.[67] As discussed earlier, negative MHC class II expression is important in relation to allogeneic MSC use. Baseline MHC class II expression in equine adipose, peripheral blood, or umbilical cord derived MSCs have not been reported.[26,80] Only equine bone marrow and umbilical cord blood derived MSCs have been

reported to respond to IFN-γ and upregulate MHC class II expression.[67,81] The study presented in Chapter 5 sought to address this knowledge gap by completing a gene expression analysis following IFN-γ stimulation of MSCs derived from bone marrow, adipose, peripheral blood, and umbilical cord blood. We hypothesized that adipose derived MSCs would be similarly responsive to IFN-γ stimulation as bone marrow derived MSCs, but that peripheral blood and umbilical cord blood derived MSCs would be more resistant to IFN-γ stimulation. Our hypothesis was based on the knowledge that adipose is an inflammatory tissue, suggesting that MSCs derived from this source may be more responsive to inflammatory stimuli. Both peripheral blood and umbilical blood are exposed to numerous cytokines in circulation, and yet have not been documented to have MHC class II expression at baseline isolation, thus we proposed they may be resistant to IFN-γ stimulation with respect to upregulation of MHC class II. This study has great clinical relevance to veterinary species, where ease of isolation often guides MSC source selection, and further outlines the concept that MSCs derived from different sources respond differently to inflammatory stimuli.

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CHAPTER 2

BONE MARROW CONCENTRATE AND PLATELET RICH PLASMA DIFFER IN CELL DISTRIBUTION AND INTERLEUKIN 1 RECEPTOR ANTAGONIST PROTEIN CONCENTRATION.

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Abstract:

Purpose

Bone marrow concentrate (BMC) and platelet-rich plasma (PRP) are used extensively in regenerative medicine. The aim of this study was to determine differences in the cellular composition and cytokine concentrations of BMC and PRP and to compare two commercial BMC systems in the same patient cohort.

Methods

Patients (29) undergoing orthopedic surgery were enrolled. Bone marrow aspirate (BMA) was processed to generate BMC from two commercial systems (BMC-A, BMC-B). Blood was obtained to make PRP utilizing the same system as BMC-A. Bone marrow derived samples were cultured to measure colony forming units and flow cytometry was performed to assess mesenchymal stem cell (MSC) markers. Cellular concentrations were assessed for all samples. Catabolic cytokines and growth factors important for cartilage repair were measured using multiplex ELISA.

Results

Colony forming units were increased in both BMCs compared to BMA ($p<0.0001$). Surface markers were consistent with MSCs. Platelet counts were not significantly different between BMC-A and PRP, but there were differences in leukocyte concentrations. TGFβ1 and PDGF were not different between BMC-A and PRP. IL-1ra concentrations were greater ($p=0.0018$) in BMC-A samples (13,432 pg/ml) than in PRP (588 pg/ml). The ratio of IL-1ra:IL-1β in all BMC samples was above the value reported to inhibit IL-1β.

Conclusions

The bioactive factors examined in this study have differing clinical effects on musculoskeletal tissue. The differences in the cellular and cytokine composition differ between PRP and BMC and between BMC systems should be taken into consideration by the clinician when choosing a biologic for therapeutic application.

Level II evidence: Clinical Level II

Introduction:

Biologic solutions to address the pathologic process of osteoarthritis (OA) have been investigated as potential treatments for focal cartilage lesions, osteochondral lesions, and generalized OA [1]. Mesenchymal stem cells (MSCs) appear promising for the treatment of OA, but are subject to regulatory restrictions, and are not currently approved by the FDA as well as many other countries' regulatory bodies for clinical use [6, 36]. Bone marrow concentrate (BMC) has been used to deliver MSCs to damaged cartilage [23], to augment microfracture [17, 26-28, 48], and as a direct joint injection method for cartilage repair and early OA [33]. BMC is generated by centrifugation of bone marrow aspirate (BMA) allowing for immediate administration to a patient, and many regulatory bodies have approved various companies' products for clinical use. While BMC contains fewer MSCs than what can be obtained through culture expansion [11, 53], the number of MSC needed for effective therapy has yet to be determined. In addition to MSCs, BMC contains numerous bioactive molecules and cell types including lymphocytes, neutrophils, monocytes, and platelets in various stages of differentiation [21, 23, 34]. In orthopedics, cytological analysis of BMC is rarely performed. In one study, a 4 fold increase in platelets, total nucleated cells, and CD34+ cells in BMC compared to BMA was reported [34]. The concentration of growth factors in BMC has not been quantified, despite their role in the regenerative potential of BMC [40, 47].

Platelet-rich plasma (PRP) is gaining popularity as a biologic treatment for focal cartilage defects and OA [13, 20]. PRP is generated by centrifugation of peripheral blood resulting in increased platelet concentration. The platelets provide multiple growth factors with known roles in target cell activation, cell recruitment, cartilage matrix production, and modulation of the inflammatory response [22]. Currently the only established difference between PRP and BMC is

the presence of MSCs in BMC, with no other information available on the component differences to guide clinicians. In current orthopedic practice, it is commonly regarded that BMC is essentially PRP with additional stem cells, however this has not been fully elucidated. Therefore, one aim of this study was to determine how BMC differs from PRP. There is known variability in PRP due to differences between patients and manufacturers, and the same premise is assumed for BMC, resulting in the second aim of this study: to compare BMCs generated from two different commercial systems.

Materials and Methods:

This study was prospective and supervised by the biologics committee as a quality assessment project. Between 11/2013 and 07/ 2014, 29 consecutive patients, within an age range of 18-70 years, consented for participation. All patients were under the care of a single surgeon (JGK). Patients were excluded if there was a history of blood disorders, hematological malignancy, use of immunosuppressive drugs, or drugs with bone marrow suppressive effects.

Blood and Bone Marrow Aspirate Collection

Blood (25 mL) was drawn into a syringe containing 4mL acid citrate dextrose (ACD). One mL was removed for the study and the remainder was processed to generate 3mL PRP (Magellan®, Arteriocyte Medical Systems Inc., Hopkinton, MA). One mL of PRP was removed for study purposes. Bone marrow was aspirated from the iliac crest into a 30mL syringe containing 4mL ACD. The needle was advanced 1cm and rotated 90 degrees after each 5mL aspirate until a total of 120mL was aspirated in 4 syringes. The total aspirate was mixed, 1mL was retained as the BMA sample for the study, and the remainder was separated into two 60mL

samples and processed in two systems; Magellan® (BMC-A) (Arteriocyte Medical Systems Inc., Hopkinton, MA), and SmartPrep® 2 (BMC-B) (Harvest Technologies Corp., Plymouth, MA). All aspirations were performed by the same surgeon (JGK). One mL of each BMC-A and BMC-B was retained for the study. Representatives from both BMC companies observed aspiration and concentration techniques. Samples were shipped de-identified to prevent bias in sample processing and analysis. All samples were processed within 24 hours of collection.

Cytology

Automated counts were performed on whole blood (WB), PRP, BMA, BMC-A, and BMC-B samples to assess platelets, red blood cells (RBC), and nucleated cell counts (NCC), which included myeloid precursors as well as white blood cells (WBC; neutrophils, monocytes, lymphocytes, eosinophils, basophils). Cytological smears were evaluated of all samples to verify automated counts.

Colony Forming Units and Flow Cytometry

Equal volumes of BMCs were used for colony forming unit (CFU) assays, rather than a known number of nucleated cells. This reflects clinical practice where volume, as opposed to cell count, would be the unit of BMC application. Samples of BMC were cultured in duplicate with DMEM containing 10% FBS, penicillin/streptomycin, and 1ng/mL basic fibroblastic growth factor [50]. Media were changed every 48 hours. After two weeks, CFUs were circled with a 1.8 cm diameter self-inking marker. A colony was counted if it was ≥1 mm in diameter. This adapted technique [24, 33] allowed for retention of viable cells and further analyses.

Cells were then passaged once and then lifted with Accumax (Sigma-Aldrich, St. Louis, MO) when confluent at passage 1 for flow cytometry of MSCs markers [16], including positive (CD146, CD73, CD271) and negative markers (CD34, CD45). Conjugated primary monoclonal antibodies and isotype controls were used as recommended by the manufacturer (BD Biosciences, San Jose, CA). Cells were analyzed on a FACS Canto II (BD Immunocytometry Systems, San Jose, CA) flow cytometer and using FlowJo software (TreeStar Inc., Ashland, OR).

Growth Factor and Cytokine Analyses

Multiplex assays were performed according to manufacturer directions to measure vascular endothelial growth factor (VEGF), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), interleukin-1 receptor antagonist protein (IL-1ra), and interferon-γ (IFN-γ) (Fluorokine MAP Multiplex Human Cytokine Panel A, R&D Systems, Minneapolis, MN). Transforming growth factor-beta 1 (TGF-β1), TGF-β2, and TGF-β3 were measured using the Fluorokine MAP Mulitplex TGF-beta 3-plex Kit (R&D Systems, Minneapolis, MN). Platelet derived growth factor-isoform BB (PDGF-BB), and acidic fibroblast growth factor (FGF-1) were quantified using the Fluorokine MAP Multiplex Human Angiogenesis Panel A (R&D Systems, Minneapolis, MN). Multiplex assays were performed using the Luminex 200 instrument (Luminex Corp., Austin, TX).

Institutional Review Board Approval

This study was approved by the Hospital for Special Surgery's Institutional Review Board (ID #29055).

Statistical Analyses

BMA was obtained from 29 patients. Blood was also obtained from the last 14 of the 29 patients. In BMC-B from the first 6 patients, no MSCs were observed in the cultures. The company was contacted, and they replaced the machine, which resulted in growth of MSCs in all subsequent samples. The authors regarded this as mechanical failure and elected to exclude these patients from the study. Four BMA and BMC samples were excluded because they were not processed within 24 hours of collection due to a shipping delay as the result of inclement weather. Further inclusion/exclusion criteria for statistical analysis were set to confirm that BMC and PRP were generated. For BMC, this was defined as an increase in NCC or CFU compared to BMA, resulting in exclusion of 1 sample. For PRP, inclusion was defined as an increase in platelet or PDGF concentration compared to that measured in whole blood, resulting in exclusion of 2 patients [13]. To account for the individual variability inherent in biologics, paired statistical methods were used. Data were tested for normality using a Shapiro-Wilk test. A two sided paired t-test was used for normally distributed differences between groups, otherwise, a Wilcoxon Signed Rank test was used, with an alpha of 0.05. There were no available studies using a multiplex of BMA/BMC data to perform a power analysis.

Results:

The mean age of patients was 48.2 years with a range of 23-68. Ten were female and the remainder (9) male. There were no reported complications associated with collection of bone marrow or blood.

Cytology and platelet concentration in PRP compared to BMC

BMC-A was used for comparison to PRP because both were generated from the same system, thus allowing for a comparison based on biologic differences rather than preparation methods. The concentration of total WBCs and all subtypes of WBCs were greater in BMC-A compared to PRP (Table 2.1). There was an 11.8 fold increase in WBC concentration in BMC-A compared to PRP. Neutrophils were increased in BMC-A by 19 fold, monocytes by 11 fold, and lymphocytes by 7 fold compared to PRP. The platelet concentration in PRP was increased by 2.5 fold compared to WB, however, there was no difference in platelet concentration between BMC-A and PRP.

Cytology and platelet concentration in BMA compared to BMC

The NCC in both BMC-A and BMC-B was increased compared to BMA verifying that both systems concentrated nucleated cells (Table 2.2). The NCC in BMC-A was increased by 3.3 fold, and by 4.1 fold in BMC-B compared to BMA. There was no difference in NCC between BMC-A and BMC-B. The total WBC concentration was increased in both BMC-A and BMC-B compared to BMA, but there was no difference between BMC-A and BMC-B. All subtypes of WBCs were increased in BMC-A and BMC-B compared to BMA. The average neutrophil concentration in BMC-B was 1.6 fold greater than BMC-A. In contrast, the average concentration of lymphocytes was 1.3 fold greater in BMC-A compared to BMC-B. However, there was no significant difference between lymphocyte concentration in BMC-A and BMC-B. There were no statistically significant differences in monocyte, eosinophil, or basophil concentrations between BMC-A and BMC-B. Platelet concentration was increased by 4.8 fold in

	WB, PRP, BMC-A Cytology			
	Mean ± S.D. (range)			
	WB	**PRP**	**BMC-A**	**p-value**
WBC thou/ul	4.2 ± 1.6 * (1.9-6.4)	3.1 ± 1.2 *† (0.7-5.1)	36.7 ± 16.9 † (16.8-77.2)	* 0.031 † 0.0001
Neutrophils thou/ul	2.6 ± 1.3 * (0.7-4.5)	0.9 ± 0.4 *† (0.1-1.8)	17.5 ± 9.8 † (5.4-42.7)	* 0.0003 † 0.0004
Monocytes thou/ul	0.2 ± 0.09 * (0.1-0.4)	0.3 ± 0.2 *† (0.1-0.7)	3.3 ± 2.3 † (0.8-10.5)	* 0.41(n.s) † 0.0010
Lymphocytes thou/ul	1.2 ± 0.4 * (0.6-2.2)	1.7 ± 0.8 *† (0.4-2.8)	12.6 ± 5.4 † (5.3-27.3)	* 0.07(n.s) † 0.0001
Eosinophils thou/ul	0.07 ± 0.05 * (0-0.1)	0.0 ± 0.0 *† (0.0-0.0)	0.6 ± 0.4 † (0.1-1.3)	* 0.0039 † 0.0015
Basophils thou/ul	0.01 ± 0.04 * (0.0-0.1)	0.04 ± 0.05 *† (0.0-0.1)	0.2 ± 0.2 † (0.04-0.7)	* 0.50(n.s) † 0.0015
Platelets thou/uL	80.3 ± 55.5 * (14-178)	202.3 ± 86.1 *† (73-368)	151.6 ± 111.2 † (25-362)	* 0.0010 † 0.92(n.s)

Table 2.1. Comparison of cellular composition and differential cell count in WB (whole blood), PRP (platelet rich plasma), and BMC-A (bone marrow concentrate from commercial system A). White blood cells (WBC) and all cell differential means are expressed with standard deviation to nearest whole unit, thousands of cells per microliter (thou/uL), with the range within the parentheses. A shared symbol (* or †) between two groups corresponds to the shared p-value based on a one sided paired T test.

	BMA, BMC-A, BMC-B Cytology			
	Mean ± S.D. (range)			
	BMA	**BMC-A**	**BMC-B**	**p-value**
NCC thou/ul	11.6 ± 5.8 *† (4.9-23.2)	38.3 ± 20.6 *‡ (10.5-94)	47.4 ± 20.1 †‡ (15.2-81.4)	* <0.0001 † <0.0001 ‡ 0.14(n.s)
WBC thou/ul	10.7 ± 5.3 *† (4.7-21.4)	36.7 ± 16.9 *‡ (16.8-77.2)	44.3 ± 18.6 †‡ (14.4-77.6)	* <0.0001 † <0.0001 ‡ 0.19(n.s)
Neutrophils thou/ul	7.1 ± 4.1 *† (2.8-15.5)	17.5 ± 9.8 *‡ (5.4-42.7)	27.4 ± 12.8 †‡ (9.0-53.5)	* <0.0001 † <0.0001 ‡ 0.0023
Monocytes thou/ul	0.6 ± 0.4 *† (0.2-1.6)	3.3 ± 2.3 *‡ (0.8-10.5)	2.9 ± 1.2 †‡ (0.3-5.0)	* <0.0001 † <0.0001 ‡ 0.33(n.s)
Lymphocytes thou/ul	2.0 ± 0.6 *† (1.0-3.5)	12.6 ± 5.4 *‡ (5.3-27.3)	9.7 ± 3.6 †‡ (1.6-20.8)	* <0.0001 † <0.0001 ‡ 0.054(n.s)
Eosinophils thou/ul	0.2 ± 0.2 *† (0.04-0.8)	0.6 ± 0.4 *‡ (0.1-1.3)	0.8 ± 0.5 †‡ (0.2-1.9)	* <0.0001 † <0.0001 ‡ 0.15(n.s)
Basophils thou/ul	0.04 ± 0.03 *† (0-0.1)	0.2 ± 0.2 *‡ (0.04-0.7)	0.2 ± 0.2 †‡ (0.0-0.9)	* <0.0001 † 0.013 ‡ 0.51(n.s)
Platelets thou/uL	31.5 ± 25.4 *† (3-94)	151.6 ± 111.2 *‡ (25-362)	111.1 ± 97.4 †‡ (11-300)	* <0.0001 † <0.0001 ‡ 0.031

Table 2.2. Comparison of cellular composition and differential cell count in BMA (bone marrow aspirate), BMC-A (bone marrow concentrate from commercial system A), and BMC-B (bone marrow concentrate from commercial system B). Nucleated cell counts (NCC), white blood cells (WBC) and all cell differential means are expressed with standard deviation to nearest whole unit, thousands of cells per microliter (thou/uL), with the range within the parentheses. A shared symbol (*, or †, or ‡) between two groups corresponds to the shared p-value based on a one sided paired T test.

BMC-A and 3.5 fold in BMC-B compared to BMA, resulting in a significantly greater platelet concentration in BMC-A compared to BMC-B.

Colony Forming Units

The number of CFUs was not different between duplicates ($p=0.44$), so duplicates were averaged for analyses. The mean number of CFUs was 7.8 ± S.D. 12.3 (range 0-46) in BMA, 41.4 ± 27.4 (range 9-90) in BMC-A, and 32.7 ± 30.3 (range 2-90) in BMC-B. CFUs were significantly increased in both BMCs compared to BMA ($p<0.0001$), and BMC-A and BMC-B were not significantly different ($p=0.079$).

Flow Cytometry of MSCs from BMC

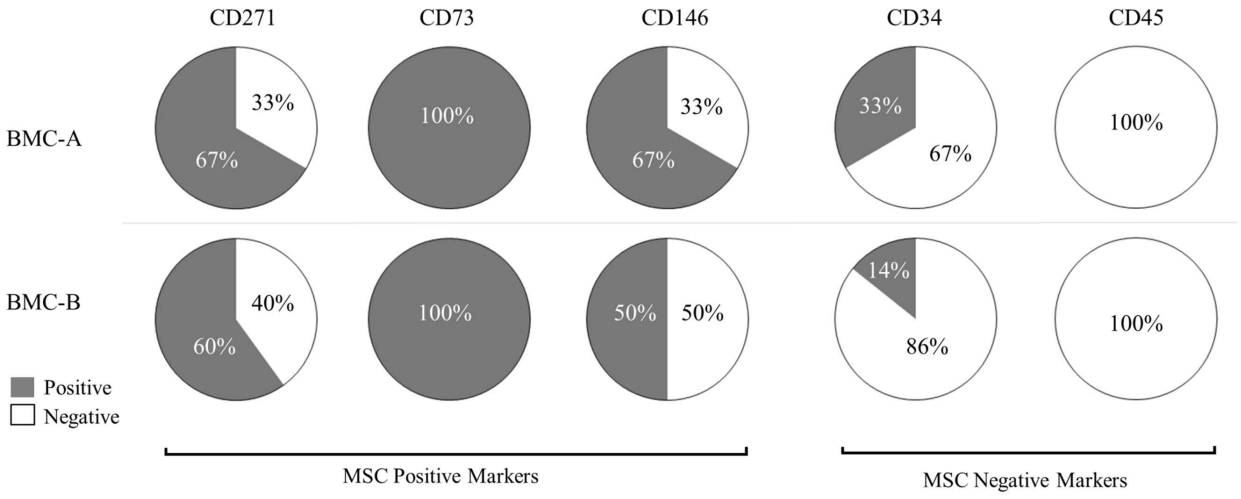
The minimum number of $2.5x10^5$ cells needed for flow cytometry of each molecule limited the ability to measure all five MSC markers in every sample, so CD45 and CD271 were prioritized based on the literature [14, 45]. There were insufficient CFUs in BMA samples for comparison to BMC. All MSCs from BMC-A and BMC-B were negative for the lymphocyte marker CD45 (Figure 2.1). The majority of BMC-A and BMC-B-derived MSCs were positive for CD271, and only a small percentage were positive for the hematopoietic stem cell marker CD34. All MSCs were positive for CD73 and variably positive for CD146.

Growth Factors and Cytokines

PDGF-BB

The concentration of PDGF was not significantly different between PRP and BMC-A (Table 2.3). As expected, PDGF was increased (1.7 fold) in PRP compared to WB. PDGF was

Figure 2.1: Cell surface marker expression based on flow cytometry of cell samples cultured from BMC-A (bone marrow concentrate from commercial system A) and BMC-B (bone marrow concentrate from commercial system B). CD271, CD73, and CD146 are markers expressed on mesenchymal stem cells (MSCs). MSCs are negative for CD34, a hematopoetic stem cell marker and CD45, a leukocyte marker.



	WB, PRP, BMC-A Cytokines			
	Mean ± S.D. (range)			
	WB	**PRP**	**BMC-A**	**p-value**
PDGF pg/mL	3530 ± 2078 * (842-7698)	5975 ± 2644 ***†** (2277-10680)	7549 ± 3601 † (2433-15056)	* 0.0075 † 0.51(n.s)
TGF-β 2 pg/mL	<17.5 *	65± 70 ***†** (0-218)	219 ± 219 † (0-779)	* N/A † 0.049
VEGF pg/mL	31 ± 40 * (0-152)	10 ± 19 ***†** (0-66)	276 ± 273 † (5-941)	* 0.0093 † 0.0010
IL-8 pg/mL	< 3.50 *	< 3.50 ***†**	273 ± 300 † (0-1176)	N/A
IL-1β pg/mL	< 1.50 *	< 1.50***†**	7 ± 13 † (0-48)	N/A

Table 2.3. Comparison of growth factor and cytokine concentration in WB (whole blood), PRP (platelet rich plasma), and BMC-A (bone marrow concentrate from commercial system A). Means are expressed with standard deviation to nearest whole unit, picograms per milliliter (pg/ml), with the range within the parentheses. PDGF=platelet-derived growth factor; TGF-β = Transforming growth factor beta; VEGF= vascular endothelial growth factor; IL-1β= interleukin-1 beta; IL-8= interleukin-8; N/A= not able to be calculated or not applicable. A shared symbol (* or †) between two groups corresponds to the shared p-value based on a one sided paired T test.

also increased in both BMC-A and BMC-B compared to BMA, with concentrations greater in BMC-A compared to BMC-B (Table 2.4).

TGF-β1, 2, 3

There was no significant difference in TGF-β1 concentration between BMC-A and PRP (Figure 2.2). Not surprisingly, TGF-β1 was increased in PRP compared to WB and in both BMC-A and BMC-B compared to BMA. BMC-A had significantly more TGF-β1 compared to BMC-B. TGF-β2 was below limit of detection (17.5 pg/mL) in all BMA and WB samples. TGF-β2 was present in 15 out of 18 BMC-A samples, 4 out of 19 BMC-B samples, and 9 out of 12 PRP samples. The concentration of TGF-β2 was greater in BMC-A compared to PRP (Table 2.3) and BMC-B (Table 2.4). TGF-β3 concentrations were below the limit of detection (62.2 pg/mL) in all samples.

VEGF

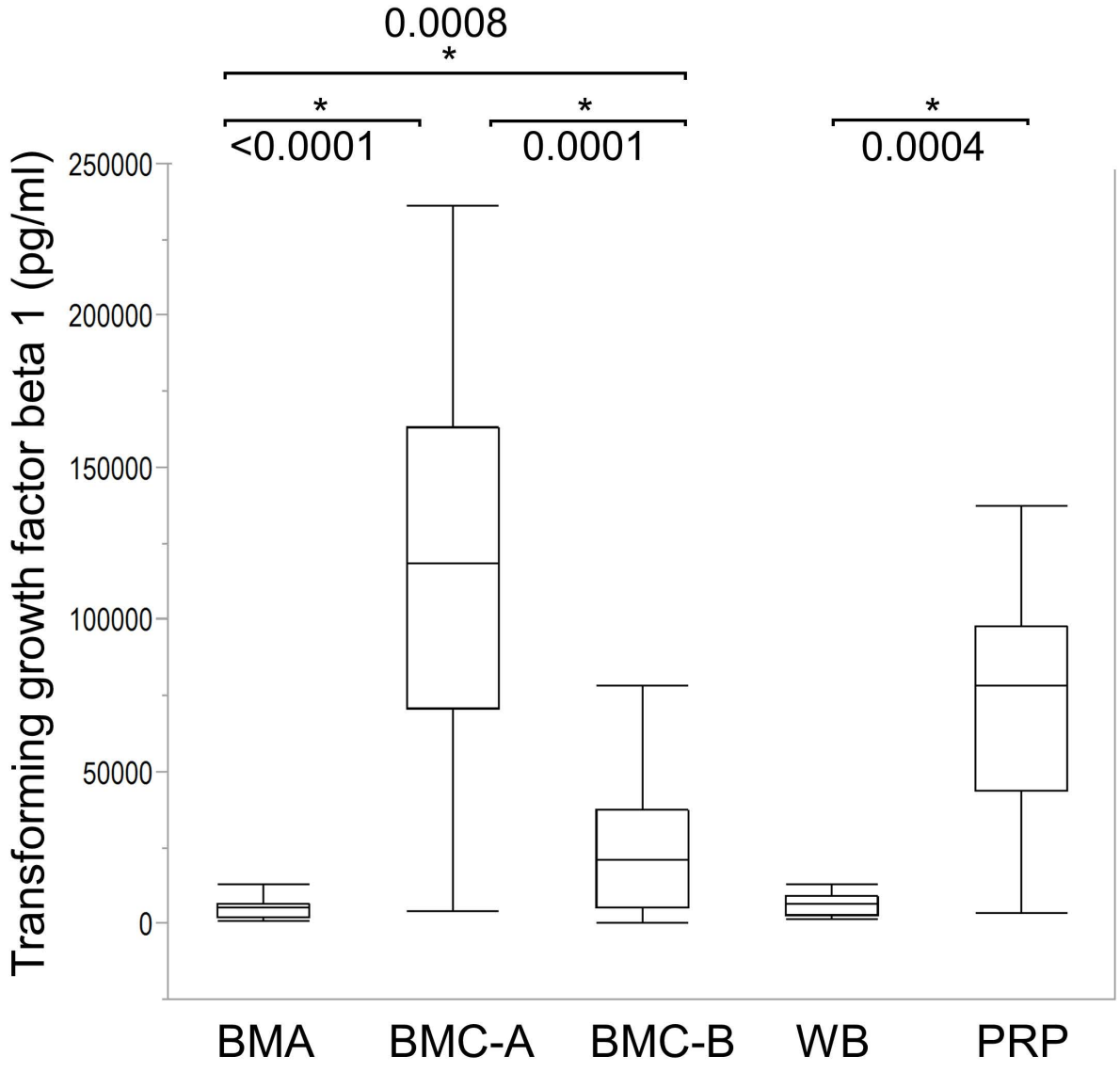
VEGF concentrations were lowest in PRP, WB, and BMA samples (Tables 2.3 and 2.4). In BMC-A, VEGF was increased compared to PRP (Table 2.3). Compared to BMA, VEGF concentration was increased 4 fold in BMC-A and 7 fold in BMC-B (Table 2.4).

