OPTIMIZING MESENCHYMAL STEM CELL THERAPY FOR LIGAMENT REPAIR:

EFFECTS OF DOSAGE AND CELL PRIMING ON INFLAMMATION, CELLULAR RESPONSE AND MECHANICAL PROPERTIES

by

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Dedicated to my wonderful family for their unwavering support and my amazing friends for their constant enthusiasm throughout this process. I am so grateful.

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Abstract

This dissertation is composed of three sections each identifying integral biological events associated with improved ligament healing while using allogeneic MSCs. The first section compares two doses of MSCs to determine key cellular and cytokine expression levels that result in stronger ligaments, the second part builds on these findings by enhancing allogeneic MSC therapy through cell priming, and the third section defines local and systemic cytokine patterns that characterize an improved ligament healing environment from an inflammatory perspective.

In the first section, two different cell doses (low dose $1x10^6$ and high dose $4x10^6$ MSCs) were administered at the time of injury and compared with normal ligament healing at days 5 and 14 post-injury. The lower dose of MSCs resulted in improved ligament mechanics at day 14 and showed an improved cellular profile with fewer pro-inflammatory cells and cytokines compared to the high dose of MSCs. This study outlined cellular responses that correlated with improved ligament functional properties as well as demonstrated the potential of allogeneic MSCs (higher dose) to create a more inflammatory healing environment illustrating the importance of cell dosage.

The second section examined the role of priming cells with a pro-inflammatory cytokine (polyinosinic and polycytidylic acid) prior to administration into an injured ligament. Healing was studied at days 4 and 14 post-injury in ligaments that received unprimed MSCs ($1x10^6$ cells), primed MSCs ($1x10^6$ cells), and controls (no MSCs). Priming cells yielded stronger ligaments compared to the unprimed MSC group at day 14 along with increased type 2 macrophage infiltration and procollagen 1α deposition at day 4 of healing. Both primed and

unprimed MSCs were detected in the healing region 14 days post-application, however, significantly fewer remained in the primed group. There was a pattern of co-localization of MSCs with both endothelial cells and pericytes in the healing region suggesting a strong interaction and role in angiogenesis for both primed and unprimed MSCs.

The third section measured cytokine fluctuations with the goal of identifying critical patterns contributing to the improved outcome from the use of primed MSCs. This analysis examined local and systemic changes in cytokine expression upon primed and unprimed MSC application. Systemic changes were monitored days 1-4 post-injury via serum collection. Local changes were measured at day 4 post-injury within the ligament homogenate. The primed cells resulted in a general dampening of several pro-inflammatory cytokines including GM-CSF, IL-6, TNFα, and IL-12 during the first few days after injury. However, there was also a general dampening of anti-inflammatory cytokine IL-10 in this group. Locally, the primed cells lead to increased anti-inflammatory IL-1Ra within the healing matrix and decreased pro-inflammatory IL-1α. Summarizing the results, priming MSCs appeared to alter both the systemic and local healing environments by reducing inflammation leading to an overall improvement in ligament healing.

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Chapter 1: Background

Ligaments and Ligament Damage

Ligaments are a fibrous tissue that connect bone to bone and provide support to joints throughout the body. They are mostly comprised of collagen which contributes to 75% of their dry weight. Several collagen types are found in ligaments and include type I, III, V, VI, XI, and XIV. Type I is the most abundant and accounts for 85% of the matrix[1]. Elastin and proteoglycans are also found within ligaments, and all components combined contribute to the mechanical properties of ligaments. Injuries to these structures can lead to joint instability, pain, and eventually more chronic conditions such as osteoarthritis.

Musculoskeletal injuries account for 33 million of the injuries in the US annually with 50% involving soft tissue such as ligament and tendon[2]. Ligament injuries affect all age groups and often require an intervention for healing, depending on the location and healing environment. The healing process fills the void, yet the ligament portrays more scar-like extracellular matrix architecture compared to the native tissue. This tissue can become problematic in ligaments because it consists of less organized, smaller collagen fibrils[3–5] that are associated with decreased tissue strength and compromised joint function. Subsequently, the normal healing cascade places the ligament at risk for re-injury. Thus, there is a growing interest in finding therapeutic interventions to minimize scar-like tissue and increase the tissue's regenerative potential.