IL-8

IL-8 concentrations were below the limit of detection (3.5 pg/mL) in WB and PRP samples (Table 2.3). IL-8 was increased 3 fold in BMC-A and 5 fold in BMC-B samples compared to BMA (Table 2.4). There was no difference in IL-8 concentration between the two BMC groups.

Figure 2.2: Transforming growth factor beta 1 in BMA (bone marrow aspirate), BMC-A (bone marrow concentrate from commercial system A), BMC-B (bone marrow concentrate from commercial system B), WB (whole blood), and PRP (platelet rich plasma).

* indicates a significant difference ($p<0.05$) between two groups based on a one sided paired T test. Data are presented in an outlier plot with whiskers extending to the farthest point within 1.5 interquantile range lengths.



	BMA, BMC-A, BMC-B Cytokines			
	Mean ± S.D. (range)			
	BMA	**BMC-A**	**BMC-B**	**p-value**
PDGF pg/mL	1820 ± 976 *† (380-3819)	7549 ± 3601 *‡ (2433-15056)	5036 ± 3645 †‡ (618-16030)	* <0.0001 † 0.0006 ‡ 0.0013
TGF-β 2 pg/mL	< 17.5 *†	219 ± 219 *‡ (0-779)	33 ± 83 †‡ (0-284)	* N/A † N/A ‡ 0.0023
VEGF pg/mL	68 ± 53 *† (0.8-172)	276 ± 273 *‡ (5-941)	502 ± 508 †‡ (0-1958)	* 0.0001 † <0.0001 ‡ 0.041
IL-8 pg/mL	82 ± 110 *† (0-448)	273 ± 300 *‡ (0-1176)	404 ± 387 †‡ (0-1328)	* 0.0003 † <0.0001 ‡ 0.24(n.s)
IL-1β pg/mL	< 1.50 *†	7 ± 13 *‡ (0-48)	31 ± 40 †‡ (0-110)	* N/A † N/A ‡ 0.011

Table 2.4. Comparison of growth factor and cytokine concentration in BMA (bone marrow aspirate), BMC-A (bone marrow concentrate from commercial system A), and BMC-B (bone marrow concentrate from commercial system B). Means are expressed with standard deviation to nearest whole unit, picograms per milliliter (pg/ml), with the range within the parentheses. PDGF=platelet-derived growth factor; TGF-β = Transforming growth factor beta; VEGF= vascular endothelial growth factor; IL-1β= interleukin-1 beta; IL-8= interleukin-8; N/A= not able to be calculated or not applicable. A shared symbol (*, or †, or ‡) between two groups corresponds to the shared p-value based on a one sided paired T test.

IL-1β

There was no measureable IL-1β in WB, PRP, or BMA (Tables 2.3 and 2.4). Additionally, IL-1β was undetectable in nine BMC-A and six BMC-B samples. In samples that were above the lower limit of detection (1.5 pg/mL), IL-1β was greater in BMC-B compared to BMC-A (Table 2.4).

IL-1ra

All samples contained measurable IL-1ra. IL-1ra was significantly increased in BMC-A compared to PRP (Figure 2.3). In WB, IL-1ra was greater than in PRP. Compared to BMA, IL-1ra was significantly increased in both BMC-A (3 fold) and BMC-B (5 fold), and BMC-B was significantly greater than BMC-A. The ratios of IL-1ra to IL-1β were calculated for all samples with detectable concentrations of IL-1β (Figure 2.4). The average ratio of IL-1ra:IL-1β for the 6 patients with BMC-A and BMC- B samples with detectable IL-1β was not significantly different ($p=0.09$).

TNF-α, IL-6, IFN-γ, FGF-1

All four of these cytokines were undetectable in all samples. Minimum detectable concentrations based on the standard curves were: TNF-α (5.1 pg/mL), IL-6 (5.2 pg/mL), IFN-γ (2.9 pg/mL), and FGF-1 (5.7 pg/mL).

Figure 2.3: Interleukin 1 receptor antagonist in BMA (bone marrow aspirate), BMC-A (bone marrow concentrate from commercial system A), BMC-B (bone marrow concentrate from commercial system B), WB (whole blood, and PRP (platelet rich plasma).

* indicates a significant difference ($p<0.05$) between two groups based on a one sided paired T test. Data are presented in an outlier plot with whiskers extending to the farthest point within 1.5 interquantile range lengths.

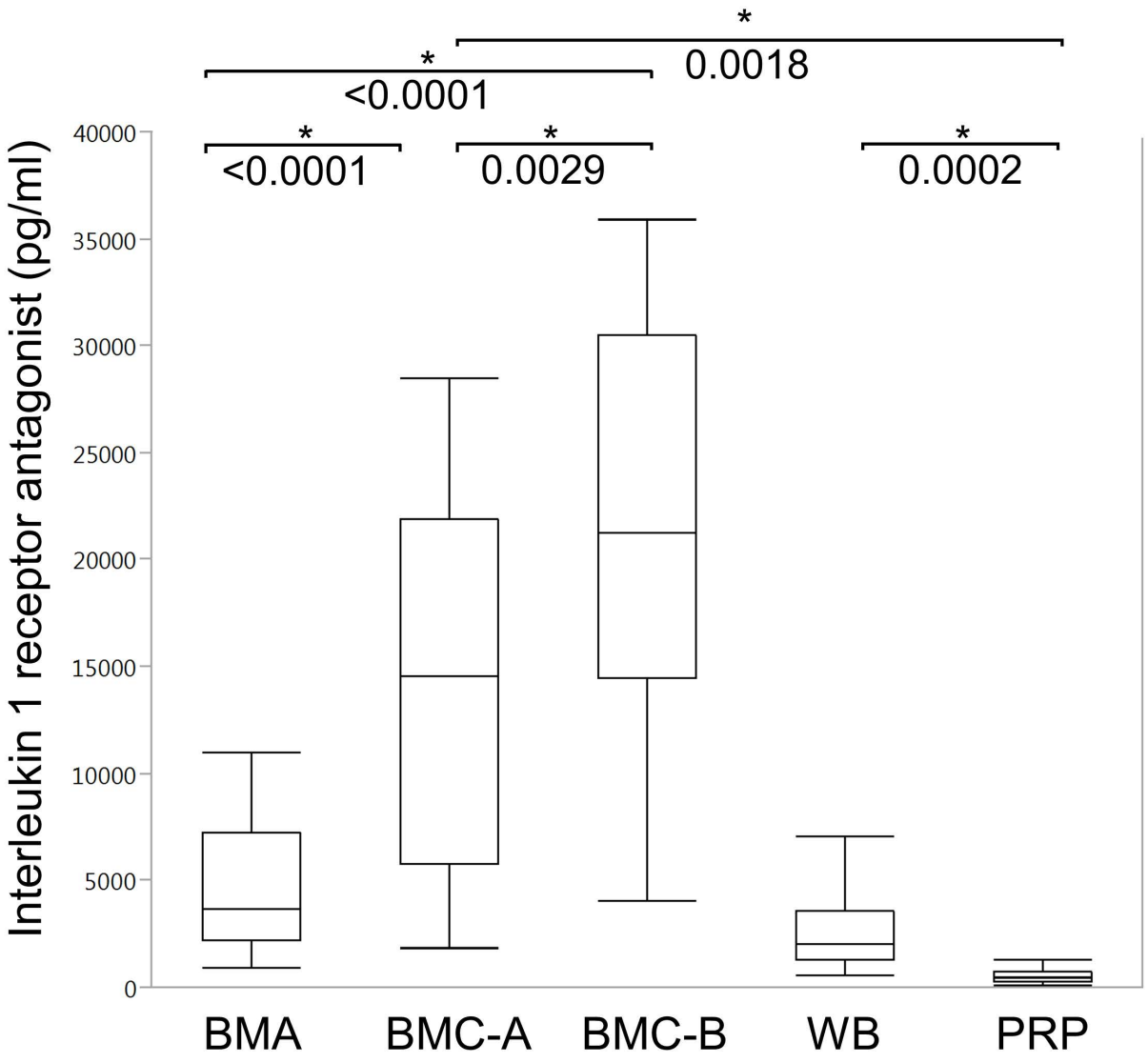
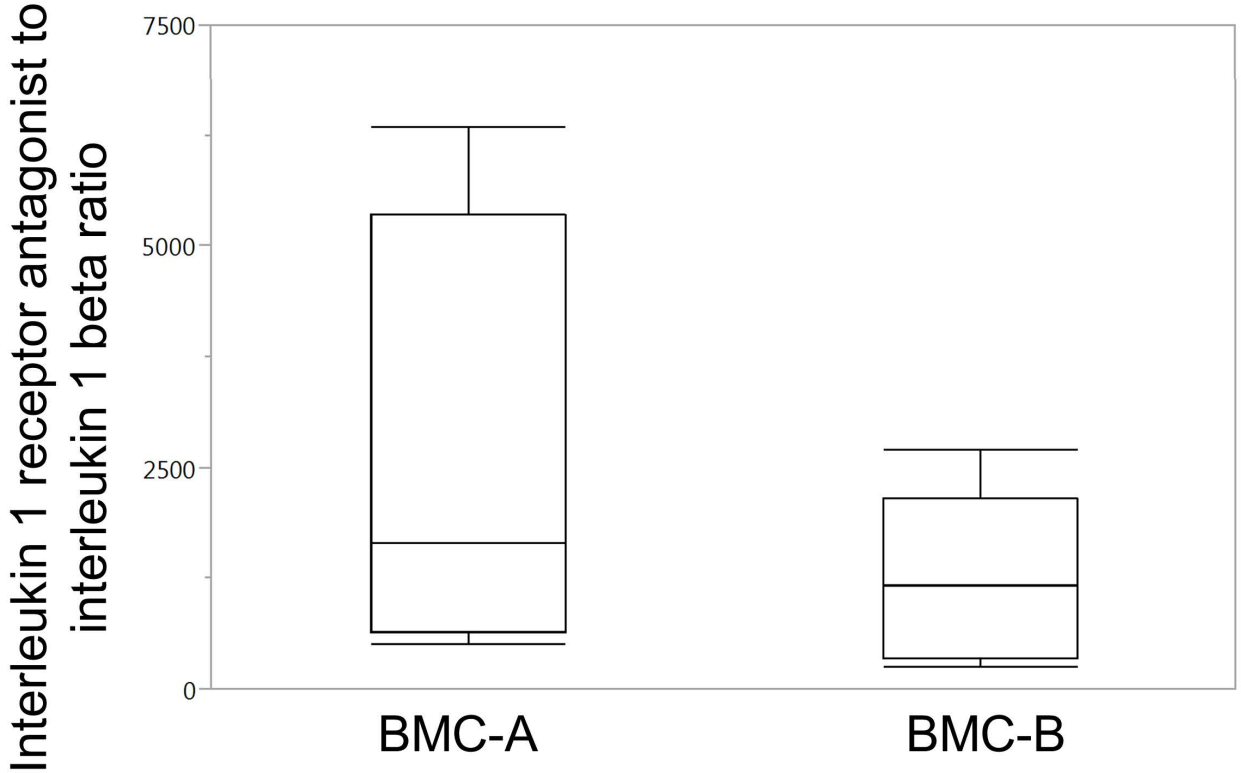


Figure 2.4: Ratio of interleukin 1 receptor antagonist to interleukin 1 beta in BMC-A (bone marrow concentrate from commercial system A) and BMC-B (bone marrow concentrate from commercial system B). Ratios represented are from the n= 9/18 BMC-A samples and n= 13/19 BMC-B samples that had detectable levels of IL-1b. The samples with IL-1b levels below the limit of detection (1.5 pg/mL) would have an even greater positive biological effect on competing with endogenous IL-1b in clinical use. * indicates a significant difference ($p<0.05$) between two groups based on a one sided paired T test. Data are presented in an outlier plot with whiskers extending to the farthest point within 1.5 interquantile range lengths.



Discussion:

The most important finding of this study is the discovery that bone marrow derived biologics contain clinically relevant concentrations of IL-1ra. BMC provides growth factors found in PRP in addition to stem cells. This study shows several significant differences in the cellular and molecular composition of PRP and BMC (Table 2.5), and differences in cytology and bioactive molecules between BMC manufacturing systems. Both BMC manufacturing systems effectively concentrated BMA as demonstrated by an increased total NCC in most BMC samples. BMC-B initially failed to generate BMC due to a malfunction of the centrifuge. Further, there was one failure to generate BMC in BMC-A and two failures to generate PRP from blood with no known machine malfunction. This might be expected based on similar results when generating PRP [10], but it has not been previously documented. Failure to generate BMC or PRP would not be visually recognized by the surgical team which underscores the importance of counting cells and platelets in biologics when reporting outcome data.

Although there was an increase in NCC in the BMC-B group compared to BMC-A, BMC-A and BMC-B did not have significantly different CFUs. This suggests that NCC alone may not be predictive of MSC concentration. In a previous study, higher NCCs and CFUs were found in BMC-B compared to BMC-A [33]. These conflicting results are likely explained by different study methodologies. In the current study, a uniform volume of BMC was used for culture rather than a defined number of nucleated cells [33]. This method was chosen to reflect clinical practice where a doctor would administer a specified volume of BMC rather than a defined number of nucleated cells. All MSCs had flow cytometry markers consistent with MSCs. The heterogeneity in surface markers of MSCs observed in this study has been previously reported and is not surprising given the lack of cell selection processes and the short culture

	BMA	BMC-A	BMC-B	WB	PRP
IL-1ra	++	+++	++++	++	+
IL-1ra :IL-1β	-	++	+	-	-
MSC	+	+++	++	-	-
TGF-β1, PDGF	+	+++	++	+	+++
Il-1β, IL-8	+	++	++	-	-

Table 2.5: Summary of the defining characteristics of BMA (bone marrow aspirate), BMC-A (bone marrow concentrate from commercial system A), BMC-B (bone marrow concentrate from commercial system B), whole blood (WB), and platelet rich plasma (PRP). The + symbols depict the presence of and relative concentration of the anabolic cytokine interleukin 1 receptor antagonist (IL-1ra), mesenchymal stem cells (MSCs), transforming growth factor beta-1 (TGF-β1) platelet derived growth factor (PDGF), and interleukin 1 beta (IL-1β). A – symbol indicates the cytokine was below the limits of detection or MSCs were not assessed.

duration (analysis at passage 1) as some of the characteristic markers are not uniformly expressed until later passages [3, 38].

Several growth factors have positive effects on cartilage repair and in the treatment of OA. The role of PDGF in cartilage repair and homeostasis has been extrapolated from its function during chondrogenesis [2]. PDGF induces MSC proliferation [22, 25] and inhibits IL-1β induced chondrocyte apoptosis and inflammation [42]. PDGF was present in all samples in varying concentrations and corresponding to platelet concentrations. All three TGF-β isoforms examined have roles in chondrogenesis [7, 35, 39, 43, 44, 51, 56]. TGF- β1 has been shown to stimulate chondrogenesis of synovium and bone marrow-derived MSCs [18, 37], inhibit IL-1β -mediated inflammation [9], and enhance cartilage healing [12]. TGF-β1 was increased in PRP and BMC, paralleling increased platelet concentrations. PRP and BMC-A did not differ in TGF-β1 concentration, however increased TGF-β1 was present in BMC-A compared to BMC-B. These findings are likely related to platelet, monocyte, and neutrophil concentrations [31]. TGF-β2 was present in most BMC-A and PRP samples, but only a few BMC-B samples. In OA chondrocytes, TGF-β2 decreases collagen type 2 cleavage and chondrocyte hypertrophy through inhibition of IL-1β and TNF-α [49]. Many cell types secrete TGF-β2, thus differences in cellular distribution may be the source of the divergent TGF-β2 concentrations.

In balance with growth factors, PRP and BMC products contain pro-inflammatory cytokines. IL-1β and IL-8 are secreted by monocytes, neutrophils, and MSCs [19, 29, 52]. In WB and PRP, IL-1β and IL-8 were undetectable, but they were present in BMA and increased in both BMC groups. This is likely a result of concentration of neutrophils, monocytes, and MSCs during centrifugation [4]. IL-8 is a potent chemoattractant for neutrophils, which secrete IL-1, and in turn, can further stimulate monocytes to produce IL-8 [4, 8, 32]. This might suggest that

PRP would be a less inflammatory, more anabolic biologic. However, the surprising and significant concentration of IL-1ra found in BMC and its absence in PRP needs to be taken into consideration when choosing between PRP and BMC for any therapeutic modality.

Interleukin-1 receptor antagonist (IL-1ra) is a naturally occurring antagonist that competitively binds to IL-1β and IL-1α cell surface receptors thereby inhibiting IL-1 catabolism. IL-1ra is thought to be responsible for the beneficial effects of the biologic autologous conditioned serum (ACS) [54, 41]. Two randomized control trials in knee OA reported superiority of ACS over hyaluronan or saline injection [5, 55]. ACS is not approved for use in the United States, thus its implementation has been confined primarily to Europe. The original reported concentration of IL-1ra in ACS was 10,254 pg/mL [41], and subsequent studies documented concentrations of 2,015 pg/mL [54] and <2,000 pg/mL [46]. In the present study, the average concentration of IL-1ra in BMA was 4,510 pg/mL, which was then increased 3 fold in BMC-A and 5 fold in BMC-B. For effective therapy, an IL-1ra:IL-1 ratio of 10:1 to 100:1[15, 30] has been reported as sufficient to block IL-1. The IL-1ra:IL-1β ratio in BMC samples ranged from 249:1 to 17,568:1, indicating a net inhibitory effect on IL-1. The use of BMC products provides a patient-side method for generation of IL-1ra that would be allowed in regions such as the USA where ACS is restricted by regulatory agencies. While this study did not seek to correlate clinical outcomes with biologic components, due to the heterogeneity of surgical procedures involved, the variability of cellular and molecular components described can serve as a guide to design appropriate sample sized studies to investigate this. Although PRP, BMC, and ACS are purported to alter the healing response through the actions of specific growth factors, stem cells, and IL-1ra respectively, it is likely that the overall net effect of the various components will determine the clinical impact. Different tissues likely have different

requirements for biologic agents and the acute versus chronic disease progression will require distinct approaches. The ideal formulation for each tissue and disease state can only be determined through comparative clinical studies.

Conclusion:

Differences in the cellular and molecular composition of PRP and BMC should be considered in light of the pathology being treated. BMC provides a patient-side method to generate the potent anti-inflammatory therapy, IL-1ra, and deliver stem cells in addition to growth factors commonly found in PRP to patients where they might not be otherwise available due to regulatory restrictions.

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Ethical standards:

All procedures performed in studies involving human participants were approved by the institutional research review board and in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments or comparable ethical standards.
Informed consent was obtained from all individual participants included in the study.

Conflict of interest:

Author (JGK) is a consultant for Arteriocyte Medical Systems Inc. Author (LAF) is a consultant for Arthrex Inc. All samples were de-identified prior to shipment to prevent any potential biases from being introduced into the study analyses.

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CHAPTER 3

PRIMING THROUGH AN INFLAMMATORY MICROENVIRONMENT AND TLR LIGANDS ENHANCE MSC IMMUNOMODULATORY FUNCTION.

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Abstract:

Mesenchymal stem cells (MSCs) have the therapeutic potential to treat a variety of inflammatory and degenerative disease processes, however tissue environmental effects on MSCs have been overlooked. Our hypothesis was that TLR4 stimulation or exposure to inflammatory macrophages would impair the immunomodulatory function of MSCs, whereas TLR3 stimulation would promote their immunosuppressive properties.

MSCs were exposed to polyinosinic:polycytidylic acid (poly I:C) to stimulate TLR3 receptors or lipopolysaccharide (LPS) to stimulate TLR4 receptors. MSC phenotype was assessed by inflammatory gene expression and MSC immunomodulatory function was assessed by mitogen-stimulated T cell proliferation assays. Peripheral blood monocytes were isolated using CD14 MACs positive selection, differentiated into macrophages, and polarized using interferon-gamma (IFN-γ). Polarization was confirmed by increased gene expression of TNFα, CCL2, and CXCL10. Inflammatory macrophages were co-cultured with MSCs for 6 hours, and the resultant MSC phenotype was analyzed as described above.

TLR priming and co-culture of MSCs with inflammatory macrophages resulted in increased expression of IL-6, CCL2, and CXCL10 in MSCs. TLR priming or exposure of MSCs to inflammatory macrophages significantly ($p<0.05$) enhanced their immunomodulatory function, demonstrated by a decrease in T cell proliferation in the presence of poly I:C primed MSCs (11%), LPS primed MSCs (7%), or MSCs exposed to inflammatory macrophages (12%), compared to unstimulated MSCs. The recipient injured tissue bed in chronic injuries may not contain sufficient inflammatory signals to activate MSC immunomodulatory function. Enhancement of MSCs immunomodulatory function through inflammatory priming prior to clinical application might improve the therapeutic effect of MSC treatments.

Keywords

Mesenchymal stem cells, MSC, immunomodulation, TLR, polarization, regenerative medicine

Introduction

Mesenchymal stem cells (MSCs) have the therapeutic potential to treat a wide variety of inflammatory and degenerative disease processes in humans and animals [1–5]. However, little is known about how the recipient tissue environment affects the phenotype and function of transplanted MSCs. Contemporary evidence suggests that the regenerative effects of MSCs are through their ability to modulate the environment rather than acting as a cell source for new tissue formation. The secretome of MSCs contains numerous paracrine factors thought to produce a therapeutic effect through modulation of the tissue repair process and immunomodulation of the local tissue environment [6,7]. The clinical response to MSC therapy has been inconsistent, which may be attributed to the heterogeneous nature of an MSC population[8–10], or the complex interactions between MSCs with the transplantation recipient environment [11,12].

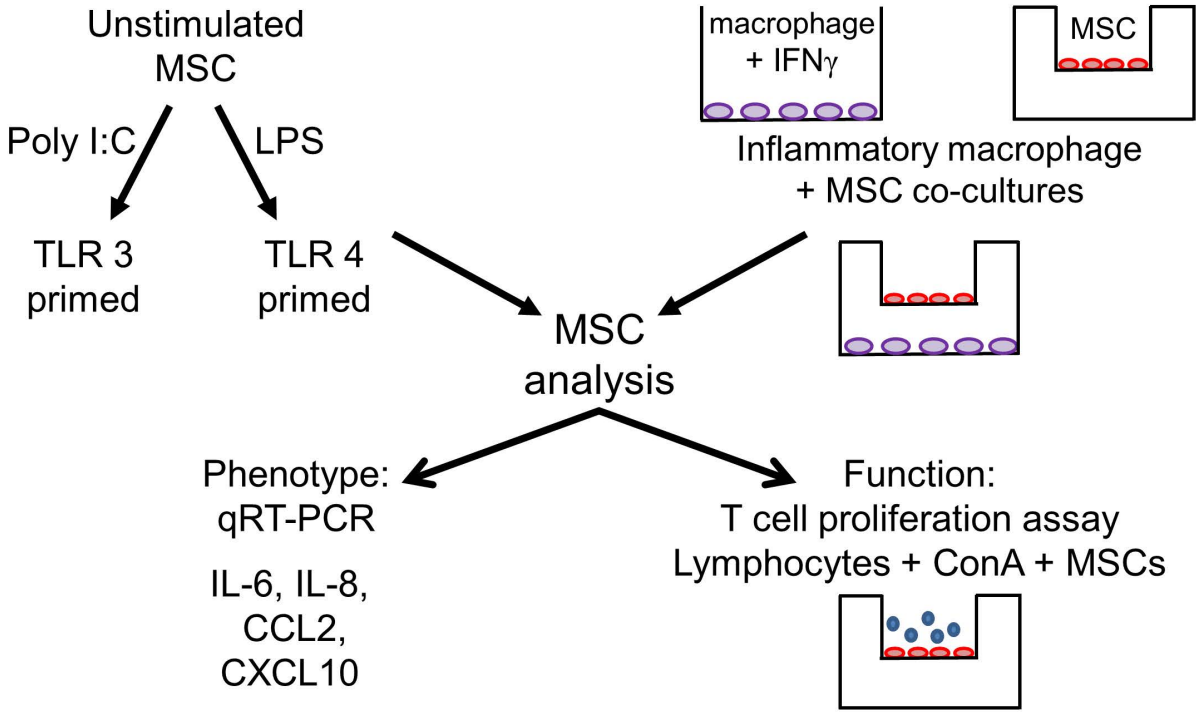
Several studies have investigated how MSCs affect the recipient environment [13–17], but less is known about the reciprocal relationship of how the tissue environment affects MSC function. Some studies suggest that resting MSCs are initially immunomodulatory, then depending on the stimuli to which they are exposed, they are polarized similar to macrophages [18,19]. The MSC1, pro-inflammatory phenotype, is induced through lipopolysaccharide (LPS) stimulation of toll-like receptor 4 (TLR4). The MSC2, immunomodulatory phenotype, is induced through polyinosinic:polycytidylic acid (poly I:C, synthetic double stranded RNA analog) stimulation of TLR3. Toll-like receptors are conserved

proteins that recognize pathogen associated molecular patterns (PAMPs) in order to turn on general responses to classes of pathogens. Other studies suggest that MSCs exist in a resting state, and stimulation with pro-inflammatory cytokines, such as interferon gamma (IFN-γ) or tumor necrosis factor alpha (TNF-α), are essential to induce the immunomodulatory capacity of MSCs [20,21]. In order to improve MSCs clinical benefit in the recipient environment, it is essential to further understand how inflammatory stimuli impact MSC function.

Macrophages are present in every tissue bed targeted for MSC transplantation, and they play an essential role in injury and repair [22,23]. Paracrine signals from the environment regulate macrophage polarization and the ratio of M1:M2 cells, and macrophages secrete numerous cytokines that could alter the function of transplanted MSCs [24]. It has not been previously investigated how macrophages can impact MSCs, and these important inflammatory cell mediators would directly interact with MSCs in the recipient tissue bed.

To harness the full capacity of MSCs for regenerative approaches, an understanding of how the recipient environment affects MSC function is warranted. To better understand the effect of inflammatory stimuli on MSC function, MSCs were exposed to TLR ligands and inflammatory macrophages and resultant changes in MSC immunomodulatory function were assessed (Figure 3.1). Our hypothesis was that TLR4 stimulation or exposure to inflammatory macrophages would impair the immunomodulatory function of MSCs, whereas TLR3 stimulation with poly I:C would promote their immunosuppressive properties.

Figure 3.1: Study design overview. Macrophages were isolated, matured, and polarized to a pro-inflammatory phenotype on the bottom of transwell co-culture wells. MSCs were cultured on the inserts and then exposed to macrophage secretome via the transwells. After co-culture, MSCs were removed, placed in a new well, and lymphocytes were added to allow direct MSC-lymphocyte contact. MSCs were exposed to polyinosinic:polycytidylic acid (poly I:C) to stimulate TLR3 receptors or lipopolysaccharide (LPS) to stimulate TLR4 receptors. Gene expression of inflammatory cytokines (IL-6, IL-8) and chemokines (CCL2, CXCL10) was analyzed to characterize MSC phenotype. T cell proliferation assays with the mitogen concavalinA (ConA) characterized functional changes in MSC immunomodulatory capabilities.



Results

Macrophage polarization

Macrophage isolation and polarization produced consistent gene expression changes. Polarization of macrophages with exposure to IFN-γ resulted in a significant increase in TNF-α, CCL2, and CXCL10 expression (Figure 3.2; p=0.00070, p=0.012, p=0.0069, respectively) compared to unstimulated macrophages. There was a 0.45 fold decrease in IL-10 expression (p=0.00010), and no significant change in expression of IL-6 (p=0.29).

MSC priming

Gene expression

Both TLR3 and TLR4 priming of MSCs resulted in increased expression of cytokines and chemokines compared to unstimulated MSCs. Both TLR3 and TLR4 priming resulted in increased expression of CCL2 and IL-6 (Figure 3.3). There was no difference in expression in CCL2 or IL-6 as a result of the type of TLR priming. TLR3 and TLR4 priming resulted in increased expression of IL-8, with expression in TLR4 primed MSCs significantly greater than that in TLR3 primed MSCs. TLR priming increased CXCL10 expression, and expression in TLR3 stimulated MSCs was greater than in TLR4 stimulated MSCs. MHC class II status did not appear to affect gene expression response with the only noted trend of MHC class II positive MSCs having the highest expression of CXCL10 of all unstimulated MSCs.

T cell proliferation

TLR3 and TLR4 stimulation of MSCs resulted in a similar enhanced ability of MSCs to suppress mitogen stimulated T cell proliferation compared to unstimulated MSCs (Figure 3.4). The lymphocytes were derived from different horses than the MSCs, meaning that the MSCs

Figure 3.2: Gene expression changes in macrophages following exposure to IFN-γ are consistent with a pro-inflammatory macrophage profile. Macrophages were exposed to 100 ng/ml IFN-γ for 6 hours and resultant changes in gene expression were measured and compared to unstimulated macrophages using the $2^{-\Delta\Delta CT}$ method. In IFN-γ polarized macrophages, TNF-α, CCL2, and CXCL10 expression was upregulated compared to unstimulated macrophages. IL-10 expression was decreased in IFN-γ stimulated macrophages compared to unstimulated macrophages. IL-6 expression was unchanged following IFN-γ exposure. Box and whisker plots represent n=8. * indicates a p value < 0.05: one-sided paired t-test.

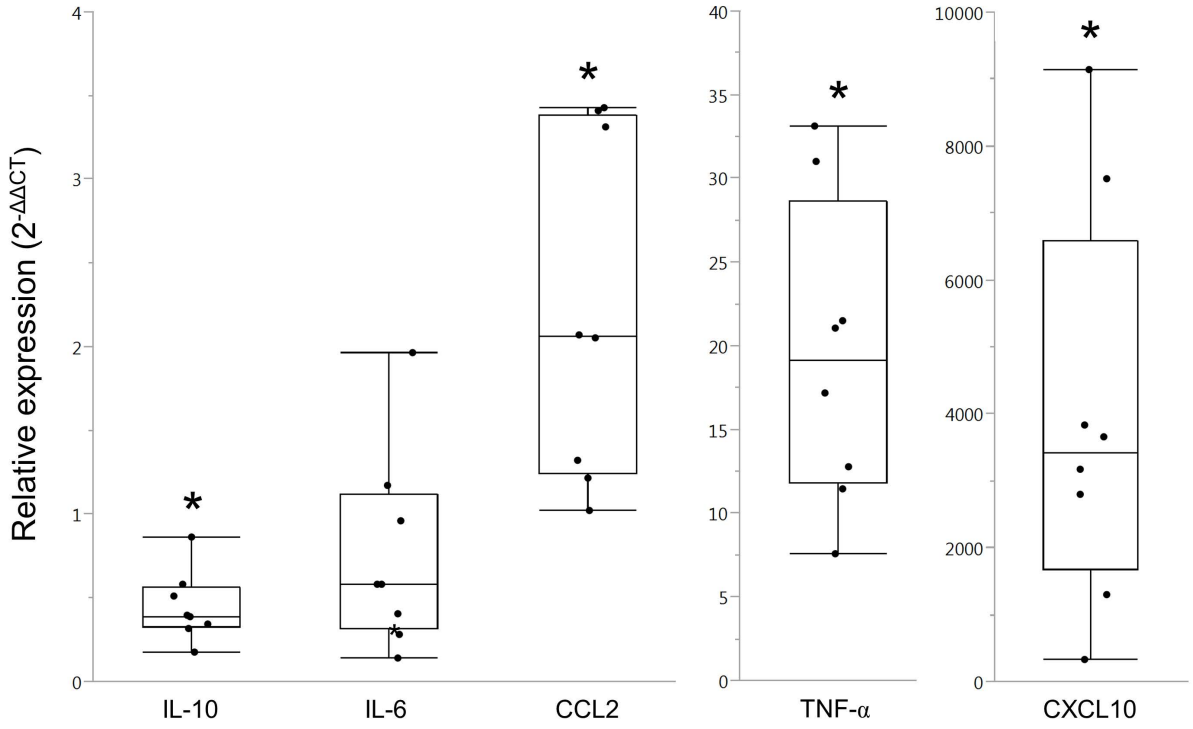


Figure 3.3: Gene expression changes in MSCs following TLR priming**.** MSCs were exposed for 1 hour to 10 μg/ml polyinosinic:polycytidylic acid (poly I:C) to stimulate TLR3 receptors or 100 ng/ml lipopolysaccharide (LPS) to stimulate TLR4 receptors. Resultant changes in gene expression was measured and compared to untreated, control MSCs using the $2^{-\Delta\Delta CT}$ method. IL-6, IL-8, CCL2, and CXCL10 expression was significantly increased in all TLR primed MSCs compared to the untreated MSCs. Box and whisker plots represent n=8. TLR primed MSC groups that do not share the same letter are significantly different in expression of that gene. $P < 0.05$; repeated measures analysis of variance (ANOVA), with the horse as a random effect in the model, followed by a Tukey HSD test for multiple comparisons.

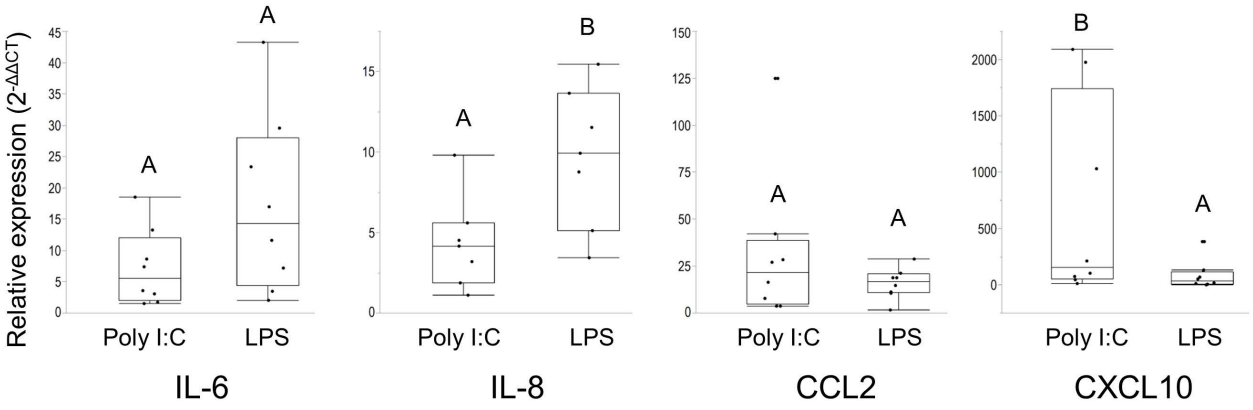
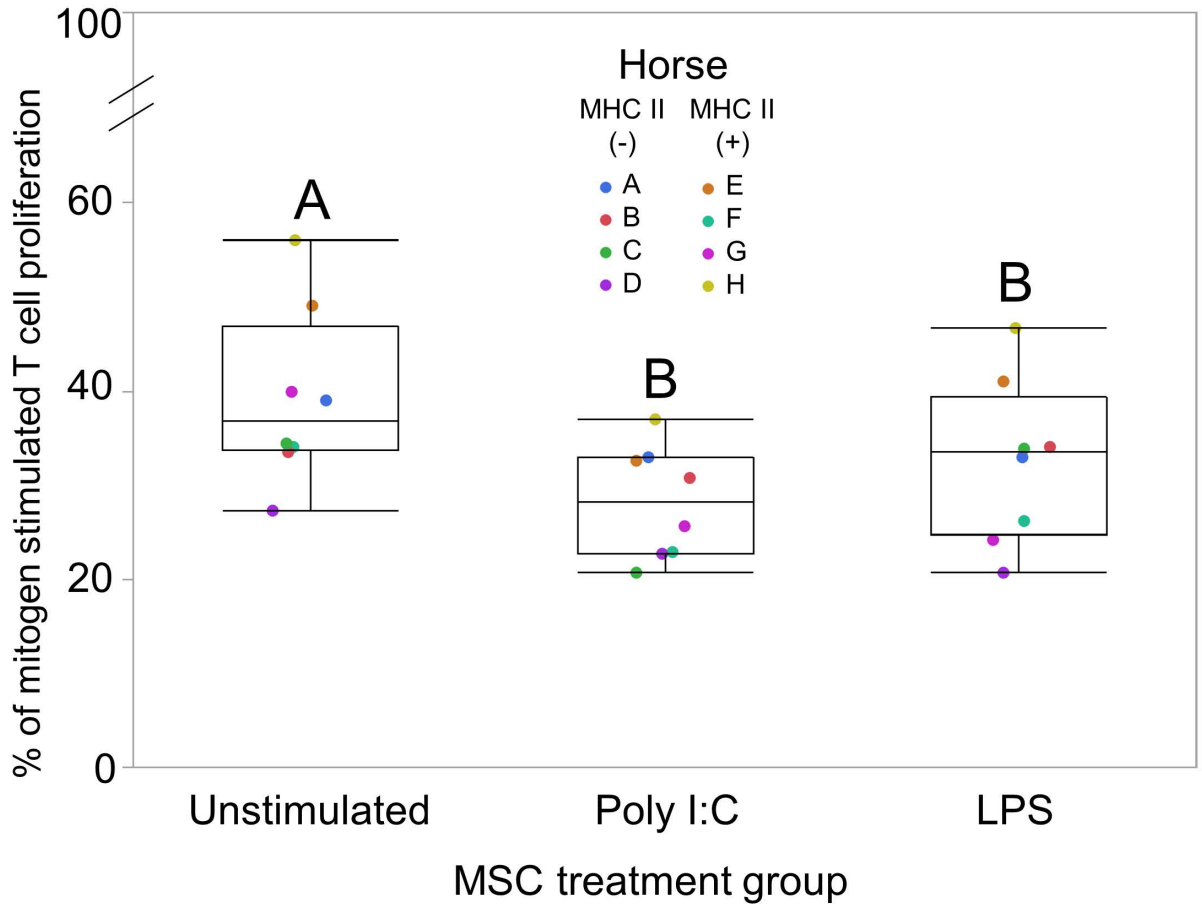


Figure 3.4: Poly I:C primed MSCs and LPS primed MSCs have an increased ability to suppress T cell proliferation compared to unstimulated MSCs**.** MSCs ability to control mitogen stimulated T cell proliferation was compared to mitogen stimulated lymphocytes alone (positive control) from that experiment, which was set at 100% T cell proliferation. T cell proliferation in the presence of TLR3 stimulated (poly I:C) MSCs and TLR4 stimulated (LPS) MSCs was significantly decreased compared to unstimulated MSCs. MSCs from horses A, B, C, and D were uniformly MHC-II negative (MHC-II (-)), whereas MSCs from horses E, F, G, and H were variably MHC-II positive (MHC-II (+)). Box and whisker plots represent n=8. Groups that do not share the same letter are significantly different. $P<0.05$: repeated measures analysis of variance (ANOVA), with the horse as a random effect in the model, followed by a Tukey HSD test for multiple comparisons.



were acting on allogeneic lymphocytes. Unstimulated MSCs reduced mitogen stimulated T cell proliferation to 27 to 56% of mitogen stimulated lymphocytes alone. TLR3 stimulation resulted in an average 11% reduction in lymphocyte proliferation, and TLR4 stimulation lead to a 7% reduction compared to unstimulated MSCs. MHC class II positive MSCs appeared to have a greater increase in their immunomodulatory capabilities in response to TLR stimulation. MHC class II positive MSCs stimulated with TLR3 led to an average 15% reduction in lymphocyte proliferation, while MHC class II negative MSCs resulted in only a 7% decrease compared to unstimulated MSCs. Similarly, TLR4 stimulation of MHC class II positive MSCs produced a 10% reduction in lymphocyte proliferation, while stimulation of MHC class II negative MSCs resulted in only a 3% decrease in lymphocyte proliferation compared to unstimulated MSCs.

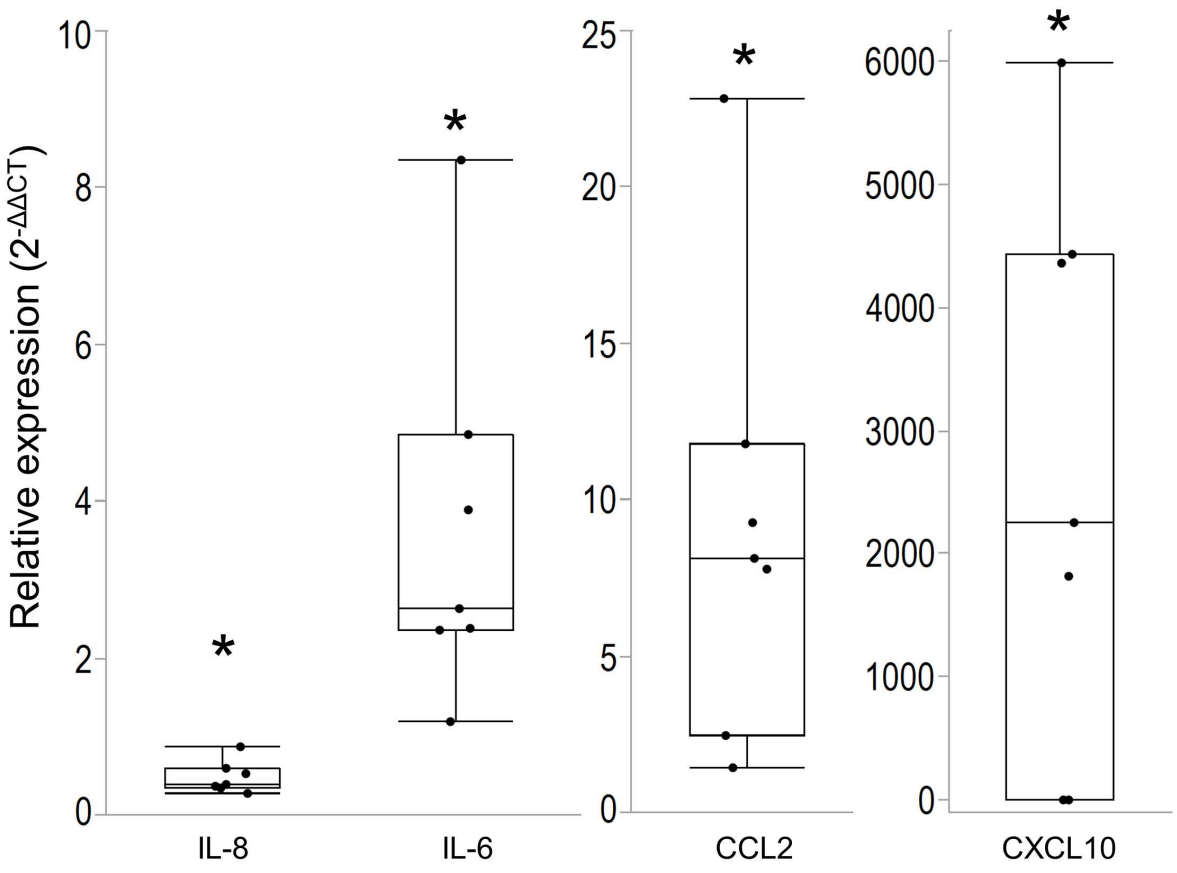
MSC and inflammatory macrophage co-culture

This study sought to utilize a pro-inflammatory macrophage population for modeling the recipient environment that might be present after trauma or surgery. Exposure of MSCs to a cell capable of producing a variety of cytokines, as well as responding to signals from MSCs, is a closer approximation to the in vivo scenario than exposing MSCs to a single cytokine.

Gene expression

Co-culture of MSCs with inflammatory macrophages resulted in upregulation of cytokines and chemokines in the MSCs (Figure 3.5). Exposure of MSCs to IFN-γ polarized/inflammatory macrophages resulted in a 3.7 fold increase in IL-6 expression in MSCs (Figure 3.5; $p=0.025$). Expression of CCL2 was increased by an average of 9.1 fold (Figure 3.5, $p=0.023$), and CXCL10 was increased by 2693 fold (Figure 3.5, $p=0.0216$). In contrast, IL-8 expression was decreased by an average of 0.5 fold (Figure 3.5, $p=0.0005$). MHC class II status

Figure 3.5: Gene expression changes in MSCs following exposure to pro-inflammatory macrophages. $2^{-\Delta\Delta CT}$ values describe the fold change in expression in MSCs exposed to inflammatory macrophages compared to the unexposed control MSCs. IL-6, CCL2, and CXCL10 expression was significantly upregulated in inflammatory macrophage exposed MSCs compared to control MSCs. IL-8 expression was downregulated following MSC exposure to inflammatory macrophages. Box and whisker plots represent n=7. * indicates a p value < 0.05: one sided paired t test.



did not markedly affect gene expression responses; with the only noted trend in MHC class II positive MSCs having the highest expression of CXCL10 in control MSCs.

T cell proliferation

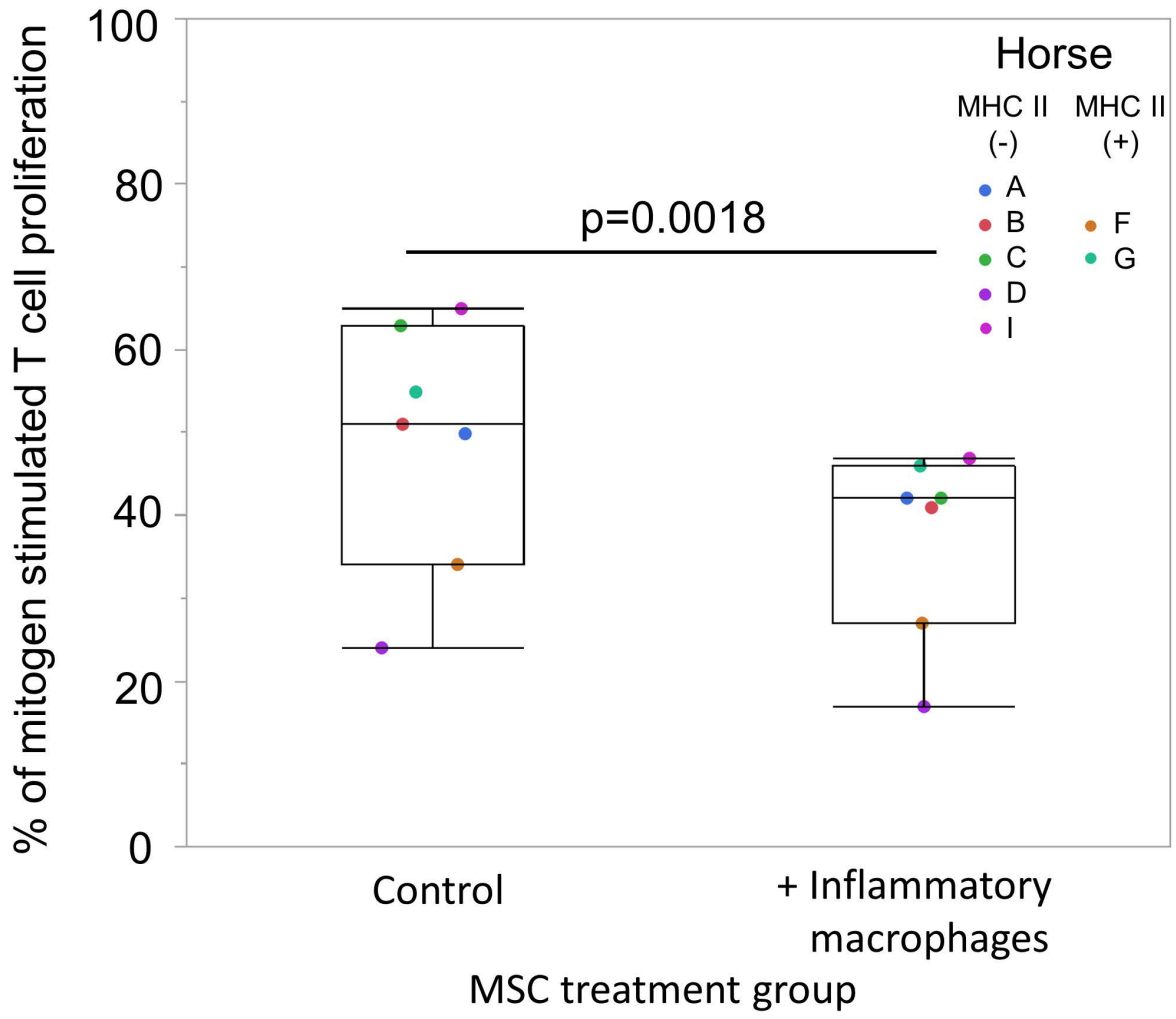
The ability of MSCs to suppress mitogen stimulated T cell proliferation was significantly increased in MSCs that had been previously cultured with inflammatory macrophages (Figure 3.6). MSCs exposed to IFN-γ polarized macrophages decreased lymphocyte proliferation by an additional 12% compared to control MSCs that were not exposed to macrophages. There were no appreciable trends in lymphocyte proliferation due to MHC class II status of MSCs.

Discussion

This study sought to determine how inflammatory stimuli such as TLR ligands and inflammatory macrophages affected the phenotype and function of MSCs. Our hypothesis was that TLR4 stimulation and exposure to inflammatory macrophages would impair the immunomodulatory function of MSCs, whereas TLR3 stimulation with poly I:C would promote their immunosuppressive effects.

TLR4 stimulation of MSCs further enhanced the ability of MSCs to suppress lymphocyte proliferation beyond that observed with unstimulated MSCs. This finding was in contrast to our hypothesis, which proposed that TLR4 stimulation would impair the immunomodulatory function of MSCs. While on average, LPS stimulated MSCs further suppressed lymphocyte proliferation, MSCs from a few individual animals led to no change or a slight increase in lymphocyte proliferation compared to lymphocytes exposed to untreated MSCs. This inconsistency in response could be due to a variety of factors, including the natural heterogeneity of any given MSC population or the heterogeneity in the response of MSCs to LPS stimulation.

Figure 3.6: MSCs have an increased ability to suppress T cell proliferation following exposure to pro-inflammatory macrophages compared to control MSCs. The ability of MSCs to suppress mitogen stimulated T cell proliferation was compared to mitogen stimulated lymphocytes alone (positive control) from that experiment, which was set at 100% T cell proliferation. Box and whisker plots represent n=7. * indicates a p value < 0.05: one-sided paired t-test.



One of the components of LPS signaling is CD14, which is expressed at variable levels on the cell surface of equine MSCs [25]. This could also result in differing responses of MSCs to LPS stimulation.

In Waterman's studies of MSC priming in mouse and human cells, LPS primed MSCs lost their ability to suppress T cell proliferation [19]. In the present study, LPS primed MSCs maintained their immunosuppressive capabilities and had an enhanced ability to suppress T cell proliferation compared to untreated MSCs. This could be due to methodological differences with Waterman using a complete PBMC population, whereas phagocytes (neutrophils, monocytes) were removed with carbonyl iron in the present study. With the presence of phagocytes in the proliferation assay, more non-specific lymphocyte proliferation occurs due to an increased production of inflammatory cytokines. This increase in background proliferation could have decreased the sensitivity in measuring the effects of MSCs on T cell proliferation. Another difference between studies was that that proliferation of all T cells was measured in the present study, whereas in the Waterman paper only cytotoxic T cells were analyzed. Because of these differences, the findings of the present study may not be in opposition to the previous priming work. However, a loss of immunomodulatory ability might not reflect a switch to a pro-inflammatory phenotype as suggested [19]. LPS primed MSCs did not induce proliferation of unstimulated lymphocytes in the previous work [19], or in the present study, which further questions a pro-inflammatory phenotype.

Similar to LPS, poly I:C priming significantly increased the immunomodulatory ability of MSCs compared to unstimulated MSCs, which supported our hypothesis. This is consistent with the ideas put forth by Waterman [19] and Bunnell [18], where exposing MSCs to this TLR3 ligand produced a homogenous immunomodulatory population. The superior immunomodulatory

ability of poly I:C stimulated MSCs compared to unstimulated MSCs found could be due to an increase in sensitivity in the T cell proliferation assay as described above, or to the higher concentration of poly I:C used. In vivo studies support the superior ability of poly I:C primed MSCs to decrease inflammation compared to unstimulated MSCs [26], highlighting the potential of TLR3 priming of MSCs for clinical applications. While the TLR3 primed and TLR4 primed MSCs were not significantly different from each other in their ability to suppress T cell proliferation, TLR3 priming conferred a more homogenous ability to suppress T cells. Poly I:C primed MSCs lymphocyte suppression had a smaller standard deviation (±3.5) compared to LPS primed MSCs (±8.5) and the unstimulated MSCs (±7.2). It appears that poly I:C activates MSCs immunosuppressive potential in a way that is less dependent on individual variability compared to LPS stimulation. For these reasons, poly I:C might be a superior activator of MSC immunosuppressive capabilities compared to LPS.

In contrast to our predictions, which were based on the polarization paradigm [18], exposure of MSCs to inflammatory macrophages enhanced the ability of MSCs to suppress T cell proliferation (Figure 3.6). Our findings are more consistent with the inflammatory licensing theory which suggests that exposure of MSCs to inflammatory cytokines activates their immunomodulatory capabilities [21,27–30]. Polchert found that IFN-γ treatment of MSCs prior to application, improved their ability to treat graft versus host disease (GVHD) [29]. While IFN-γ has repeatedly been shown to be necessary in induction of MSC immunomodulatory capabilities, it is not sufficient for activation and requires the presence of TNF-α, IL-1α, or IL-1β [27]. A recent study found that exposure of MSCs to IFN-γ and TNF-α abolished the innate immunomodulatory heterogeneity of MSC clones in vitro and in vivo [21]. Based on gene expression results, our inflammatory macrophages are producing TNF-α. IFN-γ could also be present in the

inflammatory macrophage co-culture milieu, either through production by macrophages [31] or a few contaminating T cells. Since the required duration and order of stimulation of these cytokines is currently unknown, the MSCs in our experiments could have been stimulated sequentially by TNF-α in the macrophage co-culture, then IFN-γ during the T cell mitogen assay or stimulated simultaneously by both cytokines in the macrophage co-culture.