Normal Ligament Healing

Ligament healing, like wound healing, can be divided into 3 overlapping phases consisting of inflammation, proliferation, and remodeling. During these phases, various cell types infiltrate

the healing ligament at differing time points and locations. Chamberlain et al.[6] examined 8 time points during rat medial collateral ligament (MCL) healing and characterized cellular spatial and temporal patterns during normal healing. They found that neutrophils are key players during the inflammatory phase and infiltrate the injured area 1 day post-injury. Endothelial cells emerge during the transition from inflammation to proliferation and continue to have a presence through the proliferative phase. Circulating monocytes/macrophages peak around day 5 during the proliferative phase to assist in the clean up of cellular debris. The proliferative phase transitions into the remodeling phase around days 14-21, which is when an increase in apoptotic cells can be detected (Fig. 1.1)[6]. Remodeling of the ligament may continue for months to years without fully regaining its original mechanical properties[7–10].

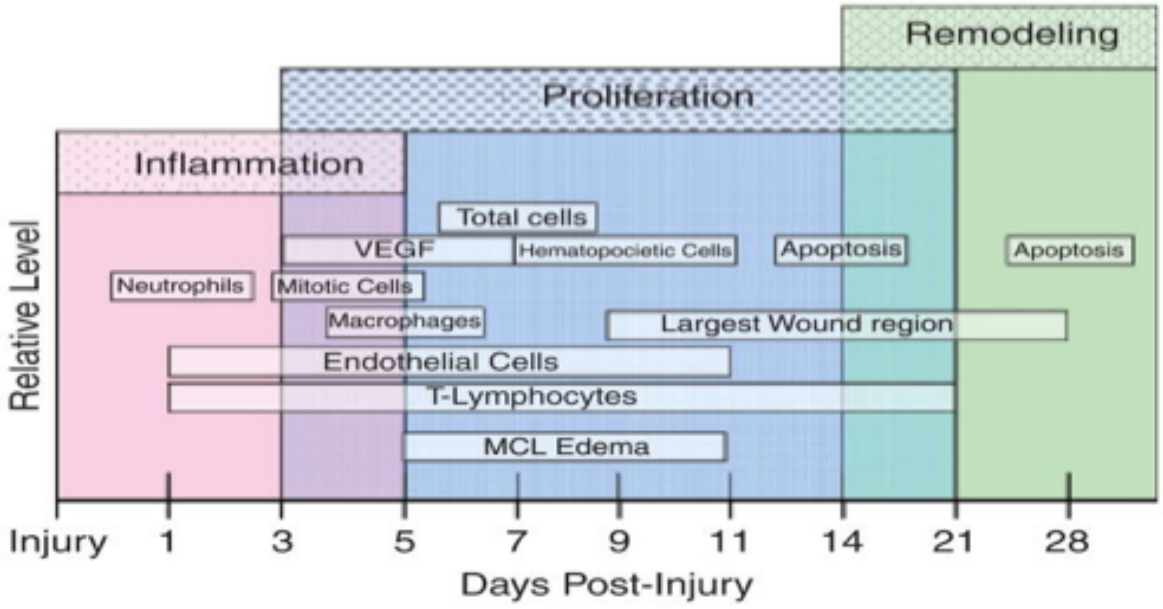


Fig. 1.1 (Reprinted with permission) Temporal cellular response during the 3 phases of rat medial collateral ligament healing.

Possible causes of diminished mechanical properties in healed ligament may be due to smaller collagen fibers, less organized extracellular matrix (ECM), and altered ECM protein composition. Provenzano et al.[11] showed increased bifurcations of collagen fibrils in the healing region along with larger collagen fibrils dividing into smaller diameter fibrils at the transition zone from normal ligament to the injured area. Several other researchers have examined mechanisms of fibrillogenesis in order to determine ways to achieve healed ligaments with similar ECM characteristics to that of uninjured ligaments[5,12–14]. Chamberlain et al.[13] showed that procollagen I and decorin are down-regulated in an injured MCL compared to normal ligaments. They also reported an up-regulation in collagen III, myofibroblasts and fibromodulin through day 28 of healing. This increase in collagen III combined with a decrease in procollagen I (precursor to collagen I) substantially altered the matrix compared to normal ligaments which consist mainly of collagen type I[15].

After a ligament heals, the ECM is more scar-like and fibrotic in nature. There is consistent evidence of involvement of macrophages, other immune cells and fibroblasts in regulating fibrotic conditions[16–18]. Lymphocytes and macrophages can activate myofibroblasts (which participate in collagen synthesis and matrix remodeling) via paracrine signaling and promote fibrotic healing[18]. Other pro-fibrotic mediators involved in the development of scar-like ECM are cytokines produced by immune and inflammatory cell types[18–20] and some of the earliest genes that are up-regulated upon injury (day 3) in a healing rat MCL are related to immune factors and inflammation[14]. This provokes an interest in finding ways to modulate the immune and inflammatory response during early ligament healing with the goal of minimizing scar tissue formation and allowing for more regenerative healing.