TLR3 and TLR4 stimulation of MSCs resulted in consistent upregulation in inflammatory cytokine (IL-6, IL-8) and chemokine (CCL2, CXCL10) gene expression. Increased gene expression of IL-8 after LPS, compared to poly I:C stimulation, is consistent with both the MSC priming paradigm [19] and in vivo inflammatory responses where TLR4 and IL-8 are upregulated together .[32] With the exception of MSCs with high MHC class II expression, CXCL10 expression was very low in unstimulated MSCs. CXCL10 was markedly upregulated in response to TLR stimulation, producing very large (range 1-2693) fold changes in expression. Since TLR3 stimulation mimics viral infection, the increased expression of CXCL10 (IP-10) in response to poly I:C priming is consistent with documented in vivo production of this chemokine in response to viral infections in both the horse and human [33,34]. Poly I:C primed human MSCs were also found to secrete increased concentrations of CXCL10 compared to LPS primed MSCs; knockdown studies revealed that TLR3 stimulation was specifically related to CXCL10 secretion [19]. These cytokines and chemokines may be important in MSC-mediated chemotaxis of immune cells, where it has been suggested that MSCs recruit specific subsets of immune cells in order for the MSCs to exert immunomodulatory effects on the cells in their proximity [28]. Based on the environment signals MSCs receive, they may differentially recruit and regulate the immune system in vivo.

Exposure of MSCs to either poly I:C, LPS, or inflammatory macrophages produced similar increases in modulation of T cell proliferation. This indicates that poly I:C or LPS stimulation alone is similar to the effect of an inflammatory microenvironment on MSCs. It is difficult to predict the inflammatory cytokines and cells present in the recipient tissue bed of an individual. For example, in an osteoarthritic joint, synovial fluid cytokine concentrations vary significantly depending on the duration and etiology of the disease [36–38]. Because the inflammatory status of the recipient environment cannot be accurately predicted, it might be beneficial to activate MSCs prior to clinical use through priming with stimuli, such as poly I:C, in order to produce a predictable response to therapy.

Methods

Horses

Fifteen adult horses were used for collection of blood and bone marrow aspirate with approval from the Institutional Animal Care and Use Committee of Cornell University.

Cell Isolation and Culture

*MSC*s

Bone marrow aspirate was collected aseptically from the sternum of 11 horses[10]. Aspirates were cultured using the direct plating method [39] in MSC media: low glucose (1 g/dl) DMEM containing 10% fetal bovine serum (FBS, Atlanta biologicals, Flowery Branch, GA, endotoxin concentration <0.1 EU/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and basic fibroblastic growth factor (bFGF, 1 ng/ml). MSCs were expanded one passage and cryopreserved [10] for subsequent experiments. MHC class I and class II expression was analyzed

on flow cytometry[10], and all MSCs used in these studies were MHC class I positive. MSCs from 4 out of 9 horses' were variably MHC class II positive (50-99% of total MSCs).

Lymphocytes

Lymphocytes were obtained and stained with 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE, Sigma-Aldrich) [10]. They were then suspended in lymphocyte proliferation media: RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% FBS, 0.1 mM 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 μg/ml). Unstimulated and concavalinA (ConA, 5 μg/ml; Sigma-Aldrich) stimulated lymphocytes were used in subsequent immunomodulation (T-cell proliferation) studies.

Macrophages

Monocytes were isolated using adapted protocols[10,40]. Briefly, plasma was allowed to separate from red blood cells. Peripheral blood mononuclear cells (PBMCs) were then isolated from the plasma using Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ, USA) gradient centrifugation. Monocytes were isolated from the PBMC pool by magnetic activated cell sorting utilizing the MidiMAC system (Miltenyi Biotec, Inc., Auburn, CA) with a mouse anti-equine CD14 antibody (Courtesy of Dr. Bettina Wagner, Cornell University, clone 105) and rat anti-mouse IgG MACS microbeads (Miltenyi Biotec), according to manufacturer directions for LS columns for positive selection. After MACS selection, CD14 positive cells were counted, resuspended in macrophage media (DMEM containing 10% normal horse serum (NHS, HyClone Laboratories, Logan, UT), L-glutamine (2mM), penicillin (100 units/ml), and streptomycin (100 μg/ml)), and plated at $1.8x10^6$ cells per well in 6 well plates. Macrophage media was added every 48 hours during culture. Monocytes were allowed to spontaneously differentiate into

macrophages over 6-7 days of culture before being used in experiments. This isolation technique achieved 99% CD14 positive cells on flow cytometry after 6 days in culture.

Macrophages were washed with warm phosphate buffered saline (PBS) and then stimulated with IFN-γ (100 ng/mL; recombinant equine interferon gamma, R&D Systems, Minneapolis, MN) for 6 hours. Macrophages were washed with PBS, followed by macrophage media, and then used to stimulate MSCs, or were used to confirm macrophage inflammatory polarization by gene expression.

Experimental Design

MSC Priming

MSCs were primed with LPS or poly I:C as previously described, with modifications in ligand concentration [19]. Based on a series of optimization experiments, 100 ng/ml LPS for 1 hour, or 10 μg/ml poly I:C for 1 hour were selected for producing robust changes in gene expression and MSC modulation of mitogen stimulated T cell proliferation without affecting MSC viability.

For analysis of gene expression changes following MSC priming, MSCs were plated at $2x10^5$ cells/well in 6 well plates 24 hours before priming. After priming, MSCs were washed two times with MSC media, cultured an additional 4 hours, lifted with trypsin, and snap frozen for subsequent RNA isolation. To assess the ability of primed MSCs to modulate mitogen stimulated T cell proliferation, MSCs were thawed and plated 24 hours prior to priming in 24 well plates [10]. MSCs were exposed to priming reagents for 1 hour, were washed two times with MSC media and then lymphocytes were then added as described below for T cell proliferation assays.

Co-Culture

To assess the phenotypic changes in MSCs following exposure to inflammatory macrophages, MSCs were co-cultured with macrophages in transwells (Figure 3.1). MSCs were thawed and plated at $2.5x10^5$ cells/well on the insert of a transwell (Corning® Transwell® polyester 0.4 μM pore membrane, 6 well plates). Macrophages were polarized, as described above, in the base of co-culture wells. MSCs adhered to transwell inserts were washed once with PBS and then transferred to wells containing inflammatory polarized macrophages in fresh media. Co-cultures were continued for 6 hours, then MSCs were collected for RNA isolation and gene expression analysis or transferred for use in T-cell proliferation assays. The 6 hour co-culture duration was selected after optimization experiments comparing 3 and 6 hours of co-culture. MSCs cultured alone, with no macrophages, served as controls. The MSCs and macrophages used in the co-culture were derived from different horses.

Gene Expression

RNA was extracted from macrophages according to manufacturer's directions (RNeasy mini kit, Qiagen, Valencia, CA) with an on-column DNase step. RNA was isolated from MSCs according to manufacturer's directions (E.Z.N.A.® MicroElute Total RNA Kit, Omega Bio-Tek, Norcross, GA) with an on-column DNase step. Samples were processed for cDNA synthesis using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, California) with 250ng of RNA as template. All cDNA samples had a single band of product from a beta-2-microglobulin PCR [41].

A SYBR Green (Applied Biosystems, Foster City, CA) based quantitative real time PCR assay was run on an Applied Biosystems 7900HT Fast Real Time PCR instrument (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Genes associated with

inflammatory macrophages include TNF-α, CCL2, CXCL10, and IL-6, while IL-10 is associated with alternatively activated macrophages [24,42]. Relative gene expression following IFN-γ stimulation was analyzed to confirm polarization of macrophages to an inflammatory phenotype. IL-6, IL-8, CCL2, and CXCL10 were selected for MSC analysis based on microarray analysis of differentially expressed genes in primed MSCs [43], and on our optimization experiments. All primers for these genes of interest have been previously described: IL-6 [44], IL-8 [32], IL-10 [44], TNF-α [44], CCL2 [33], CXCL10 [33] as well as the housekeeping gene SCAMP3 [41]. Determination of a single PCR product was made by DNA melting curve analysis [45]. Primers for SCAMP3, IL-8, CCL2, and CXCL10 span intron boundaries and genomic DNA contamination would yield two peaks in the melt curve analysis. All reactions had a melting curve with a single peak. The cycle threshold (CT) values for duplicate samples were averaged and data were analyzed with the ΔCT method where fold change is expressed as $2^{-\Delta\Delta CT}$ [46].

T cell proliferation / immunomodulation

To assess the ability of primed MSCs to modulate mitogen-stimulated T cell proliferation, a T cell proliferation assay was performed in duplicate using LPS and poly I:C primed MSCs, as well as unstimulated MSCs. After priming, $1.5x10^6$ lymphocytes +/- ConA were added to each well..

To determine how the immunomodulatory function of MSCs changed following exposure to inflammatory macrophages, the ability of MSCs to modulate mitogen stimulated T cell proliferation was assessed. After co-culture, MSCs on transwell inserts were transferred into clean wells. The insert and bottom of the transwells were washed with PBS. Lymphocyte proliferation media +/- ConA was added to the bottom of each well and $3.0 x10^6$ lymphocytes +/-

ConA were added to the transwell insert to allow for cell contact with MSCs. Again, the lymphocytes were derived from different horses than the MSCs.

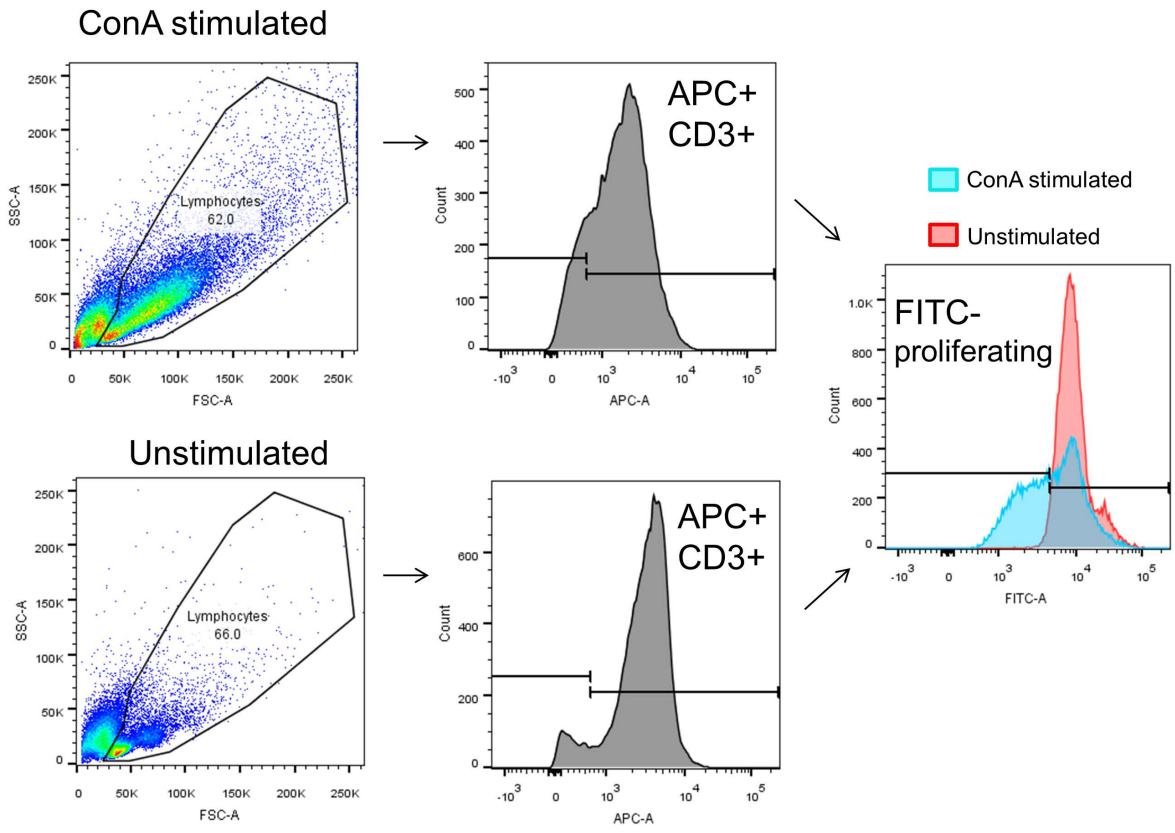
The resultant ratio of lymphocytes to MSCs (approximately 15:1) was based on previously published equine lymphocyte proliferation experiments [10,47] and was determined to be optimal in our preliminary experiments. Cultures were maintained for 72 hours, and media were not exchanged over the 3 days. After culture, lymphocytes were aspirated from the wells, blocked with 10% normal goat serum (NGS), and stained with primary mouse anti-horse CD3 antibody (clone UCF6G-3.3; University of California Davis, Davis, CA, USA) and secondary goat anti-mouse allophycocyanin (APC) antibody (BD Biosciences).

Proliferation of T cells was evaluated using CFSE dilution measured by flow cytometry. Events were gated on forward scatter (FSC) and side scatter (SSC) to exclude debris (Figure 3.7). CD3-positive cells (T cells) were identified by APC fluorescence. Unstimulated T cells were used to set the boundary of nonproliferating cells, such that all cells to the left (lower fluorescence intensity on fluorescein isothiocyanate, FITC) of that boundary were determined to be proliferating. The percentage of events in the proliferating T-cell gate was determined and compared to the percentage of all APC+ events. Differences in lymphocyte mitogen response can be caused by naturally occurring variation between horses and over time in an individual. To account for this, the effect of MSCs on lymphocyte proliferation was calculated by comparing all samples to mitogen stimulated lymphocytes alone (positive control) from that experiment, which was set at 100% lymphocyte proliferation.

Statistical analyses

Data were tested for normality using a Shapiro-Wilk test. Macrophage gene expression data ($2^{-\Delta\Delta CT}$), macrophage exposed MSCs gene expression data ($2^{-\Delta\Delta CT}$), and T cell proliferation

Figure 3.7: Measuring T cell proliferation with CFSE dilution flow cytometry. Lymphocytes were gated on forward scatter (FSC) and side scatter (SSC) to exclude debris. ConcavalinA (ConA) stimulation induces proliferation and an increase in lymphocyte size and granularity. CD3-positive cells (T cells) were identified by APC fluorescence. Unstimulated T cells (shown in red) were used to set the boundary of nonproliferating cells, such that all cells to the left (lower fluorescence intensity on fluorescein isothiocyanate, FITC) of that boundary were determined to be proliferating. ConA stimulated cells (shown in blue) have cells in both the proliferating and nonproliferating gate. The percentage of events in the proliferating T-cell gate was determined and compared to the percentage of all APC+ events.



data in the presence of macrophage exposed MSCs were analyzed using a two-sided paired t-test. MSC priming gene expression data (ΔCT values), and T cell proliferation data in the presence of primed MSCs were analyzed using a repeated measures analysis of variance (ANOVA), with a fixed effect of treatment group and the horse as a random effect in the model, followed by a Tukey HSD test for multiple comparisons. All analyses were performed by using JMP Pro 11 software (SAS Institute, Cary, NC, USA), and significance was set at $P < 0.05$.

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Contributions

All authors conceived of the study and participated in the planning and coordination of experiments. JMC, LVS, and LAF were primarily responsible for the priming and co-culture study design. JMC and MBG performed all cell isolation, MSC priming and macrophage co-culture experiments. JMC performed the gene expression assays and T cell proliferation assays. All authors contributed to data analysis and interpretation. JMC and LAF were primarily responsible for writing the manuscript. All authors edited the draft manuscript and read and approved the final manuscript. LAF is the guarantor for the manuscript.

Competing interests

The authors declare that they have no competing interests.

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CHAPTER 4

INFLAMMATORY LICENSING EQUINE MSCS IMPROVES IMMUNOMODULATORY FUNCTION AND DOES NOT INDUCE A PRO-INFLAMMATORY MSC SECRETOME.

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Abstract:

Introduction: Inflammatory licensing of MSCs can improve their ability to modulate the environment and decrease inflammation to promote functional tissue repair. This study sought to understand how inflammatory licensing equine MSCs with IFN-γ and poly I:C affected their immunomodulatory function when followed with exposure to inflammatory macrophages, and to determine how inflammatory licensed MSCs secretome affects the recipient environment.

Methods: MSCs were primed with either IFN-γ or poly I:C. Macrophages were isolated and polarized to an inflammatory phenotype using IFN-γ. Inflammatory licensed MSCs were co-cultured with inflammatory macrophages and resultant gene expression changes in both the MSC and macrophages were analyzed. The immunomodulatory potential of MSCs was assessed in a T cell proliferation assay. MSCs were analyzed for changes in immunogenicity (MHC-I, MHC-II), immunomodulatory (IDO, COX-2, TGF-β1), cytokines (IL-6, IL-8) and chemokines (CCL2, CXCL10). Macrophages were assessed for changes in cytokines (IL-6, IL-10, TNF-α, IFN-γ) and chemokines (CCL2, CXCL10). IFN-γ and poly I:C primed MSCs were used to generate conditioned media which was then used as a surrogate for an inflammatory primed MSCs secretome, and applied to untreated and IL-1β stimulated chondrocytes. Chondrocyte gene expression was analyzed in respect to inflammatory status (IL-6, TNF-α, CCL2, CXCL10, MMP-13, COX-2) and function (aggrecan, Col2) following exposure to the secretome of inflammatory licensed MSCs.

Results: IFN-γ primed MSCs have a superior ability to suppress T cell proliferation compared to naïve MSCs, and this ability was maintained following MSC-macrophage co-culture. Inflammatory macrophage co-culture caused MHC-I and MHC-II gene expression upregulation in naïve and inflammatory licensed MSCs. Conditioned media from inflammatory licensed

MSCs induced minimal changes in untreated chondrocyte gene expression and this conditioned media downregulated inflammatory gene expression in IL-1β stimulated chondrocytes. Conclusion: In vitro inflammatory licensing agents enhanced the immunomodulatory ability of MSCs exposed to inflammatory macrophages, with little evidence that licensing creates a pro-inflammatory MSC secretome. Compared to the licensing agents, inflammatory macrophage exposure alone produced an inconsistent change in immunomodulatory function. This suggests that in vitro inflammatory licensing prior to clinical use could result in more consistent induction of immunomodulatory function, compared in vivo inflammatory licensing by the recipient environment.

Keywords

Mesenchymal stem cells, MSC, immunomodulation, inflammatory licensing, polarization, regenerative medicine

Introduction

Mesenchymal stem cells (MSCs) have the therapeutic potential to treat a wide variety of inflammatory and degenerative disease processes in humans and animals [1–5] through their ability to modulate the local tissue environment and stimulate a regenerative healing response.[6,7] To generate a homogenous population of immunomodulatory MSCs, and improve the consistency in responses of patients to MSC therapy, inflammatory licensing of MSCs by priming with either IFN-γ, or the TLR3 ligand polyinosinic:polycytidylic acid (poly I:C), has been suggested.[8–11] Priming MSCs with IFN-γ enhances their immunomodulatory function in vitro[8] and markedly improves their ability to treat graft versus host disease.[10] Similarly, priming MSCs with poly I:C

priming improves their immunomodulation ability,[12] and improves their capacity to attenuate symptoms of diabetic neuropathy.[11] However, little is known about how the recipient environment affects the function of inflammatory-licensed MSCs, or if the MSCs maintain their immunomodulatory capacity when transplanted into an inflammatory recipient environment.

Macrophages are present in the recipient tissue environment of all tissue sites targeted for MSC therapy, and can be used as a resource to model the inflammatory environment into which MSCs will tranplanted.[13,14] Paracrine signals from the tissue environment regulate macrophage polarization status resulting in secretion of numerous cytokines that could alter the function of transplanted MSCs.[15] To gain insight as to how IFN-γ and poly I:C primed MSCs might react in an inflammatory recipient environment, and how the primed MSCs affect inflammatory macrophages, primed MSCs and inflammatory macrophages were co-cultured and gene expression changes were studied. As tests of functional significance, the immunomodulatory potential of inflammatory-licensed MSCs was measured, and their secretome was tested on chondrocytes for regulation of genes associated with joint health.

Methods

Horses

Fifteen adult horses were used for collection of blood and bone marrow aspirate with approval from the Institutional Animal Care and Use Committee of Cornell University.

Cell Isolation and Culture

*MSC*s

Bone marrow aspirate was collected aseptically from 8 horses[19]. Aspirates were cultured using the direct plating method [20] in MSC media: low glucose (1 g/dl) DMEM containing 10%

fetal bovine serum (FBS, Atlanta biologicals, Flowery Branch, GA, endotoxin concentration <0.1 EU/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and basic fibroblastic growth factor (bFGF, 1 ng/ml). MSCs were expanded and cryopreserved [19] for subsequent experiments. All MSCs were used at passage 4 or less.

Lymphocytes

Lymphocytes were obtained as previously described, and stained with 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE, Sigma-Aldrich) [19]. They were then suspended in lymphocyte proliferation media: RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% FBS, 0.1 mM 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 μg/ml). Unstimulated and concavalinA (ConA, 5 μg/ml; Sigma-Aldrich) stimulated lymphocytes were used in subsequent immunomodulation (T-cell proliferation) studies.

Macrophages

To generate inflammatory macrophages, monocytes were isolated according to previously described protocols[19,21]. Using MACS selection, CD14 positive cells were isolated, counted, resuspended in macrophage media (DMEM containing 10% normal horse serum (NHS, HyClone Laboratories, Logan, UT), L-glutamine (2mM), penicillin (100 units/ml), and streptomycin (100 μg/ml)), and plated at $1.8x10^6$ cells per well in 6 well plates. Macrophage media was added every 48 hours during culture. Monocytes were allowed to spontaneously differentiate into macrophages over 6-7 days of culture before use in experiments. Using this isolation technique, 99% of cells were confirmed CD14 positive by flow cytometry after 6 days in culture. Macrophages were washed with warm phosphate buffered saline (PBS) and then stimulated with IFN-γ (100 ng/mL; recombinant equine interferon gamma, R&D Systems,

Minneapolis, MN) for 6 hours. Macrophages were washed with PBS, followed by macrophage media, and then they were used to stimulate MSCs, or to confirm polarization to an inflammatory macrophage phenotype by gene expression.

Experimental Design

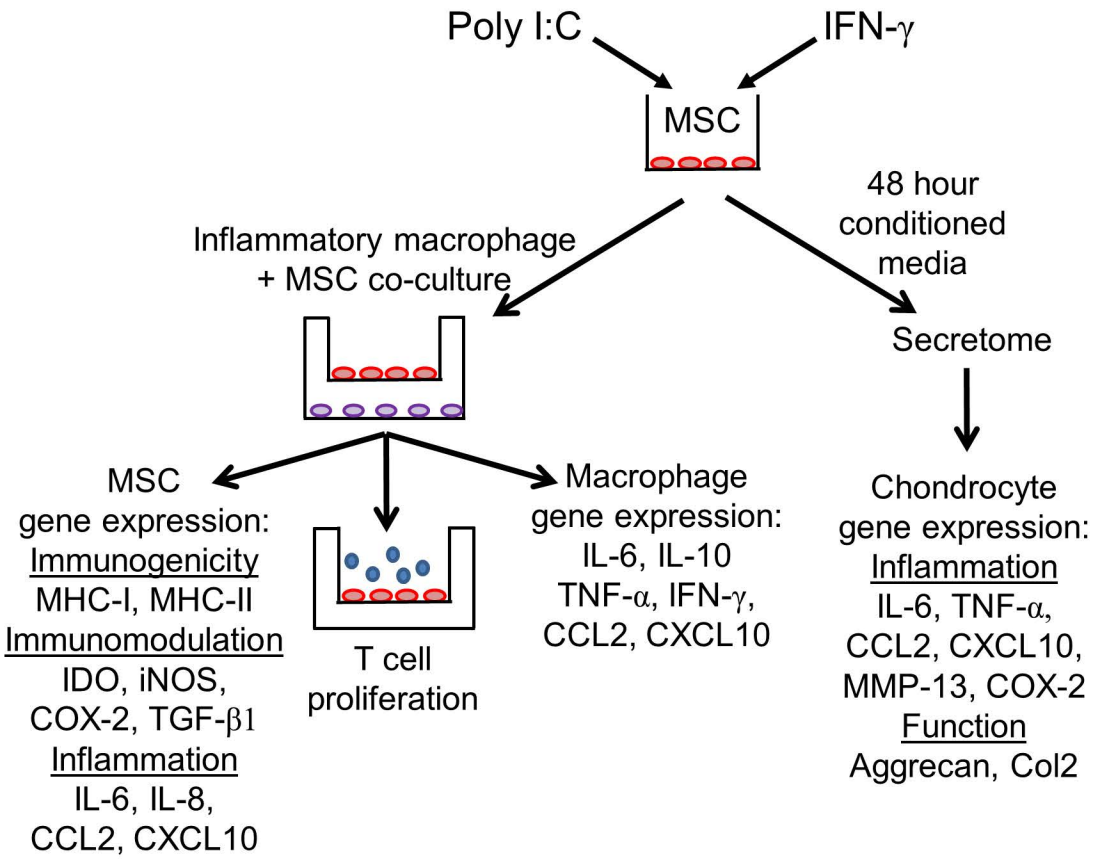
MSC Priming

MSCs were primed with poly I:C as previously described[12], with modifications in ligand concentration. Based on a series of optimization experiments, 10 μg/ml poly I:C stimulation for 1 hour was selected to produce robust changes in gene expression and modulation of mitogen stimulated T cell proliferation by MSCs, without affecting MSC viability. MSCs were also primed with 100 ng/ml IFN-γ for 24 hours as previously described.[10]

Co-Culture

In the following experiments, MSCs and macrophages used in co-culture were derived from different horses. To assess the effect of priming and/or exposure to inflammatory macrophages on MSC gene expression and immunomodulation function, MSCs were plated on transwell inserts (Corning® Transwell® polyester 0.4 μM pore membrane, 6 well plates) and primed with IFN-γ for 24 hours, or with poly I:C for 1 hour (Figure 4.1). MSCs were then washed twice with MSC media, and the inserts were transferred to wells containing inflammatory macrophages. Co-cultures were continued for 6 hours, and MSCs were collected for RNA isolation and gene expression analysis as described below, or transferred to empty wells for use in T-cell proliferation assays. To determine how primed MSCs affect gene expression in inflammatory macrophages, RNA was isolated from macrophages at the end of the co-culture period and analyzed as described below. The 6 hour co-culture duration was selected after

Figure 4.1: Study design overview. MSCs were primed with polyinosinic:polycytidylic acid (poly I:C) to stimulate TLR3 receptors or interferon-gamma (IFN-γ) to induce inflammation. MSCs on co-culture inserts were exposed to pro-inflammatory macrophages contained in the bottom of the transwells. After co-culture, MSCs were removed, placed in a new well, and lymphocytes were added to allow direct MSC-lymphocyte contact. Gene expression of immunogenicity (MHC-I, MHC-II), immunomodulation (IDO, COX-2, TGF-β1), and inflammatory mediators (IL-6, IL-8, CCL2, CXCL10), were analyzed. T cell proliferation assays with the mitogen concavalinA (ConA) characterized functional changes in MSC immunomodulation. Macrophage gene expression of cytokines (IL-6, IL-10, TNF-α, IFN-γ) and chemokines (CCL2, CXCL10) was assessed to characterize the effect of inflammatory primed MSCs on macrophages. To analyze the functional significance of the MSC secretome after priming, MSCs conditioned media over a period of 48 hours. This primed MSC secretome was then placed on IL-1β stimulated or untreated chondrocytes, and the effect of the conditioned media was assessed by measuring inflammatory (IL-6, TNF-α, CCL2, CXCL10, MMP-13, COX-2) and matrix (aggrecan, Col2) gene expression changes in the chondrocytes. The primed secretome was also analyzed to determine prostaglandin E_2 (PGE_2) concentration.



optimization experiments comparing 3 and 6 hours of co-culture, based on the production of robust changes in MSC gene expression and modulation of mitogen stimulated T cell proliferation by MSCs.

Functional significance of primed MSCs secretome

Chondrocytes were plated in chondrocyte media[22] with or without 10ng/ml IL-1β (recombinant equine IL-1β, R&D) at 200,000 cells/ well in a 24 well plate (Figure 4.1). After 24 hours, the media was removed and primed-MSC conditioned media was added. Stimulation with IL-1β was continued with medium exchange in the IL-1β stimulated group. After 24 hours, chondrocytes were lysed for subsequent RNA analysis.

Gene Expression analyses in MSCs, macrophages, and chondrocytes

RNA was isolated from MSCs and chondrocytes according to manufacturer's directions (E.Z.N.A.® MicroElute Total RNA Kit, Omega Bio-Tek, Norcross, GA) with an on-column DNase step. RNA was extracted from macrophages according to manufacturer's directions (RNeasy mini kit, Qiagen, Valencia, CA) with an on-column DNase step. Samples were processed for cDNA synthesis using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, California) with 250ng of RNA as template. All cDNA samples had a single band of product from a beta-2-microglobulin PCR. [23]

A SYBR Green (Applied Biosystems, Foster City, CA) based quantitative real time PCR assay was run on an Applied Biosystems 7900HT Fast Real Time PCR instrument (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. All genes were analyzed with a SYBR Green assay except for MMP13 and aggrecan which were analyzed with a Taqman

primer and probe assay, using 18s as a housekeeping gene and Taqman Gene Expression Master Mix (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

Genes associated with inflammatory macrophages include TNF-α, CCL2, CXCL10, IL-6, while IL-10 is associated with alternatively activated macrophages.[15,24] iNOS is associated with inflammatory macrophages in some species,[25] and inflammatory macrophages have also been documented to secrete IFN-γ.[26] Relative gene expression following IFNγ stimulation was analyzed to confirm polarization of macrophages to an inflammatory phenotype and macrophage gene expression was also assessed following co-culture with MSCs. IL-6, IL-8, CCL2, and CXCL10 were selected for MSC analysis based on microarray analysis of differentially expressed genes in primed MSCs [27], and on our optimization experiments. Additionally, expression of MHC-I, MHC-II, IDO, and iNOS was also assessed to further evaluate the antigenicity and immunomodulatory properties of MSCs. To determine the effect of primed MSC conditioned media of IL-1β stimulated and untreated chondrocytes, IL-6, IL-8, CCL2, CXCL10, MMP13, aggrecan, and Col2 were selected for analysis.[28]

All primers are listed and have been previously validated for these genes of interest: IL-6, [29] IL-8, [30] IL-10, [29] TNF-α, [29] CCL2, [31] CXCL10, [31] IFN-γ,[29] MHC-I[32], MHC-II (personal communication Doug Antczak), IDO,[33] iNOS,[33] Col2a1,[34] MMP-13,[35] Aggrecan[35] as well as the housekeeping gene SCAMP3 [23] (Table 4.1 and 4.2). For Sybr green reactions, determination of a single PCR product was made by DNA melting curve analysis.[36] The cycle threshold (CT) values for duplicate samples were averaged and data were analyzed with the ΔCT method where fold change is expressed as $2^{-\Delta\Delta CT}$.[37]

Gene	Abbreviation	Function	Forward 5'-3'	Reverse 5'-3'
Secretory carrier membrane protein 3	SCAMP3	Housekeeping gene	CTGTGCTGGG AATTGTGATG	ATTCTTGCTGG GCCTTCTG
Major histocompatibility complex class I	MHC-I	Self recognition	ACCGTGAGGT CACCCTGA	CTCCGTGTCCT GGGTCA
Major histocompatibility complex class II	MHC-II	Antigen presentation	TCCCTATGCT GGGACTTTTC	CGCCAGGCTTC AGATAGAAC
Indoleamine 2,3-dioxygenase	IDO	Mediator of immunomodulation	TCATGACTAC GTGGACCCAA AA	CGCCTTCATAG AGCAGACCTTC
Prostaglandin endoperoxide synthase 2	COX-2	Mediator of immunomodulation	CAGCATAAAC TGCGCCTTTTC	AGGCGGGTAGA TCATTTCCA
Transforming growth factor beta 1	TGF-b1	Cytokine	AGAGCTGCGC CTCCTAAGG	ACGACTCCGGT GACATCAAAG
Interleukin 6	IL-6	Cytokine	TGCTGGCTAA GCTGCATTCA	GGAAATCCTCA AGGCTTCGAA
Interleukin 8	IL-8	Cytokine	GCTGGCTGTT GCTCTCTTG	CCGAAGCTCTG CAGTAATTCTT
Interleukin 10	IL-10	Cytokine	GCCTTGTCGG AGATGATCCA	TTTTCCCCCAG GGAGTTCAC
Tumor necrosis factor-alpha	TNF-α	Cytokine	AAAGGACATC ATGAGCACTG AAAG	GGGCCCCCTGC CTTCT
Interferon-gamma	IFN-γ	Cytokine	CCAGCGCAAA GCAATAAGTG	GGCCTCGAAAC GGATTCTG
C-C motif chemokine 2	CCL2	Chemokine	GGCTCAGCCA GATGCAATTA	GCTTTCTTGTCC AGCTGCTT
C-X-C motif chemokine 10	CXCL10	Chemokine	GACTCTGAGT GGAACTCAAG GAAT	GTGGCAATGAT CTCAACACG
Collagen type 2	COL2A1	Cartilage extracellular matrix	TCAAGTCCCT CAACAACCAG ATC	GTCAATCCAGT AGTCTCCGCTC TT

Table 4.1: Overview of Sybr green primers used in gene expression analysis

Gene	Abbreviation	Function	Forward 5'-3'	Reverse 5'-3'	Probe
18s ribosomal RNA	18s	Ribosome structural unit	GGCGTCCCCCAACTTCTT	AGGGCATCACAGACCTGTTATTG	TGGCGTTCAGCCACCCGAGATT
Collagenase 3	MMP-13	Matrix metallo-proteinase	TGAAGACCCGAACCCTAAACAT	GAAGACTGGTGATGGCATCAAG	CAAAACACCAGACAAATGCGATCCTTCCTTA
Aggrecan	ACAN	Cartilage extracellular matrix	GATGCCACTGCCACAAAACA	GGGTTTCACTGTGAGGATCACA	CCGAGGGTGAAGCTCGAGGCAA

Table 4.2: Overview of Taqman primers and probes used in gene expression analysis

T cell proliferation / immunomodulation

After primed MSC/inflammatory macrophage co-culture, MSCs on transwell inserts were transferred to clean wells. Lymphocyte proliferation media +/- ConA was added to the bottom of each well, and 2.5 $x10^6$ lymphocytes +/- ConA were added to the transwell insert to allow for cell-cell contact with MSCs. The resultant ratio of lymphocytes to MSCs (approximately 15:1) was based on previously published equine lymphocyte proliferation experiments [19,33] . Cultures were maintained for 72 hours, and media were not exchanged over the 3 days. After culture, lymphocytes were aspirated from the wells, blocked with 10% normal goat serum (NGS), and stained with primary mouse anti-horse CD3 antibody (clone UCF6G-3.3; University of California Davis, Davis, CA, USA) and secondary goat anti-mouse allophycocyanin (APC) antibody (BD Biosciences).

Proliferation of T cells was evaluated using CFSE attenuation measured by flow cytometry. Events were gated on forward scatter (FSC) and side scatter (SSC) to exclude debris. CD3-positive cells (T cells) were identified by APC fluorescence. Unstimulated T cells were used to set the boundary of nonproliferating cells, such that all cells to the left (lower fluorescence intensity on fluorescein isothiocyanate, FITC) of that boundary were determined to be proliferating. The percentage of events in the proliferating T-cell gate was determined and compared to the percentage of all APC+ events. Differences in lymphocyte mitogen response can be caused by naturally occurring variation between horses and over time in an individual. To account for this, the effect of MSCs on lymphocyte proliferation was calculated by comparing all samples to mitogen stimulated lymphocytes alone (positive control) from that experiment, which was set at 100% lymphocyte proliferation.

Statistical analyses

Macrophage gene expression data ($2^{-\Delta\Delta CT}$) was analyzed using a two-sided paired t-test. T cell proliferation data and MSC gene expression data following priming and macrophage exposure (ΔCT values) were analyzed using a repeated measures analysis of variance (ANOVA), with a fixed effect of treatment group and the horse as a random effect in the model, followed by a Tukey HSD test for multiple comparisons. Macrophage gene expression data following MSC co-culture (ΔCT values) and chondrocyte gene expression data following MSC secretome exposure (ΔCT values) were analyzed using a repeated measures ANOVA with a fixed effect of treatment group and the MSC horse source as a random effect in the model, followed by a Tukey HSD. 95% confidence intervals were calculated for each macrophage and chondrocyte gene expression group $2^{-\Delta\Delta CT}$ values to determine if the group was significantly different from the control. Data were tested for normality using a Shapiro-Wilk test. All analyses were performed by using JMP Pro 11 software (SAS Institute, Cary, NC, USA), and significance was set at $P < 0.05$.

Results

Generation of inflammatory macrophages

This study sought to utilize an inflammatory macrophage population to model an inflammatory recipient environment such as might be present after trauma or surgery.

Stimulation of macrophages with IFN-γ resulted in an inflammatory macrophage population. Gene expression of CXCL10 (average 5859 $2^{\Delta\Delta CT}$ range (30 –22584; p=0.0078), TNF-α (average 6 $2^{\Delta\Delta CT}$ (3.5 –12; p=0.0078), and IFN-γ (average 6.8 $2^{\Delta\Delta CT}$ (0.6 –22; p=0.0391) were significantly upregulated following IFN-γ treatment (macrophages from 4 horses).

Expression of IL-10 (average 0.5 $2^{\Delta\Delta CT}$ (0.2 –1.1; p=0.0038) was significantly down-regulated in IFN-γ stimulated macrophages and there was no change in CCL2 (average 1.5 $2^{\Delta\Delta CT}$ (0.7 –4.1; p=0.25) or IL-6 (average 1.0 $2^{\Delta\Delta CT}$ (0.3 –2.0; p=0.95) expression with IFN-γ stimulation.

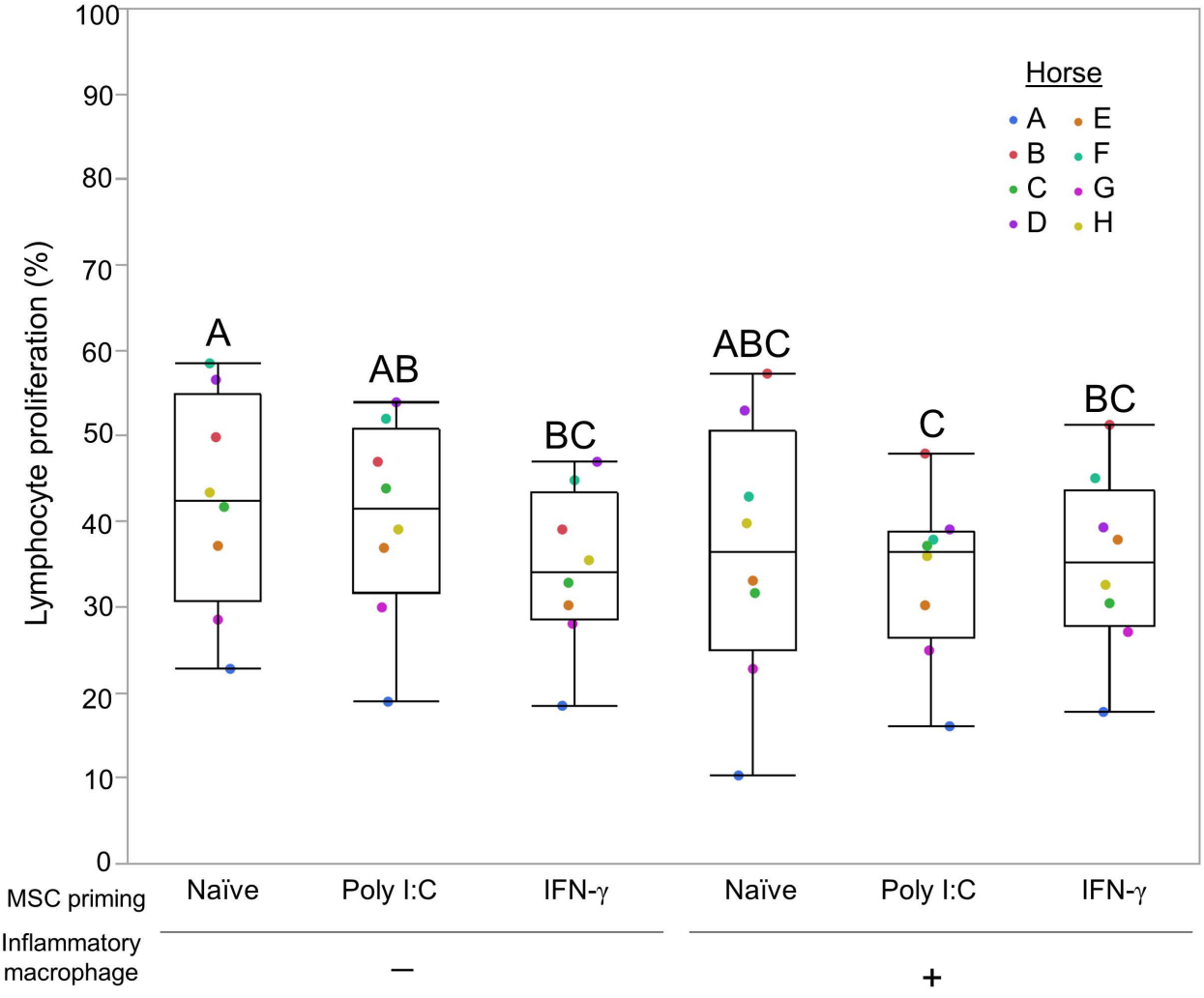
Immunomodulatory properties of MSCs after inflammatory priming and exposure to inflammatory macrophages

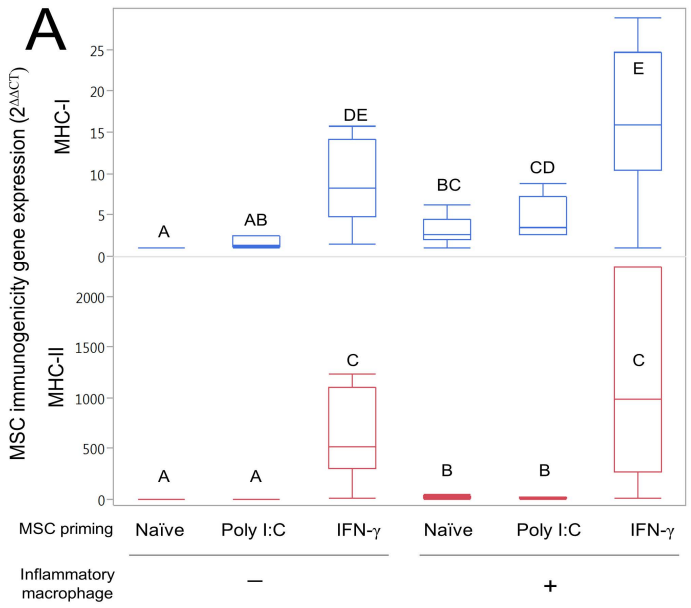
To determine how the immunomodulatory function of inflammatory licensed MSCs changed following exposure to inflammatory macrophages, the ability of MSCs to modulate mitogen-stimulated T cell proliferation was assessed using a T cell proliferation assay. Lymphocytes used in these studies were derived from different horses than the MSCs, resulting in an allogeneic study design. The immunomodulatory function of inflammatory primed MSCs, determined by T cell proliferation assay, was improved or unchanged with exposure to inflammatory macrophages (Figure 4.2). MSCs treated with IFN-γ priming alone, priming MSCs with IFN-γ or poly I:C followed by exposure to inflammatory macrophages, resulted in an increased ability of MSCs to suppress T cell proliferation compared to untreated MSCs. The immunomodulatory ability of MSCs was not significantly changed with exposure to inflammatory macrophages alone or poly I:C priming alone. No treatment decreased the immunomodulatory capacity of MSCs.

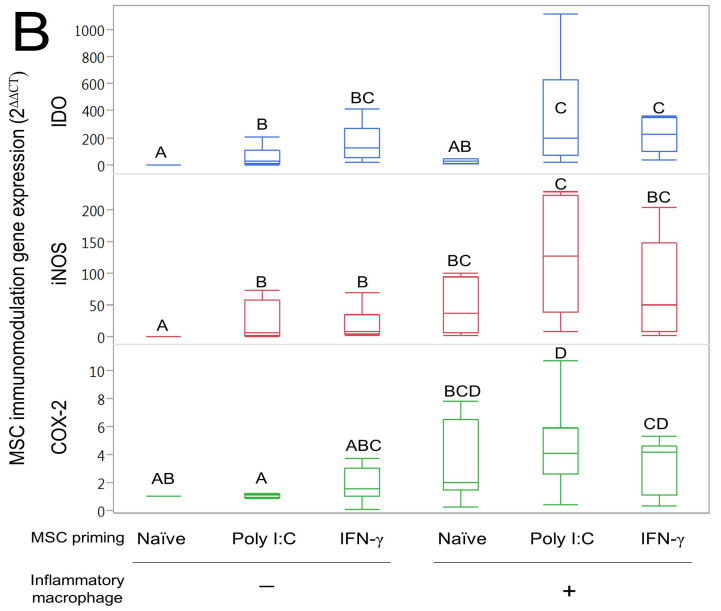
MSCs gene expression after inflammatory priming or exposure to inflammatory macrophages

Inflammatory-priming with IFN- γ, but not with poly I:C, and exposure to inflammatory macrophages resulted in significant upregulation of MHC class I and II expression in MSCs (Figure 4.3A). All primed MSC and macrophage exposure groups, with the exception of inflammatory macrophage exposure alone, resulted in significant induction of iNOS and IDO expression in MSCs compared to untreated MSCs (Figure 4.3B). COX-2 expression in MSCs

Figure 4.2: Inflammatory priming and/or exposure to inflammatory macrophages does not impair MSC immunomodulatory function. The ability of MSCs to diminish mitogen stimulated T cell proliferation was compared to mitogen stimulated lymphocytes alone (positive control), which was set to 100% T cell proliferation. An increase in MSC immunomodulation was found in IFN-γ primed MSCs, MSCs primed with IFN-γ followed by inflammatory macrophage exposure, or MSC priming with poly I:C followed by inflammatory macrophage exposure. Box and whisker plots represent n=8. Groups that do not share the same letter are significantly different. P<0.05: repeated measures ANOVA, with the horse as a random effect, followed by a Tukey HSD test for multiple comparisons.







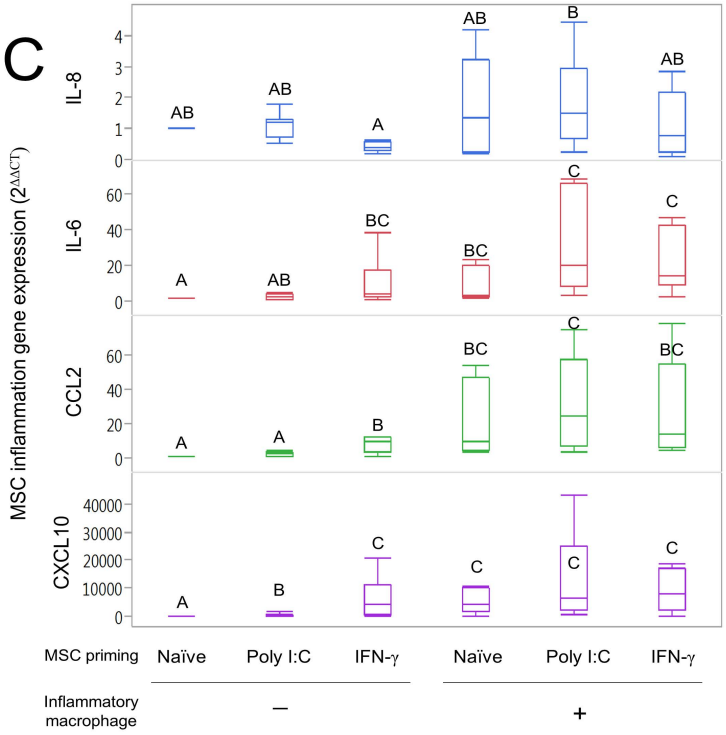


Figure 4.3: Gene expression changes in MSCs following inflammatory priming and/or inflammatory macrophage exposure. MSCs were exposed to polyinosinic:polycytidylic acid (poly I:C) or interferon-gamma (IFN-γ), and then to inflammatory macrophages. Resultant changes in expression of A) immunogenicity, B) inflammation, and C) immunmoulatory genes were measured and compared to untreated, control MSCs using the 2-ΔΔCT method. Priming with IFN-γ and or inflammatory macrophage exposure induced significant increases in A) MHC-I, MHC-II, B) IL-6, CCL2, and CXCL10 expression. Priming with poly I:C alone produced few significant changes in gene expression with the exception of C) IDO, and sequential exposure to poly I:C then inflammatory macrophages induce significant upregulation of IDO compared to inflammatory macrophage exposure alone. Box and whisker plots represent n=8 individual horses. Groups that do not share the same letter are significantly different, $p < 0.05$: repeated measures ANOVA, with the horse as a random effect, followed by a Tukey HSD test for multiple comparisons.

was significantly upregulated in all groups treated with macrophages compared to untreated MSCs (Figure 4.3B).

Gene expression of IL-6, CCL2, and CXCL10 was significantly upregulated in IFNγ primed MSCs, and in all MSC groups exposed to macrophages compared to untreated MSCs (Figure 4.3C). Following poly I:C priming, CXCL10, but not CCL2 and IL-6 expression was significantly upregulated in MSCs. Expression of IL-8 in MSCs was significantly lower following IFN-γ priming compared to poly I:C MSCs exposed to macrophages. There was no significant difference between untreated MSCs and any of the treatment groups in TGF-β1 expression (average 1.0 $2^{\Delta\Delta CT}$ (0.6 –1.8).

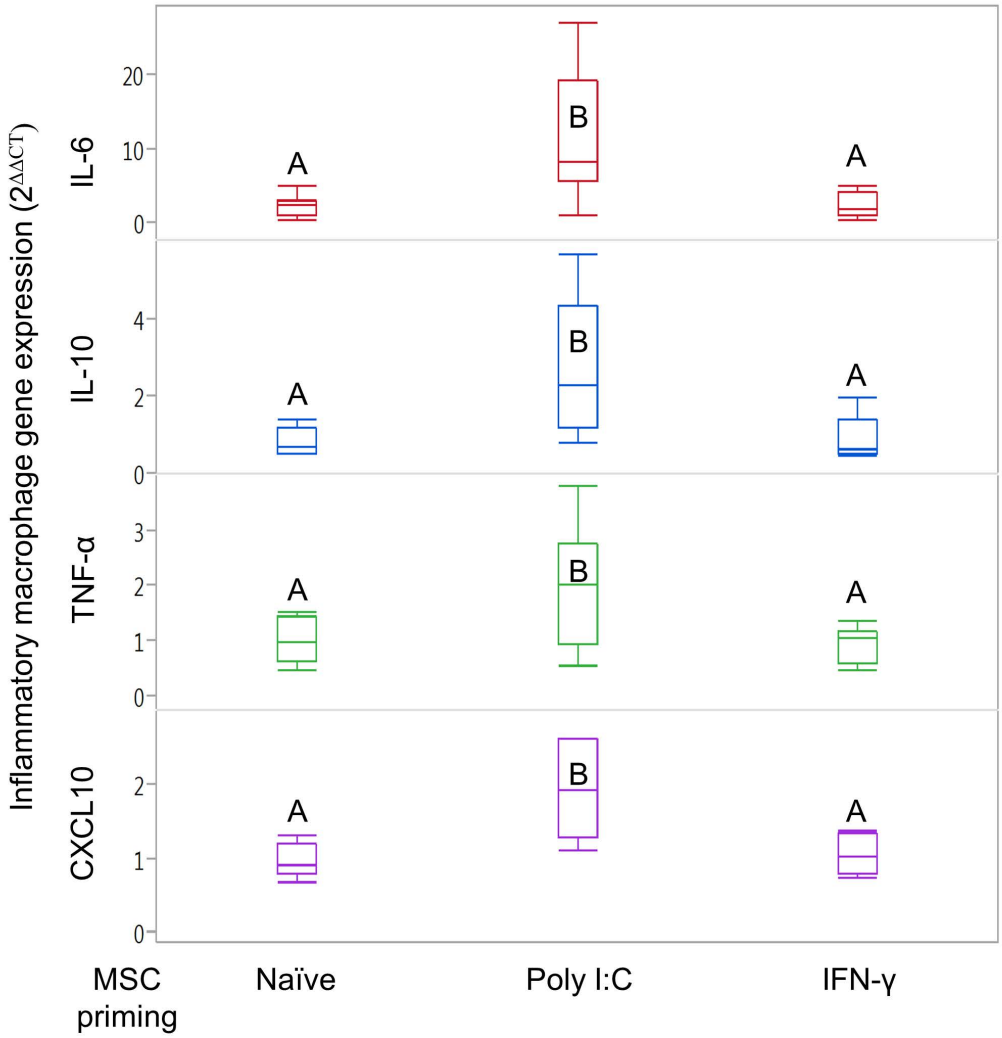
Macrophage gene expression in response to inflammatory primed MSCs

Following co-culture of inflammatory macrophages with MSCs in a transwell setup, gene expression of IL-6, IL-10, CXCL10, and TNF-α was increased only in those macrophages exposed to poly I:C primed MSCs (Figure 4.4). Expression of CCL2 and IFN-γ in inflammatory macrophages were not significantly affected by exposure to MSCs (Table 4.1s).

The secretome from MSCs diminishes the catabolic effects of interleukin-1B in articular chondrocytes

To assess the functional significance of primed MSCs on articular cartilage heath, conditioned medium from primed MSCs was generated to represent the secretome of inflammatory primed MSCS, and used to determine how the inflammatory MSC secretome affects gene expression of arthritic (IL-1β-stimulated) chondrocytes.Conditioned media from inflammatory primed MSCs was used to represent their secretome to test the functional significance of MSCs priming on genes known to be important in chondrocyte homeostasis. Conditioned media from inflammatory primed MSCs induced different changes in chondrocyte

Figure 4.4: Gene expression changes in inflammatory macrophages following exposure to inflammatory primed MSCs. Inflammatory macrophages were co-cultured in a transwell setup with MSCs primed with polyinosinic:polycytidylic acid (poly I:C) or interferon-gamma (IFN-γ). Only exposure to poly I:C stimulated MSCs produced significant changes in macrophage expression of TNF-α, CXCL10, IL-6, and IL-10. Resultant changes in gene expression was measured and compared to control inflammatory macrophages not exposed to MSCs using the $2^{-\Delta\Delta CT}$ method. Box and whisker plots represent n=8. Groups that do not share the same letter are significantly different, $p< 0.05$: repeated measures ANOVA, with the horse as a random effect, followed by a Tukey HSD test for multiple comparisons.



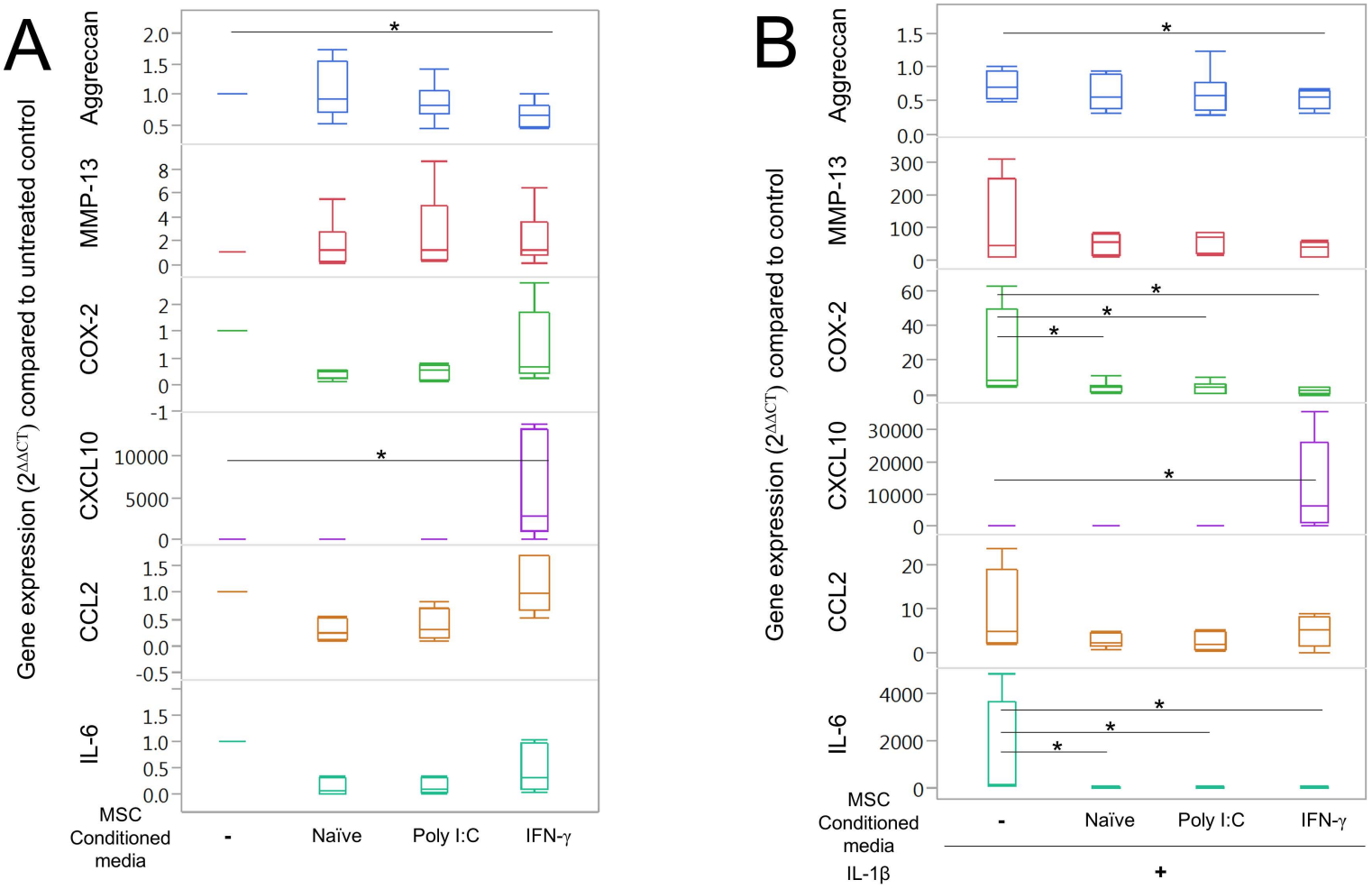
Gene	MSC Exposure		
	Ctrl MSC	Poly I:C MSC	IFN-γ MSC
IL-6	[1.1,3.5] A	[3.6,18.5]* B	[0.9,3.8] A
IL-10	[0.5,1.1] A	[1.3,4.2]* B	[0.4,1.4] A
CCL2	[1.0,1.5] A	[1.0,1.9] A	[1.0,1.6] A
CXCL10	[0.8,1.1] A	[1.2,3.2]* B	[0.8,1.3] A
TNF-α	[0.7,1.3] A	[1.1,2.9]* B	[0.6,1.2] A
IFN-γ	[0.2,5.4] A	[0.9,9.7] A	[0.5,4.8] A

Table 4.1s: Macrophage gene expression following MSC co-culture statistics. 95% Confidence intervals for $2^{\Delta\Delta CT}$ values indicate the fold change from baseline IFN-γ stimulated macrophages. * indicates confidence interval does not contain 1 and thus the change is significant from baseline. Groups that do not share the same letter are significantly different based on delta CT values. $P<0.05$: repeated measures analysis of variance (ANOVA), with the horse as a random effect in the model, followed by a Tukey HSD test for multiple comparisons.

gene expression depending on the MSC priming agent and stimulation of chondrocytes with IL-1β (Figures 4.5A, 4.5B). In non-IL-1β stimulated chondrocytes, aggrecan expression was decreased and CXCL10 expression was increased in response to IFN-γ primed MSC conditioned media compared to naïve MSC conditioned media (Figure 4.5A, Table 4.2s). IL-6, CCL2, TNF-α, COX-2, MMP-13, and Col2 expression were not significantly different in non-IL-1β stimulated chondrocytes following exposure to any of the MSC conditioned media treatment groups (Figure 4.5A, Table 4.2s).

Treatment of chondrocytes with IL-1B resulted in 0.3-0.7 fold decrease in aggrecan expression and 7-300 fold increase in MMP-13 expression compared to untreated chondrocytes verifying effective catabolic stimulation of chondrocytes. In IL1β stimulated chondrocytes (Figure 4.5B, Table 4.3s), Aggrecan expression was significantly downregulated in IL-1β stimulated chondrocytes exposed to IFN- γ primed MSC conditioned media compared to control chondrocytes, and aggrecan expression was not significantly different in chondrocytes treated with conditioned media from naïve MSCs, poly I:C primed MSCs, or IFN-γ stimulated MSCs. All MSC conditioned media groups reduced expression of COX-2 and IL-6 in IL-1β stimulated chondrocytes. CXCL10 expression was significantly increased in response to IFN-γ primed MSC conditioned media compared to control IL-1β chondrocytes that were not treated with any conditioned medium, and to IL-1β chondrocytes exposed to naïve MSC conditioned media (Figure 4.5B). CCL2, TNF-α, MMP-13, and Col2 expression was not significantly different in IL-1β stimulated chondrocytes following exposure to any of the MSC conditioned media treatment groups, compared to control IL-1β stimulated chondrocytes (Figure 4.5B, Table 4.3s)

Figure 4.5: Inflammatory primed MSC secretomes do not induce inflammatory changes in untreated chondrocytes. To analyze the functional significance of inflammatory primed MSC on cartilage health, inflammatory primed MSCs conditioned media over a period of 48 hours. This primed MSC secretome was then placed on A) untreated, or B) IL-1 β stimulated chondrocytes. A) Conditioned media from IFN-γ primed MSCs induced a downregulation in aggrecan and upregulation of CXCL10 compared to untreated control chondrocytes. B) In IL-1β stimulated chondrocytes, conditioned media from IFN-γ primed MSCs also induced a downregulation in aggrecan and upregulation of CXCL10 compared to untreated control chondrocytes. All conditioned media groups induced downregulation of IL-6 and COX-2 genes. Box and whisker plots represent n=8 individual horses. * indicates that group is significantly different from control IL-1β stimulated chondrocyte gene expression based on a 95% confidence interval.



Gene	Conditioned media exposure		
NTC Ctrl	Ctrl MSC	Poly I:C MSC	IFN-γ MSC
IL-6	[0,5.4] A	[0,6.9] AB	[0,1.8] A
CCL2	[0,2.0] A	[0,4.5] A	[0.2,3.0] B
CXCL10	[0,3.4] A	[0,415] A	[1021,10871]* B
TNF-α	[0.5,1.7] A	[0.2,1.7] A	[0.4,1.8] A
COX-2	[0,3.8] A	[0,1.7] A	[0,1.2] A
MMP-13	[0.1,3.2] A	[0,5.1] A	[0.3,3.8] A
Aggrecan	[0.7,1.5] B	[0.6,1.1] AB	[0.5,0.8]* A
Col2	[0.5,2.3] A	[0.3,2.2] A	[0.3,1.5] A

Table 4.2s: Untreated chondrocytes gene expression following MSC conditioned media exposure statistics. 95% Confidence intervals for $2^{\Delta\Delta CT}$ values indicate the fold change from baseline untreated chondrocytes. * indicates confidence interval does not contain 1 and thus the change is significant from baseline. Groups that do not share the same letter are significantly different based on delta CT values. $P<0.05$: repeated measures analysis of variance (ANOVA), with the horse as a random effect in the model, followed by a Tukey HSD test for multiple comparisons.

Gene	Conditioned media exposure		
IL-1b Ctrl	Ctrl MSC	Poly I:C MSC	IFN-γ MSC
IL-6	[0.1,0.5]* A	[0,0.4]* A	[0.1,0.7]* A
CCL2	[0.1,1.6] A	[0,1.8] A	[0.5,2.8] A
CXCL10	[0.3,2.4] A	[0,286] A	[3566,22353]* B
TNF-α	[0,1.2] A	[0,1.3] A	[0,1.6] A
COX-2	[0.3,0.7]* A	[0.1,0.8]* A	[0.2,0.5]* A
MMP-13	[0.6,2.5] AB	[1.0,2.8] B	[0.6,2.0] A
Aggrecan	[0.5,1.1] A	[0.5,1.1] A	[0.5,0.9]* A
Col2	[0.5,1.5] A	[0.3,1.8] A	[0,2.1] A

Table 4.3s: IL-1β stimulated chondrocytes gene expression following MSC conditioned media exposure statistics. 95% Confidence intervals for $2^{\Delta\Delta CT}$ values indicate the fold change from baseline IL-1β stimulated chondrocytes. * indicates confidence interval does not contain 1 and thus the change is significant from baseline. Groups that do not share the same letter are significantly different based on delta CT values. $P<0.05$: repeated measures analysis of variance (ANOVA), with the horse as a random effect in the model, followed by a Tukey HSD test for multiple comparisons.

Discussion

Inflammatory licensing of MSCs by priming with IFN-γ resulted in improved immunomodulatory function compared to untreated MSCs. Further, when IFN-γ priming was followed with exposure to inflammatory macrophages, the enhanced immunomodulation was maintained. Inflammatory licensing with poly I:C produced modest improvement in MSCs immunomodulatory function, which was correlated with the minimal changes in gene expression following poly I:C exposure compared to that observed with IFN-γ stimulation. Poly I:C priming prior to macrophage exposure does appear to improve MSCs immunomodulatory ability. This suggests that multiple types of inflammatory signals have the ability to license and improve MSCs immunomodulatory abilities,[9,12] but in vitro inflammatory stimuli priming may produce a more consistent response in MSCs than relying on the recipient environment alone to inflammatory license MSCs.

Without priming, the response of MSCs to inflammatory macrophages was highly dependent on the horse from which the MSCs were derived. For example, MSCs from Horse B were impaired in their ability to suppress T cell proliferation following exposure to inflammatory macrophages. However, MSCs from Horses B and H were exposed to the identical inflammatory macrophage population, and Horse H's MSCs responded as anticipated, suppressing T cell proliferation. This suggests that the unresponsiveness observed in Horse B is due to horse-horse MSC variability and not to macrophage differences. Horse B's response could also not be explained by MHC class II expression. Only one horse (F) had high MHC-II expression at baseline, and this horse's MSCs responded as expected to macrophage exposure. Interestingly, Horse B's MSCs were the upper data points for most of the inflammatory genes measured, further suggesting individual variability in phenotypic response between MSCs from

different individuals. The variable response of MSCs to inflammatory macrophages indicates that in vitro inflammatory licensing might be beneficial in generating a MSC population that is more consistently immunomodulatory across individuals.

Changes in expression of immunostimulatory and immunomodulatory genes have been previously reported in MSCs stimulated with IFN-γ and TNF-α.[33] The results of the present study expand on this concept and demonstrate improved immunomodulatory abilities of MSCs following inflammatory priming with IFN-γ alone, and after sequentially exposing IFN-γ stimulated MSCs to macrophages. The immunomodulatory ability of IFN-γ stimulated MSCs exposed to inflammatory macrophages are similar to what has been described previously for MSCs stimulated by both IFN-γ and TNF-α. [33] This is likely the result of TNF-α production by inflammatory macrophages as observed in this study.

Overall, IFN-γ priming appears to induce inflammatory licensing of MSCs to a greater extent than poly I:C, and the marked upregulation of inflammatory genes observed in IFN-γ primed MSCs does not appear to be associated with any deleterious effects of the resultant secretome. The IFN-γ primed MSC secretome induced similar effects on macrophages and chondrocytes as unstimulated MSCs. Use of primed MSC conditioned media as a surrogate for the secretome did not upregulate catabolic cytokine gene expression, or decrease matrix gene expression in chondrocytes. In IL-1 β stimulated chondrocytes, IFN-γ primed MSC conditioned media induced a protective reduction of inflammatory gene expression. Previous studies have found the naïve, unprimed MSC secretome to have a protective effect against the inflammatory changes caused by IL-1β in chondrocytes,[28] and the result of the present study confirm that inflammatory primed MSCs continued to have a beneficial effect on chondrocytes. The fact that inflammatory licensed MSCs did not have any deleterious effect on chondrocytes, and maintained beneficial

anti-inflammatory effects on IL-1β stimulated chondrocytes, support the investigation of inflammatory licensed MSCs for clinical use in diseases such as arthritis. This concept is further supported by previous studies showing that inflammatory synovial fluid alone is insufficient to induce MSC inflammatory licensing.[40] Taken together these results suggests that to fully harness MSC immunomodulatory benefits in joint disease, in vitro inflammatory licensing should be considered.

Co-culture of inflammatory macrophages with poly I:C, but not IFN-γ primed MSCs produced the only significant changes in macrophage gene expression. The induction of IL-10 and IL-6 in macrophages following co-culture is a shift from the initial profiles noted after stimulation. Poly I:C primed MSCs have been previously found to induce a net increase in IL-10 concentration media from poly I:C primed MSCs and cancer cells.[39] Poly I:C stimulated MSCs also upregulated TNF-α and CXCL10 in macrophages, which is a continuation of the trend found after initial IFN-γ macrophage polarization. Since seemingly divergent pathways are induced in the time frame investigated, it is difficult to determine what the net effect of poly I:C stimulated MSCs is on macrophages.

As expected, priming of MSCs with IFN-γ markedly upregulated expression of MHC-I and MHC-II, which was further increased by inflammatory macrophage exposure. This increase in MHC expression might translate to an increase in the antigenicity of MSCs, which would be contradictory to allogeneic use in patients. This suggests that regardless of the initial status of MHC II expression, when placed in an inflammatory environment there is the potential for upregulation of MHC II expression. This is consistent with observations by our laboratory that when a primary culture of MSCs contains macrophages, the MSCs often have high baseline MHC class II expression. Additionally, our laboratory found that regardless of the initial MHC

class II status, donor-recipient equine leukocyte antigen mismatched MSCs induced an antibody response.[41] This indicates that in vivo antigenicity of MSCs may be increased in an inflammatory recipient environment, calling into question the timeframe an allogeneic MSC population would have to exert a therapeutic effect prior to targeting by the recipient immune system. For allogeneic purposes, poly I:C inflammatory licensing may be preferred to IFN-γ because it does not appear to increase the antigenicity of MSCs. However, if the in vitro inflammatory licensing does not induce MSC antigenicity, the inflammatory environment likely will.

Priming MSCs in vitro could start the process of inflammatory licensing prior to MSCs being transplanted and exposed to a recipient environment. Without in vitro priming, induction of MSC immunomodulatory function would depend on the environment providing sufficient inflammatory signals. Some reports indicate that MSCs are capable of acting in a pro-inflammatory manner,[12,39] so relying on the environment to have the necessary signals and in the appropriate time frame in order to activate the immunomodulatory pathway, adds a component of uncertainty as to whether or not the transplanted MSCs would be effective. The natural heterogeneity of immunomodulatory function within an individual MSC population has been shown to be reduced with inflammatory priming[8] further supporting the benefits of in vitro inflammatory priming of MSCs prior to application in regenerative therapies.[10,40]

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CHAPTER 5

EQUINE MESENCHYMAL STEM CELLS (MSCS) FROM DIFFERENT TISSUE SOURCES DISPLAY SIMILAR IMMUNE-RELATED GENE EXPRESSION PROFILES IN RESPONSE TO IFN-γ STIMULATION.

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Abstract

Introduction: Mesenchymal stem cells (MSCs) have the therapeutic potential to improve tissue repair and decrease inflammation due to their immunomodulatory properties. MSCs can be isolated from numerous tissue sources, but it is unknown how the tissue source affects MSCs response to inflammatory stimuli. This study sought to evaluate the immune-related gene expression profiles of equine MSC from different tissue sources in response to IFN-γ stimulation. If MSCs from one or more sources were resistant to this immunomodulatory cytokine, then that source might be preferable for clinical application in regenerative medicine.

Methods: MSCs from 4 horses were isolated from peripheral blood (PB), adipose tissue (AT) and bone marrow (BM). These matched tissue sets were stimulated with IFN-γ (1 ng/mL or 100 ng/mL) for 48 hours, unstimulated MSCs served as controls and provided baseline gene expression data. Following stimulation, MSCs were analyzed for changes in gene expression related to immunogenicity (MHC-I, MHC-II), immunomodulatory function (IDO, COX-2, iNOS), cytokines (IL-6, TGF-β1) and chemokines (CCL2, CXCL10). To further explore these findings, 17 additional unmatched MSC isolations, including 5 umbilical cord blood (UCB) - MSC samples, were stimulated with 1 ng/mL IFN-γ for 48 hours, and gene expression changes were analyzed.

Results: Baseline expression of all immune related genes, with the exception of COX-2, was variable depending on MSC tissue source of origin. Baseline gene expression of PB-MSCs and AT-MSCs were most similar to each other, and different from gene expression profiles of BM-MSCs and UCB-MSCs. Regardless of the source, MSCs responded to IFN-γ and had increased expression of MHC-I, MHC-II, IDO, IL-6 and CXCL10. Following IFN-γ stimulation, gene

expression profiles were more similar across all tissue types, with no significant difference due to tissue source in expression of MHC-I, IDO, or CXCL10.

Conclusions:

MSCs from all sources responded in a similar fashion to IFN-γ induced gene upregulation. Both before and after stimulation, PB-MSCs and AT-MSCs gene expression profiles clustered together and BM-MSCs and UCB-MSCs were most similar to each another. The similarity in response and in expression following IFN-γ stimulation, suggests that when selecting a source of MSCs for clinical use in an allogeneic or inflammatory environment, the MSCs from different sources will likely respond similarly to the inflammatory stimuli.

Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that show great promise for their use in regenerative medicine due to their immunomodulatory properties.[1–3] In order for cells to be characterized as MSCs, they must be able to differentiate into three lineages (adipocytes, chondrocytes, and osteocytes), express a panel of cell surface markers while being negative for other markers, and be plastic adherent.[4] MSCs can be derived from a variety of tissue sources such as peripheral blood, adipose tissue, bone marrow, umbilical cord blood, Wharton's jelly, and muscle.[5] While all MSCs share the minimum defining characteristics, they have been shown to have various functional differences dependent on their tissue source of origin. MSCs from different sources vary in their trilineage differentiation capacities, and some sources contain MSCs that are able to differentiate into additional lineages such as hepatocytes.[6,7] The immunomodulatory capabilities of MSCs are also variable based on the tissue source,[5,8] as well as the levels of cell surface expression of markers of immunogenicity, such as for example the

major histocompatibility complex (MHC) proteins.[9]

Allogeneic MSCs are highly desired for clinical use since they can be administered acutely and avoid patient-associated factors such as age, genetic diversity, and clinical disease, all of which can negatively affect the quality and quantity of autologous MSCs.[10] MSCs are known to express varying levels of major histocompatibility (MHC) class I, a marker that the immune system uses to distinguish self from non-self, and were initially reported to have low to absent MHC class II expression, a protein used for self-recognition and immune activation.[4,11] However with further MSC characterization, MHC class II expression has been documented to be variable across MSC populations,[12,13] which suggests the potential for an immune response with allogeneic use. Interferon-γ (IFN-γ) is a pro-inflammatory cytokine important in the activation of the innate and adaptive immune response against pathogens and induces MHC class II upregulation.[14,15] *In vivo*, IFN-γ is secreted by T cells in response to T cell receptor activation during antigen presentation and foreign MHC recognition,[16] all of which make IFN-γ particularly relevant in allogeneic MSC applications.

In order to answer the many questions remaining prior to MSCs clinical application in humans, the equine model offers a readily translatable model for MSC research. The mouse model has been called into question for MSC research, because the mouse and human utilize different immunomodulatory pathways and phylogenetic analysis predicts that equine MSCs utilize the IDO pathway similar to humans.[17] Furthermore, MSC research in the horse has the potential to benefit both animal and human health as there are numerous analogous naturally occurring conditions in the horse and human that could benefit from MSC therapy.[1,2,18] Equine MSCs derived from the bone marrow are variably positive for MHC-II expression and are able to upregulate MHC class II expression in response to interferon (IFN)-γ, irrespective of baseline

MHC-II expression.[19] The latter has been documented in equine umbilical cord blood-derived MSCs also,[20] but nothing is documented on the expression of MHC-II and other immune-related genes in equine MSC from other sources. This is clinically important because if MSCs from a particular tissue source were found to be more resistant to IFN-γ-induced upregulation of MHC expression, improved clinical outcomes in an allogeneic setting could be expected.

The aims of the present study were to evaluate immune-related gene expression profiles of equine MSCs, obtained from different tissue sources, in response to IFN-γ stimulation, in order to determine whether MSC from one or more sources might be more resistant to this immunomodulatory cytokine. Our salient findings were that the MSCs responded similarly to IFN-γ stimulation regardless of tissue source of origin. We also noted that the gene expression profiles of peripheral blood and adipose tissue derived MSCs were the most similar both at baseline and following stimulation and different from the bone marrow and umbilical cord derived MSCs, which clustered similarly to each other.

Methods

MSC isolation and culture

Matched peripheral blood-derived MSCs (PB-MSCs), adipose tissue-derived MSCs (AT-MSCs), and bone marrow-derived MSCs (BM-MSCs) were isolated from 4 horses (Table 5.1), exactly as previously described.[19,21,22] Briefly, peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation on Ficoll (Sigma-Aldrich, St. Louis, MO) then plated to expand PB-MSCs. Adipose tissue (AT) was collected aseptically lateral to the tail head, and then fragmented into smaller pieces with a scalpel, prior to a collagenase digestion[22] The digested AT was then passed through a cell strainer, incubated with NH_4Cl to lyse the red

Horse ID	Breed	Age at PB	Age at AT	Age at BM	Sex
A	Thoroughbred	10	10	9	Female intact
B	Standardbred	1	1	1	Male intact
C	Thoroughbred	13	14	12	Male castrate
D	Thoroughbred	6	7	5	Female intact

Table 5.1: Signalment of horses used for paired sample isolations. Age in years at isolation of each of the sources is listed. PB: peripheral blood; AT: adipose tissue; BM: bone marrow.

blood cells, and then plated in a tissue culture dish for expansions of AT-MSCs. Bone marrow (BM) was collected aseptically from the sternum, then the nucleated cells were isolated using density centrifugation over Ficoll and plated to expand BM-MSCs.[19] Thirteen additional PB-MSC, AD-MSCs, and BM-MSCs were isolated, as well as umbilical cord blood (UCB)-derived MSCs[23] from non-source matched horses. Information on these horses with unpaired samples is listed in Table 5.1s.

All MSCs were cultured Dulbecco's modified Eagle medium (DMEM) (Corning Life Sciences, Acton, MA), supplemented with 20% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA,), 1% penicillin/streptomycin and 2mM L-glutamine (Invitrogen, Grand Island, NY). MSCs used for experimentation were all < passage 5.

Interferon (IFN)-γ stimulation of MSCs

MSCs were thawed and plated onto 6 well dishes 24 h prior to stimulation. MSCs were stimulated with IFN-γ (1 ng/ml or 100 ng/ml; recombinant equine interferon gamma, R&D Systems, Minneapolis, MN) for 48 hours, in duplicate. Unstimulated MSCs served as controls. After 48 h of stimulation, the MSCs were lysed on the plate and the cell lysate was stored at -80^{o}C for subsequent gene expression analyses.

Gene expression analyses

RNA was extracted from MSCs with an on-column DNase step, according to manufacturer's directions (E.Z.N.A.® Total RNA Kit, Omega Bio-Tek, Norcross, GA). Samples were processed for cDNA synthesis using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, California) with 500ng of RNA as template. A SYBR Green (Applied Biosystems, Foster City, CA) based quantitative real time PCR assay was run on an Applied

Horse ID	Breed	Age at PB	Age at AT	Age at BM	Age at UCB	Sex
A	Thoroughbred				10	Female intact
E	Thoroughbred	9		6		Female intact
F	Icelandic	8				Female intact
G	Icelandic	12			12	Female intact
H	Icelandic	8				Female intact
I	Standardbred		1	1		Male castrate
J	Arabian			13		Female intact
K	Paint Cross			14		Male castrate
L	Thoroughbred		21	21		Female intact
M	Warmblood		16			Female intact
N	Warmblood		18			Female intact
O	Holsteiner				11	Female intact
P	Oldenburg				17	Female intact
Q	Hanoverian				11	Female intact

Table 5.1s: Signalment of horses used for unpaired sample isolation. Age in years at isolation of each of the sources is listed. PB: peripheral blood; AT: BM: bone marrow; adipose tissue; UCB: umbilical cord blood. Age at UCB represents age of mare.

Biosystems 7900HT Fast Real Time PCR instrument (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

Expression of MHC class I (MHC-I), MHC class II (MHC-II), indoleamine 2,3-dioxygenase (IDO), prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2, COX-2) and inducible nitric oxide synthase (iNOS) was assessed to evaluate the antigenicity and immunomodulatory properties of MSCs.[9,17,24,25] In addition, the cytokines transforming growth factor-β1 (TGF-β1) and interleukin-6 (IL-6) and the chemokines C-C motif chemokine 2 (CCL2) and C-X-C motif chemokine 10 (CXCL10) were selected for MSC analyses based on their relevance in immunomodulatory functions and their potent induction following inflammatory stimuli exposure in MSCs.[1,9,26] All primers for these genes of interest, as well as the housekeeping gene secretory carrier membrane protein 3 (SCAMP3), have been validated previously and their sequences are listed in Table 5.2.[25,27–30]

Statistics

Matched tissue source MSC gene expression data (ΔCT values) were analyzed using a repeated measures ANOVA with a fixed effects of treatment group, tissue source group, and the interaction between the effects of treatment and tissue, and the MSC horse source as a random effect in the model, followed by a Tukey HSD. Baseline expression data, expression data following stimulation with 1 ng/ml IFN-γ, and the difference in gene expression between baseline and following 1 ng/ml IFN-γ (ΔCT values) were analyzed for all matched and additional samples using an ANOVA followed by a Tukey HSD. 95% confidence intervals were calculated for baseline expression and following 1 ng/ml IFN-γ stimulation (ΔCT values). Data analyzed (ΔCT values) represented the average of biologics replicates. Data were tested for normality

Gene	Abbreviation	Function	Forward 5'-3'	Reverse 5'-3'
Secretory carrier membrane protein 3	SCAMP3	Housekeeping gene	CTGTGCTGGGAATTGTGATG	ATTCTTGCTGGGCCTTCTG
Major histocompatibility complex class I	MHC-I	Self recognition	ACCGTGAGGTCACCCTGA	CTCCGTGTCCTGGGTCA
Major histocompatibility complex class II	MHC-II	Antigen presentation	TCCCTATGCTGGGACTTTTC	CGCCAGGCTTCAGATAGAAC
Indoleamine 2,3-dioxygenase	IDO	Mediator of immunomodulation	TCATGACTACGTGGACCCAAAA	CGCCTTCATAGAGCAGACCTTC
Prostaglandin endoperoxide synthase 2	COX-2	Mediator of immunomodulation	CAGCATAAACTGCGCCTTTTC	AGGCGGGTAGATCATTTCCA
Inducible nitric oxide synthase	iNOs	Mediator of immunomodulation	CCAACAATGGCAACATCAGGT	TGAGCATTCCAGATCCGGA
Interleukin 6	IL-6	Cytokine	TGCTGGCTAAGCTGCATTCA	GGAAATCCTCAAGGCTTCGAA
Transforming growth factor beta 1	TGF-b1	Cytokine	AGAGCTGCGCCTCCTAAGG	ACGACTCCGGTGACATCAAAG
C-C motif chemokine 2	CCL2	Chemokine	GGCTCAGCCAGATGCAATTA	GCTTTCTTGTCCAGCTGCTT
C-X-C motif chemokine 10	CXCL10	Chemokine	GACTCTGAGTGGAACTCAAGGAAT	GTGGCAATGATCTCAACACG

Table 5.2: Overview of primers used for quantitative RT-PCR (qRT-PCR)

using a Shapiro-Wilk test. All analyses were performed by using JMP Pro 11 software (SAS Institute, Cary, NC, USA), and significance was set at $P < 0.05$.

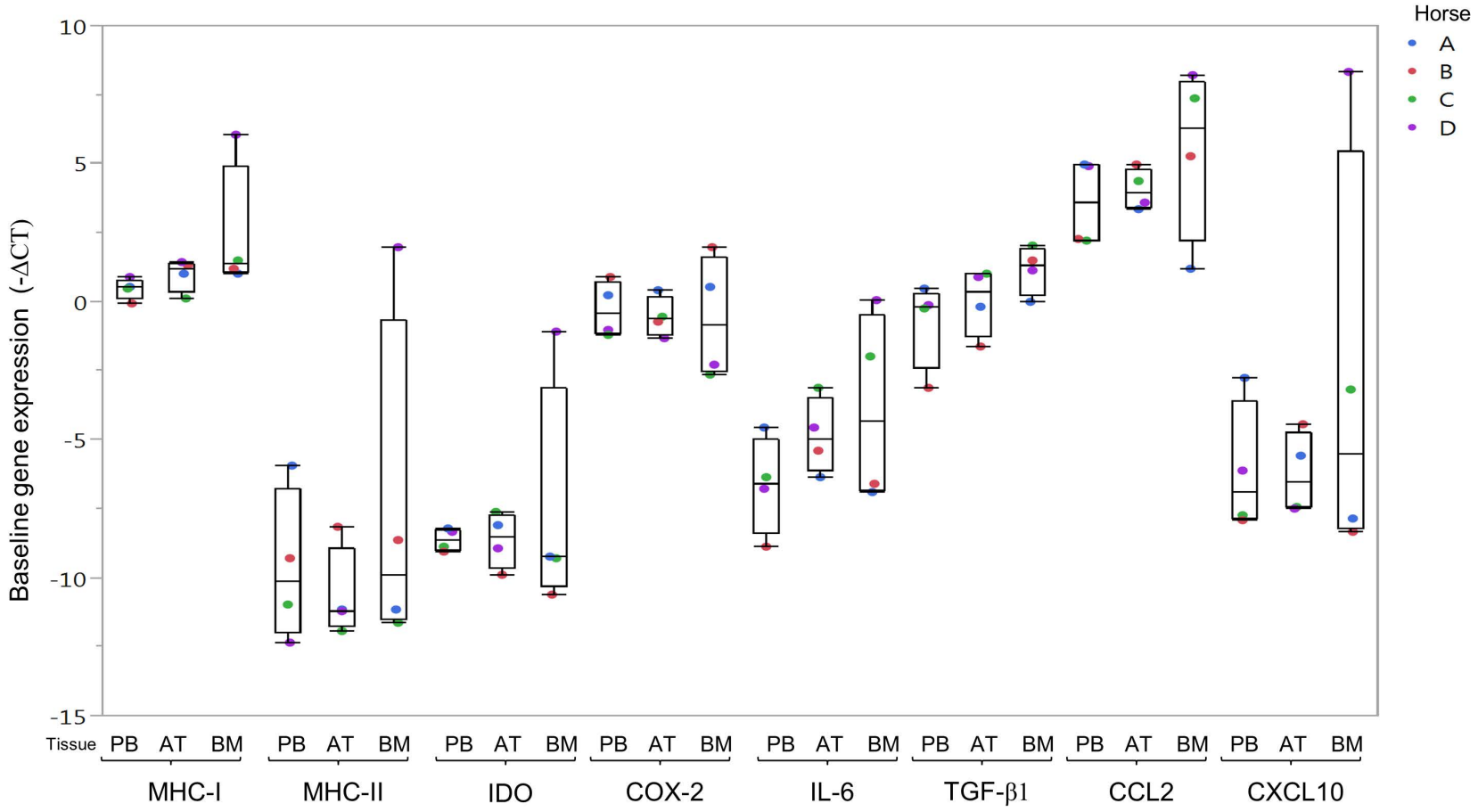
Results

To test the concept that MSCs derived from different sources respond differently to inflammatory stimuli, we utilized both matched MSC samples obtained from different sources from the same animals, as well as supplementary samples to confirm the observed trends and provide insight into umbilical cord derived MSCs.

Baseline immune-related gene expression profiles in equine MSC from different sources

To evaluate the immune-related gene expression profiles in equine MSC, we decided to start with unstimulated equine MSC from matched MSC sources from the peripheral blood (PB-MSCs), adipose tissue (AT-MSCs), and bone marrow (BM-MSCs) of 4 individual horses (Table 5.1). The following immune related genes were analyzed: antigenicity genes MHC-I and MHC-II; immunomodulatory genes IDO and COX-2; cytokines TGF-β1 and IL-6; and the chemokine genes CCL2 and CXCL10. With the exception of expression levels of TGF-β1, the highest variations in baseline expression level profiles were observed in equine BM-MSCs (Figure 5.1s). In contrast, the expression level profiles of PB-MSCs and AT-MSCs showed less variation (Figure 5.1s). More specifically, PB- and AT-MSCs (i) expressed low to absent levels of MHC-II, IDO, IL-6 and CXCL10, (ii) had low expression levels of COX-2 and TGF-β1, and (iii) had low expression levels of MHCI and CCL-2. (Figure 5.1s). As mentioned above, BM-MSCs demonstrated a high level of variation in baseline gene expression, with some samples expressing MHC-II (1/4), COX-2 (2/4), IL-6 (2/4), and CXCL10 (1/4) to a much greater magnitude than others (Figure 5.1s). When comparing expression levels of a specific gene

Figure 5.1s: Peripheral blood derived MSCs tend to have lower baseline expression of immunogencity, immunomodulatory, cytokine, and chemokine genes compared to adipose and bone marrow derived MSCs from the same horse. Variability in individual baseline expression was greatest in bone marrow (BM) derived MSCs compared to peripheral blood (PB) and adipose (AD) derived MSCs. Delta CT values represent baseline expression of genes of interest after controlling for housekeeping gene expression, the inverse is graphed so visually greater numbers correlate with greater baseline expression. Each dot represents the average of two biologic replicates. Box and whisker plots represent n=4.



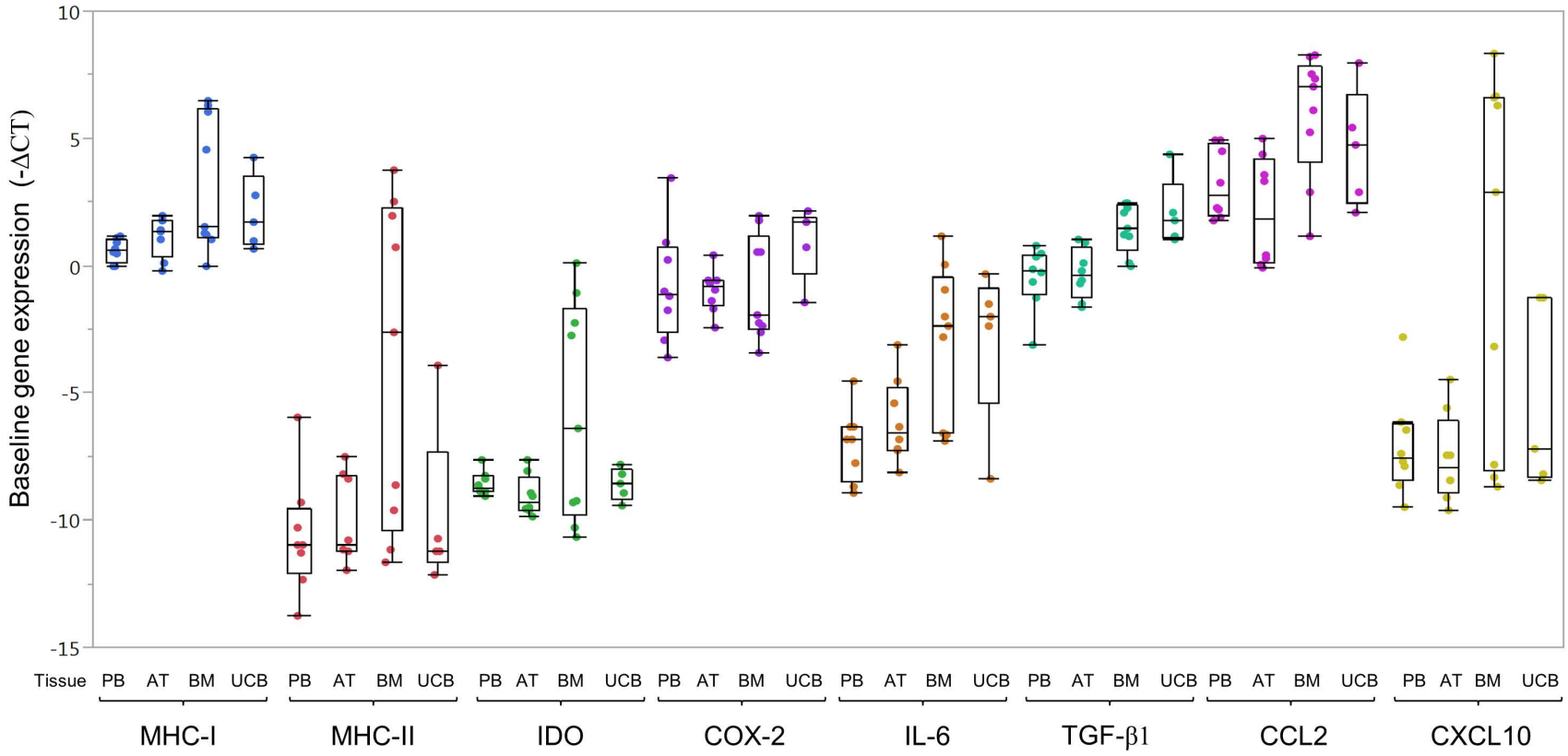
between the different sources, it was noted that PB-MSCs tended to have lower baseline expression levels of MHC-I and IL-6 when compared to MSCs isolated from the paired AT and BM tissue sources (Figure 5.1s). Inducible nitric oxide synthase (iNOS) was also included in the immune-related gene expression profiling, but no expression could be detected in any of the tested MSCs (data not shown). The absence of an signal in the iNOS qRT-PCR was not due to a primer issue since these primers were previously validated[25] and in other experiments in our laboratory the primers were successful at detecting iNOS in equine cells.

To corroborate the findings in gene expression profiles of these matched MSC samples from 4 horses, we performed qRT-PCR analyses on 17 additional unmatched MSC isolations (ie. 4 PB-MSCs, 4 AT-MSCs, 5 BM-MSCs, and 5 UCB-MSCs). With the exception of COX-2, a significant difference in baseline expression of all other genes of interest was found when combining the results from all paired and unpaired MSC isolations (Figure 5.1, Table 5.3). Overall, the results from these baseline immune-related gene expression profiles show that PB-MSCs and AT-MSCs are similar to each other, but different from BM-MSCs, which are more similar to UCB-MSCs (Figure 5.1, Table 5.3).

Immune-related gene expression profiles in equine MSCs from different sources upon interferon gamma (IFN-γ) stimulation

Next, we wanted to evaluate the effects of IFN-γ treatment on the expression levels of these immune-related genes. Initially, we used two IFN-γ concentrations, i.e. 1 and 100 ng/ml, based on what has been reported in previous literature,[19,20,31]and evaluated the effects in the 4 horse-matched equine MSC isolations from PB, AT and BM. Looking at the immune-related gene expression profiles in equine MSCs after treatment and irrespective of the source, 1 ng/ml

Figure 5.1: Baseline expression of immune related genes is variable depending on MSC tissue source. Baseline expression gene profiles of peripheral blood (PB) and adipose tissue (AT) derived MSCs were most similar and different from gene expression profiles of bone marrow (BM) and umbilical cord blood (UCB) derived MSCs. Sample baseline expression variability was greatest BM-MSCs compared to PB-, AT-, and UCB-MSCs. Delta CT values represent baseline expression of genes of interest after controlling for housekeeping gene expression, the inverse is graphed so visually greater numbers correlate with greater baseline expression. Each dot represents the average of two biologic replicates. PB (n=8), AD (n=8), BM (n=9), UCB (n=5)



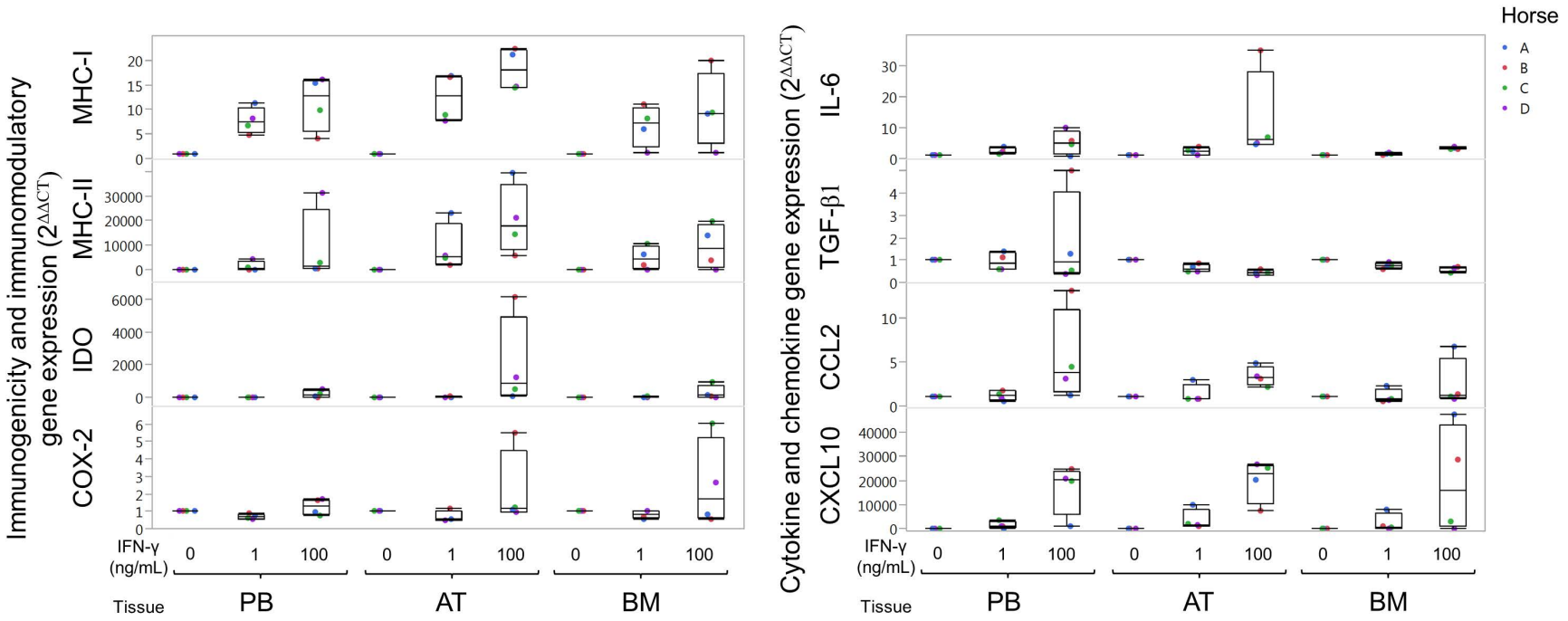
Gene	F statistic	[Delta CT Confidence Interval] Post Hoc			
		PB (n=8)	AT (n=8)	BM (n=9)	UCB (n=5)
MHC-I	0.0192*	[-1.0,-0.2] A	[-1.8,-0.5] AB	[-5.2,-1.1] AB	[-3.9,-0.3] B
MHC-II	0.0060*	[8.7,12.6] A	[8.6,11.5] A	[-1.0,8.7] B	[5.7,14.0] AB
IDO	0.0392*	[8.2,9.0] AB	[8.4,9.7] A	[2.5,9.0] B	[7.8,9.3] AB
COX-2	0.2443	[-1.1,2.6]	[0.3,1.7]	[-0.7,2.4]	[-2.8,0.8]
IL-6	0.0032*	[5.9,8.2] A	[4.7, 7.5] AB	[0.7, 5.3] B	[-1.0,6.8] B
TGF-β1	0.003*	[-0.6,1.5] A	[-0.5,1.1] A	[-2.2,-0.7] B	[-3.7,-0.4] B
CCL2	0.0056*	[-4.4,-2.1] AB	[-3.9,-0.3] A	[-7.9,-4.0] B	[-7.5,-1.7] AB
CXCL10	0.0044*	[5.4,8.8] A	[6.1,9.0] A	[-5.9, 5.3] B	[0.7,9.9] AB

Table 5.3: Baseline expression is variable based on tissue source. There were significant differences in baseline expression in all genes of interest across the different tissue sources at baseline, with the exception of COX-2. The differences between tissue sources baseline expression (ΔCT values) all paired and unpaired samples using an ANOVA followed by a Tukey HSD. Groups that do not share the same letter are statistically different from each other.

IFN-γ was sufficient to significantly upregulate expression of MHC-I, MHC-II, IDO and CXCL10 (Figure 5.2 and Table 5.4). Out of those 4 genes, IDO and CXCL10 showed a significant additional increase in expression when using 100 ng/ml. In contrast, COX-2 and IL-6 were only significantly upregulated at 100ng/ml and TGF-β1 and CCL2 were not significantly upregulated by either IFN-γ concentrations (Figure 5.2, Table 5.4). When controlling for treatment, we found that MHC-I expression was significantly higher in BM-MSCs compared to PB-MSCs (Figure 5.2, Table 5.4). IL-6 expression was significantly higher in AT-MSCs and BM-MSCs compared to PB-MSCs (Figure 5.2, Table 5.4). The third immune-related gene that showed a significant difference in source was TGF-β1, where IFN-γ treatment resulted no significant change in expression and thus similar to baseline expression, expression was significantly higher in BM-MSCs compared to PB-MSCs and AD-MSCs (Figures 5.1s and 5.2, Table 5.3). The tissue differences when controlling for treatment were not significant for the other genes tested, but some were approaching significance, like e.g. MHC-II (P value of 0.0595) and CCL-2 (P value of 0.0669) (Figure 5.2, Table 5.3).

We then expanded on these results by including the 17 additional unmatched MSC isolations and decided to focus on the 1 ng/ml IFN-γ treatment (Figure 5.3, Table 5.5 and 5.6). With the added power of the additional samples, we were able to analyze the significance of the magnitude gene expression response to 1 ng/mL IFN-γ across tissue sources. The change in MHC-I and IDO expression was significantly different between tissue sources, with a greater magnitude of response in AT-MSC compared to BM-MSCs (Table 5.5). There was also a greater magnitude upregulation of CXCL10 in PB-MSC and AT-MSC samples compared to BM-MSC (Table 5.5). The magnitude of response following IFN-γ stimulation was not significantly different depending on tissue source between any of the other genes of interest.

Figure 5.2: All samples from all sources respond to IFN-γ stimulation and upregulated MHC-I and MHC-II expression. Peripheral blood (PB) and adipose (AD) derived MSCs had similar responses to each other, compared to bone marrow (BM) derived MSCs. Variability in gene expression upregulation was greatest following stimulation with 100 ng/ml IFN-γ. IFN-γ stimulation induced great upregulation in IDO expression in AD-MSCs at both 1 1 ng/ml and 100 ng/ml IFN-γ, but 100 ng/ml was necessary to induce a robust response in MSCs from the other tissue sources. 2^ΔΔCT values represent the fold change from baseline expression (0 ng/ml of IFN-γ). Each dot represents the average of two biologic replicates. Box and whisker plots for paired samples represent n=4, for unpaired samples PB n=4, AD n=4, BM n=5, and UCB n=5.



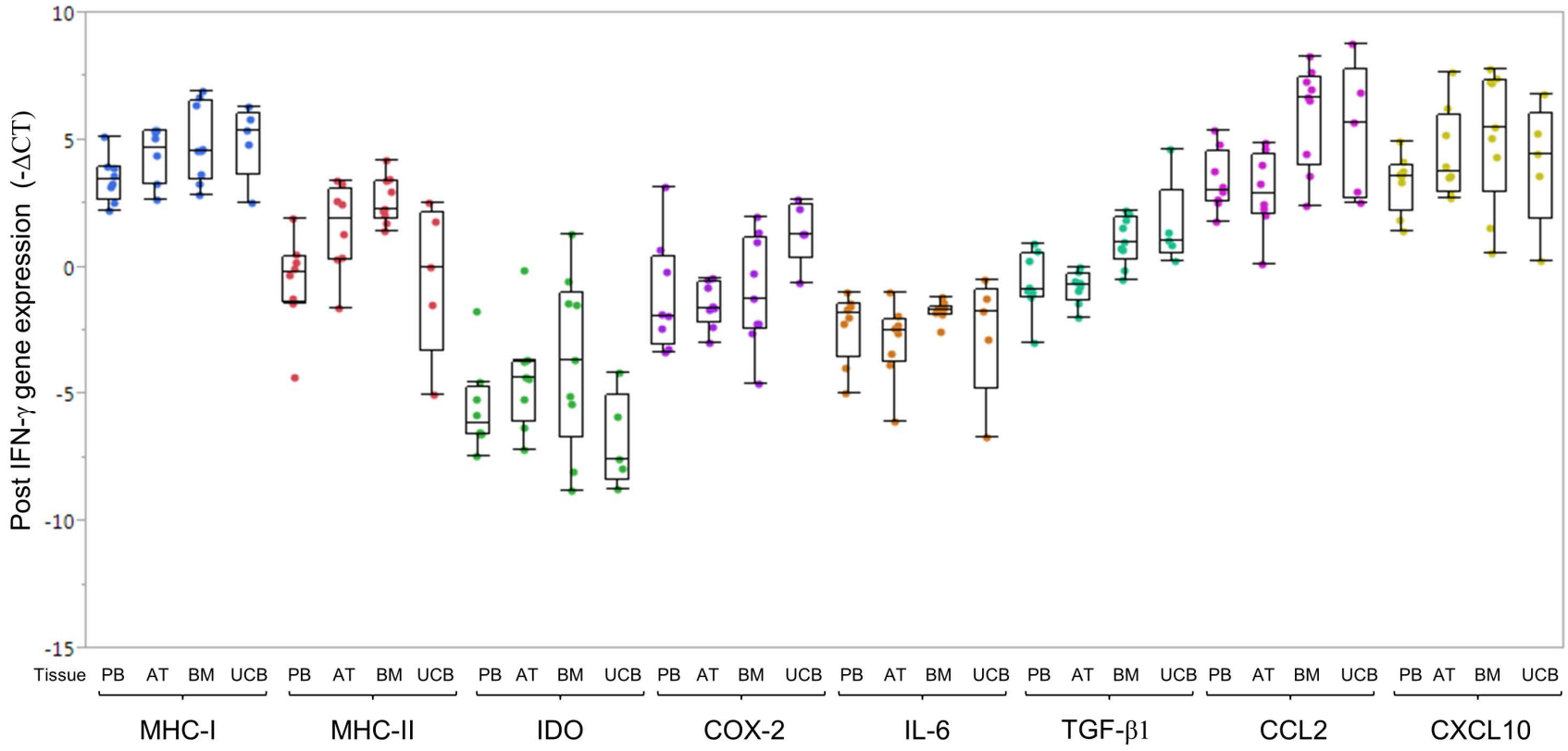
Gene	Treatment				Tissue			
	F statistic	0	1	100	F statistic	PB	AT	BM
MHC-I	<0.0001*	A	B	B	0.0025*	A	AB	B
MHC-II	<0.0001*	A	B	B	0.0595	A	A	A
IDO	<0.0001*	A	B	C	0.1558	A	A	A
COX-2	0.0052*	A	AB	B	0.9107	A	A	A
IL-6	0.0417*	A	AB	B	0.0056*	A	B	B
TGF-β1	0.221	A	A	A	0.0010*	A	A	B
CCL2	0.0502	A	A	A	0.0669	A	A	A
CXCL10	<0.0001*	A	B	C	0.2966	A	A	A

Table 5.4: Immune-related gene expression profiles in matched equine MSCs from different sources upon interferon gamma (IFN-γ) stimulation. Gene expression data (ΔCT values) from MSCs derived from different tissue sources from the same set of individuals following IFN-γ stimulation (ng/mL) was analyzed using a repeated measures ANOVA with a fixed effects of treatment group, tissue source group, and the interaction between the effects of treatment and tissue, and the MSC horse source as a random effect in the model, followed by a Tukey HSD. Treatment groups that do not share the same letter are statistically different from each other when controlling for tissue variability. Tissue groups that do not share the same letter are statistically different from each other when controlling for treatment variability.

Gene	Tissue				
	F statistic	PB	AT	BM	UCB
MHC-I	0.026*	AB	B	A	AB
MHC-II	0.0601	A	A	A	A
IDO	0.0317*	AB	B	A	AB
COX-2	0.1191	A	A	A	A
IL-6	0.4507	A	A	A	A
TGF-β1	0.5106	A	A	A	A
CCL2	0.153	A	A	A	A
CXCL10	0.0012*	B	B	A	AB

Table 5.5: The magnitude of the change in expression between baseline and following 1 ng/ml IFN-γ stimulation varies depending on sample source. Only MHC-I, IDO, and CXCL10 had a significantly different change in gene expression between baseline and following 1 ng/ml IFN-γ (ΔCT values). The differences between baseline (ΔCT values) and expression following stimulation was analyzed for all paired and unpaired samples using an ANOVA followed by a Tukey HSD. Groups that do not share the same letter are statistically different from each other.

Figure 5.3: Decreased tissue source dependent gene expression variability in MSCs following IFN-γ stimulation. Following IFN-γ stimulation gene expression profiles are more similar across all tissue types, with no significant difference due to tissue source in MHC-I, IDO, or CXCL10 expression. Regardless of MSC source MSCs responded to IFN-γ and upregulated MHC-I, MHC-II, IDO, IL-6 and CXCL10. While overall all sources became more similar in their expression profiles, peripheral blood (PB) and adipose tissue (AT) derived MSCs continued to cluster together and bone marrow (BM) and umbilical cord blood (UCB) derived MSCs remained most similar to one another. Delta CT values represent baseline expression of genes of interest after controlling for housekeeping gene expression, the inverse is graphed so visually greater numbers correlate with greater baseline expression. Each dot represents the average of two biologic replicates. PB (n=8), AD (n=8), BM (n=9), UCB (n=5)



Gene	F statistic	[Delta CT Confidence Interval] Post Hoc			
		PB (n=8)	AT (n=8)	BM (n=9)	UCB (n=5)
MHC-I	0.1201	[-4.2,-2.7]	[-5.2,-3.3]	[-6.0,-3.6]	[-6.8,-3.1]
MHC-II	0.0041*	[-0.9,2.1] A	[-3.0,0.0] AB	[-3.3,-1.9] B	[-3.3,4.2] A
IDO	0.132	[4.1,7.1]	[2.6,6.2]	[1.1,6.3]	[4.6,9.2]
COX-2	0.0493*	[-0.7,3.0] AB	[0.8,2.2] A	[-0.6,2.7] AB	[3.0,0.2] B
IL-6	0.0099*	[5.0,7.2] A	[3.4,6.1] AB	[-0.2,4.7] B	[-2.4,6.5] B
TGF-β1	0.0006*	[-0.4,1.7] A	[0.3,1.3] A	[-1.8,-0.3] B	[-3.8,0.5] B
CCL2	0.0067*	[-4.4,-2.3] A	[-4.3,-1.6] A	[-7.5,-4.4] B	[-8.6,-2.0] AB
CXCL10	0.3299	[-4.2,-2.3]	[-5.9,-2.9]	[-7.2,-3.1]	[-7.1,-1.0]

Table 5.6: Expression after 1ng/ml IFN-γ stimulation varies depending on tissue source. Many significant differences in gene expression are maintained following IFN-γ stimulation, but there is no significant difference across tissue sources in expression of MHC-I, IDO, and CXCL10. The expression following 1ng/ml IFN-γ stimulation (ΔCT values) for all paired and unpaired samples was compared between tissue sources using an ANOVA followed by a Tukey HSD. Groups that do not share the same letter are statistically different from each other.

To gain further insight into how the MSCs from different tissue sources expression profiles changed after IFN-γ stimulation, we compared all the samples gene expression levels following treatment (Figure 5.3, Table 5.6). The different magnitude of IFN-γ induced upregulation of MHC-I, IDO, and CXCL10 discussed above (Table 5.5), resulted in equivalent MHC-I, IDO, and CXCL10 expression across all tissue groups after treatment (Figure 5.3, Table 5.6). Thus, the response to IFN-γ abrogated the baseline tissue source gene expression differences. Following IFN-γ stimulation, tissue source differences were present in COX-2 expression, with UCB-MSCs having significantly higher expression than AT-MSCs (Figure 5.3, Table 5.6). The ranking of MHC-II expression across tissue source changed following IFN-γ stimulation. At baseline, BM-MSCs had the highest expression of MHC-II, followed closely by UCB-MSC samples, with PB-MSCs and AT-MSCs having the lowest levels of expression (Figure 5.1, Table 5.3). After stimulation, BM-MSC samples continued to have the highest levels of MHC-II expression, but now AT-MSC samples clustered similarly, while PB-MSC samples remained in the lowest expressing group (Figure 5.3, Table 5.6). UCB-MSC samples were very variable in expression following IFN-γ stimulation (Figure 5.3, Table 5.6). TGF-β1 and CCL2 expression did not change significantly with IFN-γ stimulation (Table 5.4), thus the tissue source expression levels clustered in the same manner as at baseline (Figure 5.1 and 5.3, Table 5.3 and 5.6). IL-6 expression was upregulated equivalently across tissue sources following IFN-γ stimulation (Table 5.5), thus the tissue source expression levels clustered in the same manner as at baseline (Figure 5.1 and 5.3, Table 5.3 and 5.6). Overall, the tissue source dependent expression differences that remained following IFN-γ stimulation (MHC-II, COX-2, IL-6, TGF-β1, CCL2) mirrored the clustering present at baseline. The PB-MSCs and AT-MSCs remained

similar in gene expression profiles to each other, and different from the profiles of BM-MSC and UCB-MSC, which were similar to one another (Figure 5.3).

Discussion

To determine if MSCs derived from different tissue sources respond differently to inflammatory stimuli, we utilized both matched MSC samples obtained from different sources (PB, AT, BM) from the same animals, as well as supplementary additional samples to confirm the observed trends and provide insight into umbilical cord derived MSCs. We found tissue source dependent gene expression differences at baseline, in the magnitude of response to stimulation, and following IFN-γ stimulation. Overall, gene expression profiles of PB-MSCs and AT-MSCs were most similar to each other and different from BM-MSCs and UCB-MSCs, which clustered together.

We hypothesized that adipose derived MSCs would be responsive to IFN-γ stimulation, similar to bone marrow derived MSCs, but that peripheral blood and umbilical cord blood derived MSCs would be more resistant to IFN-γ stimulation. Our hypothesis was based on the knowledge that adipose is an inflammatory tissue, and thus we theorized that MSCs derived from this source may be more responsive to inflammatory stimuli. Both peripheral blood and umbilical blood are exposed to numerous cytokines in circulation, and yet have not been documented to have MHC class II expression at baseline isolation, thus we predicted they would be resistant to IFN-γ stimulation with respect to MHC II upregulation. Contrary to our hypothesis, none of the MSCs tissue sources appeared to confer any “resistance” to IFN-γ stimulation and MSCs from all sources responded in a similar fashion to IFN-γ induced gene

upregulation. All MSCs have the potential to become immunogenic and upregulate MHC-II expression, and this response has not been previously documented in PB-MSCs.

One of the most striking findings of the study was the similarities in the immune related gene expression profiles of PB-MSCs and AD-MSCs compared to BM-MSCs and UCB-MSCs. Based on the MSCs equivalent ability to respond to inflammatory stimuli regardless of tissue source, we suggest that low expression of immunogenicity and inflammation genes at baseline means that the MSCs from that source have not been exposed to inflammatory stimuli in vivo. All animals utilized for sample collection were apparently healthy, thus PB-MSCs isolated would not have encountered any inflammatory stimuli in circulation. Perhaps AD-MSCs are isolated from inflammatory signaling in their tissue niche, resulting in AD-MSCs exemplifying a quiescent immune gene expression profile. Thus, maybe these two tissue sources clustered due to their protection from inflammatory stimuli prior to isolation. BM-MSCs and UCB-MSCs were similar to each other and tended to have a more inflammatory gene expression profile compared to PB-MSCs and AD-MSCs. We could theorize that the opposite holds true for BM-MSCs and UCB-MSCs, where these tissue sources do not protect the MSCs prior to isolation from inflammatory insults. Feasibly, the variable expression of inflammatory genes in BM-MSCs could be indicative of previous inflammatory insults, for the long lifespan of many of the immune progenitors present in the bone marrow could lengthen the duration of the effects of illness on BM-MSC. For instance, a past inflammatory event could have a long term effect on the bone marrow, meaning that while the horse is now healthy, the insult from a few weeks ago still impacted the isolated BM-MSCs resulting in a more activated immune gene expression profile. Similarly, subclinical infections during pregnancy could go undiagnosed and thus impact the UCB-MSCs isolated, or the inflammatory signals present at parturition could also confer a

more activated immune expression profile on the UCB-MSCs. While immunomodulation ability, proliferation rate, self-renewal ability, and differentiation capacity have been compared across tissue sources,[8,9,24] these differences in baseline profiles and following stimulation have not been realized and thus the mechanisms behind them remains unknown.

IFN-γ stimulation diminished many of the tissue dependent immune related gene expression profiles differences present at baseline. Following IFN-γ exposure, MHC-I, IDO, and CXCL10 were all equivalently expressed across tissue sources at a high level, the expression appeared to reach a maximum and plateau. In the matched sample set, some genes responded in a concentration dependent manner to IFN-γ, suggesting that with a higher concentration of IFN-γ, more gene expression differences could be dissolved as expression levels were upregulated to a maximal level. 100 ng/ml IFN-γ is often used to induce MHC-II upregulaton, however this concentration is likely supraphysiologic in relation to the environments MSCs are placed in, and much lower concentrations have been shown to have an effect.[31] While both doses were utilized in the matched MSC tissue sets, and the 1ng/ml concentration was analyzed in the additional samples based on the matched results and to more closely represent the in vivo recipient environment. However, if the goal was to create the most uniformity across tissue sources prior to clinical use, utilizing higher concentrations of IFN-γ would be indicated.

Inflammatory licensing is a multistep process by which MSCs immunomodulatory function is activated, in vitro through inflammatory priming reagents and in vivo through inflammatory recipient environments.[16,32] The most commonly utilized reagent to induce inflammatory licensing of MSCs is IFN-γ at 100 ng/ml.[16,32–34] IDO is a secreted mediator important is MSCs immunomodulatory function, and there was marked upregulation of IDO expression in all MSC sources following treatment with 100 ng/ml IFN-γ, suggesting induction of inflammatory

licensing. Since IFN-γ induced gene expression was similar across all tissue sources, one would expect the resulting immunomodulatory functional changes to manifest similarly across sources, suggesting inflammatory licensing protocols could be utilized across MSCs of different sources. Overall, the similar response indicates information regarding inflammatory licensing obtained utilizing one MSC source it likely applicable to other sources.

TGF-β1 did not increase with IFN-γ stimulation, consistent with previous findings,[24] and as a result, the tissue dependent differences found at baseline remained following IFN-γ stimulation. This highlights that while IFN-γ inducible genes could likely be normalized across tissue sources with sufficient concentrations and time, genes that do not respond to this cytokine will remain differentially expressed across MSC tissue sources. The main significance of IFN-γ induced uniformity in immune related gene expression profiles likely lies in the MSCs immunogenicity and immunomodulatory functions. It is unlikely that other known tissue dependent differences (self-renewal ability, proliferation rate, and differentiation)[8,9] would be affected by IFN-γ stimulation, however the full extent of the changes in cellular processes induced by IFN-γ in MSCs remains unknown.

To determine the in vivo significance of these in vitro findings, various experiments could address this in an animal model. One could place MSCs from different sources in recipient environments high in IFN-γ, to determine if this would also abrogate the gene profile differences in MSCs derived from different sources. A validated equine subdermal transplant model,[35,36] could be utilized to inject allogeneic MSCs from each source type into a separate injection site on the skin. This model recipient environment would provide IFN-γ exposure for the MSCs, for as discussed previously, IFN-γ is secreted by T-cells in response to foreign MHC recognition, and allogeneic MSCs injected in this manner induce a systemic response, indicative of T cell

recognition.[36] Following a period of 2-3 weeks, biopsies of the MSC injection site could be performed to determine the effect of the inflammatory environment on the MSCs gene expression profiles. If male derived MSCs were injected into a female recipient, the MSCs could be easily identified in the tissue sections utilizing fluorescent in situ hybridization with probes against the SRY gene.[37] This would enable identification of all the cells in the biopsy derived from the injected MSC population and facilitate laser capture microscopy of the sections. After isolating the MSC population from the biopsy sections, the gene expression profiles of MSCs from different sources could be analyzed and compared to the same populations prior to injection. The results of this study would be able to determine if as noted in vitro, the inflammatory exposure resulted in abatement of the tissue source dependent gene expression differences present prior to injection.

In conclusion, IFN-γ stimulation abrogates the tissue source dependent baseline differences in expression of many immune related genes present. The similarity in response and in expression following IFN-γ stimulation, suggests that when selecting a source of MSCs for clinical use in an allogeneic or inflammatory recipient environment, the immune related gene profile at baseline should not be a factor and that the cells will likely respond in a similar manner regardless of source. Whether inflammatory stimulation changes other non-immune related functions of MSCs is unknown, but warrants further investigation to determine if any other functions are altered differently dependent on tissue source. In relation to MSCs immunomodulatory properties, these results are promising for they suggest information gained from different sources can be applied across all MSCs and thus reduce the need for repetitive experiments, allowing the focus to be driving further understanding of the MSC immunomodulatory properties as a whole.

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CHAPTER 6

GENERAL DISCUSSION

Application to future research

The studies presented in this thesis have led to many important questions regarding the interactions between MSCs and the recipient environment. Our findings highlight many areas of study that require further investigation in order to develop improved regenerative medicine therapies for both humans and animals.

What determines the magnitude of response to inflammatory licensing?

Priming with inflammatory stimuli in vitro can induce inflammatory licensing, activating of MSCs immunomodulatory function. Inflammatory priming decreases the heterogeneity of MSC immunomodulatory function, both within an individual MSC population[1] and across individuals, as seen in Chapters 3 and 4. The magnitude of response that results is likely a combination of many factors. As discussed in Chapter 3, MSCs that are MHC class II positive at baseline have a greater change in their ability to suppress T cell proliferation than MSCs that are MHC class II negative at baseline following inflammatory licensing. These MHC class II positive MSCs suppressed T cell proliferation to a lesser extent than MSCs negative for MHC class II, but following inflammatory licensing were better able to suppress proliferation. As MHC class II expression is heterogeneous across the MSC population, MHC class II expression at baseline could be an indication that the MSC population is spread across various inflammatory states, with varied immunomodulatory abilities. Thus creating homogeneity in this

heterogeneous MHC class II positive MSC population with inflammatory licensing produces a greater magnitude change in the population's ability to suppress T cell proliferation.

What are the reasons behind the differences in response to inflammatory licensing with poly I:C in Chapter 3 versus Chapter 4?

When assessing the response to inflammatory licensing agents, experimental design differences can cause significant variations in results. The magnitude of response to poly I:C licensing in Chapter 3 is greater than the response noted in Chapter 4. In Chapter 3, the poly I:C primed MSCs suppressed lymphocyte proliferation in a 24 well set up, whereas in Chapter 4, poly I:C licensing was measured in a 6 well transwell assay. While the MSC to lymphocyte ratios in Chapters 3 and 4 were similar, the media volume required in the transwell setup was 4 mLs compared to 1.5 mLs utilized in the 24 well setup. This change in media volume likely affected the concentration of MSC-secreted factors, providing one explanation for why the poly I:C MSCs suppressed T cell proliferation more than untreated MSCs in Chapter 3, but not in Chapter 4. Further, as discussed above, baseline MSC class II expression appears to dictate MSCs magnitude of response to inflammatory licensing. In Chapter 3, there was a higher percentage of MHC class II positive MSCs analyzed (4 out of 8 samples) than in Chapter 4 (1 out of 8 samples). This difference in MSC sample distribution from Chapter 3 to Chapter 4 was not part of the study design, but was a result of MSC availability. Interestingly, there was a relative depletion in MHC class II positive MSC stocks, in part due to their poor viability at greater passage numbers prohibiting further expansion. These differences in Chapter 3 and

Chapter 4's findings highlight the limitations of primary cultures and the importance of fully characterizing the samples utilized in a study.

How can we block MHC expression while effectively inflammatory licensing MSCs?

If inflammatory licensing is essential for fully activating the immunomodulatory potential of MSCs, the increased immunogenicity following licensing needs to be compensated for in allogeneic applications. IFN-γ is the most commonly utilized inflammatory licensing agent,[1–4] and appears superior to poly I:C (Chapter 4). However, as documented in Chapter 5, MSCs from all tissue sources upregulated MHC class I and II expression following exposure to IFN-γ, indicating that IFN-γ inflammatory licensing would result in an increase in immunogenicity regardless of the MSC tissue source. The pathways involved in licensing MSCs and upregulating MHC expression needs to be further elucidated and then separated in order to achieve the goal of limiting the immunogenicity of primed MSCs.

MHC class II upregulation is predominately mediated through the class II transactivator (CIITA), which functions as a molecular switch that controls both constitutive and inducible MHC-II expression.[5,6] Pre-treatment with IL-1β prevents IFN-γ mediated MHC class II upregulation by decreased CIITA expression in human astroglioma cells,[7] and IL-1β treatment has been documented to downregulate expression of MHC class II in equine BM-MSCs (data from Dr. Jacqueline Hill in our laboratory). Could IL-1β be utilized to block MHC class II upregulation in MSCs during in vitro priming to achieve inflammatory licensing without an increase in antigenicity? This could be investigated in vitro by pretreating MSCs with IL-1β prior to IFN-γ licensing and licensing with IFN-γ in the presence of IL-1β, then analyzing the

changes in MHC expression as well as the ability of the MSCs to suppress T cell proliferation. Additionally, as IL-1β is a pro-inflammatory cytokine, its ability to induce inflammatory licensing in MSCs alone is worth evaluating, in light of its favorable MHC expression effects.

TGF-β is another cytokine which decreases MHC class II expression that could be used in conjunction inflammatory licensing with IFN-γ. TGF-β1 decreases IFN-γ induced MHC class II expression in both murine and human MSCs.[8] Recently, it has been found that TGF-β2 significantly downregulates MHC class I and II expression,[9–11] and partially blocks IFN-γ induced upregulation of MHC class I and II in equine BM-MSCs (Personal communication with Dr. Lauren Schnabel). In order to determine whether TGF-β2 decreases IFN-γ induced MHC upregulation without impairing the MSCs ability to be inflammatory licensed by IFN-γ, the immunomodulatory abilities of MSCs primed with IFN-γ in the presence of TGF-β2 could be compared to MSCs primed with IFN-γ in the absence of TGF-β2. The success of inflammatory licensing in the presence of TGF-β2 will depend on what IFN-γ signaling pathways are inhibited.

How can we improve isolation of an MHC-II negative population from BM-MSCs?

If BM-MSCs are desired for clinical use, how can we improve our ability to isolate MHC class II negative MSCs? In our experience, MSCs that are MHC class II positive at baseline tend to have poorer viability in upper passages compared to MSCs that begin and remain MHC class II negative. This may be that the baseline MHC class II expression is indicative of an activated phenotype, thus impairing the MSCs self-renewal function and directing the cells down a pathway of terminal differentiation. In order to expand MSCs to sufficient numbers for clinical use, it is important to maintain MSCs regenerative potential.

As discussed in chapter 4, our laboratory has noted that MSCs with high baseline MHC class II expression appear to have macrophages in the isolation culture. One option for removing the negative influence of macrophages in the initial culture is to add a negative selection sorting step to remove monocytes. Positive selection of MSCs could remove the macrophage population, but this may lead to unintentional activation of the cells, thus negative selection is likely preferred. There are various commercial sorting options available in human and murine MSCs,[12–14] but implementation of this technique in equine MSCs has been limited by commercial antibody availability. Unfortunately, while there is an effective antibody against equine CD14, a cell surface receptor expressed on monocytes,[15] this receptor is also expressed on equine MSCs.[16] CD172a, another monocyte/macrophage surface marker, is commercially available, but this is also shared with equine MSCs.[16,17] Based on the current antibody availability and the similarities in surface marker expression, separating the bone marrow monocytes from the MSCs is quite difficult. In order to search for a differentiating cell surface marker to design an antibody against, the differences in the two cell types' adherence properties could be exploited. In general, macrophages are more adherent than MSCs, so while a short incubation with trypsin is sufficient to detach MSCs, macrophages often require longer incubations and cell scraping. This also appears to be the mechanism by which macrophages are removed with subsequent passages of MSC culture. By performing RNA analysis on the two crudely separated populations, one may be able to gain insight into which cell surface markers are expressed only on macrophages and then develop an antibody for negative selection.

If the macrophages cannot be efficiently removed from the culture, could we block their upregulation of MHC expression through media additives? For example, as described above, IL-1β and TGF-β2 could be utilized for their ability to block the effect of macrophages on MSC

MHC expression by adding either cytokine to the initial culture. In one experiment in which numerous macrophage-like cells were noted in the initial bone marrow isolate, when the bone marrow isolate was cultured in the presence of TGF-β2, the MSCs were negative for baseline MHC class II expression (Personal communication Alix Berglund). The same isolate cultured without cytokine additives had moderate MHC class II expression. To elucidate whether this is blockade is repeatable, MSCs could be cultured in a transwells with inflammatory macrophages, as described in Chapters 3 and 4, in the presence or absence of TGF-β2.

Can we physically protect the inflammatory licensed MSCs from immune detection?

MSCs have both contact dependent and independent effects on leukocytes,[18,19] and MSC secretion of prostaglandin E_2 is one of the main components in their immunomodulation of T cells.[20] In order to circumvent the issues involved in the recipient immune response to allogeneic MSCs, some groups such as the Van de Walle laboratory, are investigating the potential for delivering the MSC secretome by encapsulating MSCs in alginate hydrogels.[21] MSCs encapsulated for 3 weeks remained viable, and the microencapsulation did not impair the release of bioactive factors.[21] The potential for secretome based therapy highlights another important question: does inflammatory licensing improve MSCs contact independent (secretome based) immunomodulatory capabilities? In vitro experiments could investigate inflammatory licensed MSCs ability to suppress T cell proliferation in a by separating the lymphocytes and MSCs utilizing transwells to test their contact independent effects, and compare to untreated MSCs. If inflammatory licensing confers a beneficial effect on the immunomodulatory properties of the MSC secretome, then encapsulating inflammatory licensed MSCs may provide a means to utilize

the beneficial effects of inflammatory licensing, without concerns about the increased immunogenicity conferred by the licensing agents.

Can we utilize a hypersensitivity model in the horse to test MSCs immunomodulatory function in vivo?

While we can further our understanding of MSC biology with in vitro experiments, what remains to be determined is whether of the differences induced with inflammatory licensing or between MSC tissue sources results in a difference in immunomodulatory function in vivo. A delayed hypersensitivity reaction has been utilized in the mouse to assess MSCs immunomodulatory function,[1] and this model was sensitive enough to detect differences between naïve and inflammatory licensed MSCs. To determine if MSCs can suppress an inflammatory response in vivo, a subdermal transplant model previously utilized by our group to measure immunogenicity,[22] could be modified to measure MSCs immunomodulatory function.[23] By injecting an immunostimulant, such as Complete Freund's Adjuvant, with MSCs subdermally, the MSCs could be evaluated for their ability to reduce the local response clinically (local swelling, wheel formation) and biopsies of the site could further evaluate the inflammation severity as well as the type of cells present in the infiltrate.[23] To look specifically at MSCs ability to suppress T cell proliferation in vivo to corroborate the in vitro T cell proliferation assays, concanavalinA (conA) could be injected intradermally with MSCs,[24,25] Using conA versus a more general immunostimulant such as Freund's adjuvant would provide a more precise way to measure MSCs immunomodulation in vivo without inducing multiple cell types of the immune system. If the goal was to measure MSCs effect on monocytes and macrophages in vivo, a granulomatous foreign body reaction could be induced with a surgical mesh.[26] Some

groups hope to use MSCs for their ability to modulate the immune response to surgical mesh,[27] and this technique could be used to evaluate MSCs ability to affect macrophage polarization in vivo. Selecting an immunostimulant to evaluate MSC immunomodulation in vivo should be based on the type of immune cells and immune response of interest. With any of the above described immunostimulants, since the focus would be on the local immune response, different MSC conditions (inflammatory licensing, tissue sources) could be evaluated in the same animal, strengthening the power to detect treatment group differences and reducing the number of animals required.

Identification of MSCs in the biopsy sections could allow for additional knowledge of MSC function to be gained in this experiment. By utilizing MSCs from male animals as MSC donors into female recipients, the MSCs could be easily identified in the tissue sections utilizing fluorescent in situ hybridization with probes against the SRY gene, as described in Chapter 5.[28] Alternatively, the MSCs could be labeled with the fluorophore such as CFSE,[29] or transfected with a plasmid containing GFP[30] to facilitate identification of the MSCs in the biopsy sections. By identifying MSCs in the sections, one could confirm MSC survival and potentially utilize laser capture to isolate the MSCs for gene expression profiling.[31–33]

Through completion of these dissertation studies, I have furthered our knowledge of the interactions between the recipient environment and MSCs, and highlighted the potential of utilizing inflammatory licensing to improve MSC immunomodulatory function. With the skills and knowledge gained through these studies, I am poised to address additional areas of MSC function brought to light by our findings. As we expand our understanding of MSC biology, we progress towards our goal of designing appropriate MSC therapies that account for the impact of the diseased environment of interest.

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