Mesenchymal Stem Cells and Healing

Mesenchymal stem cell (MSC) research continues to expand due to its relatively untapped potential as a therapeutic agent. MSCs are generally used for two reasons: 1) the ability for MSCs to differentiate into several different connective tissues such as cartilage, bone, muscle and fat[21–23], and 2) the capacity for MSCs to modulate immune and inflammatory responses that affect various healing environments[24,25]. This paradigm shift from differentiation to immune modulation is studied in different areas of the body[26]. Several reports suggest MSCs decrease inflammation by reducing pro-inflammatory cytokines and changing the macrophage phenotype from type 1 (M1, classically-activated) to type 2 (M2, alternatively-activated)[27–30]. The M1 phenotype is classified as pro-inflammatory, while the M2 phenotype is more reparative and anti-inflammatory[31–33]. A shift in macrophage phenotype is thought to be associated with improved healing (more regeneration of native tissue with less fibrotic scarring).

Other ways in which MSCs regulate immune responses is related to their interaction with T lymphocytes, B lymphocytes, and neutrophils. Numerous studies[24,25,34–36] have shown that MSCs can decrease proliferation and modulate the function of T cells by altering production of cytokines IL-2 and IFNγ. It has also been shown that MSCs increase the number of T regulatory cells (Tregs), which are essential for suppressing effector T cell proliferation and cytokine production[25,37]. There is some evidence that MSCs can inhibit the activation of B cells in vitro[38], and decrease proliferation and IFNγ production by natural killer T cells[39]. Finally, MSCs have been shown to inhibit neutrophil apoptosis in vitro via IL-6 production[40]. Inhibiting apoptosis did not alter neutrophil phagocytic ability and therefore could potentially result in altered healing

by maximizing neutrophil phagocytosis and minimizing reactive oxygen species production (via anti-apoptotic mechanism). The above studies outline ways in which MSCs interact with the major cell types involved during immune and inflammatory responses, however, most of these studies were performed in vitro.

A few researchers have demonstrated improved mechanics in tendons and ligaments using MSCs in vivo[41,42]. Chong et al.[41] found that administering MSCs using fibrin glue in a rabbit Achilles injury model led to improved collagen organization and mechanical properties during early stage healing. Kanaya et al.[42] used a partially torn anterior cruciate ligament model in rats and found that injecting MSCs into the joint improved both histological scores and ultimate failure loads 4 weeks post-injury. These functional improvements show great promise for the way MSCs can modulate the healing cascade. However, questions and problems remain with this approach. MSCs are not uniformly successful which was shown by Gulotta et al. using a rotator cuff tendon to bone healing model[43]. Due to the complex nature of an in vivo environment, less is known about how MSCs mechanistically exert their effects and could vary depending on the disease/injury model being studied. A better understanding of regenerative MSC mechanisms is necessary and could elucidate more effective regeneration strategies.

Priming Mesenchymal Stem Cells

MSCs have multiple functions and require specific cues from their microenvironment to promote a certain action. In order to stimulate immune modulating properties, some kind of 'licensing' is required[44] (Fig. 1.2). One way to activate MSCs is via inflammatory cytokines such as IL1-α/β, IFNγ, and TNFα. Ren et al. found that un-stimulated MSCs lack immunosuppressive

capabilities, however, upon exposure to inflammatory cytokines, they became immunosuppressive[45,46]. Waterman et al.[47] found that short-term priming with lipopolysaccharide (LPS) and polyinosinic and polycytidylic acid (poly(I:C)) polarized MSCs to be pro-inflammatory (MSC1) and anti-inflammatory (MSC2), respectively[46]. LPS and poly(I:C) act on different toll-like receptors (TLRs) that activate danger signals in cells, leading to divergent responses. Due to this being a newer area of research, there are several discrepancies in the literature related to priming and activation of specific TLRs in MSCs.

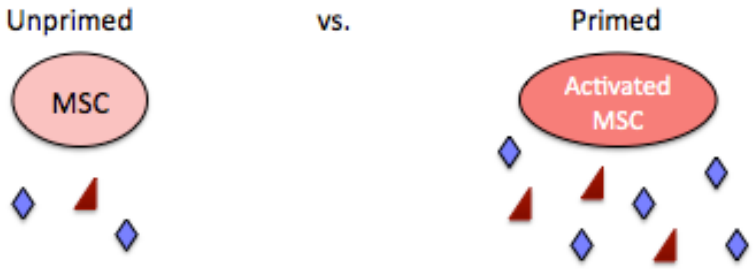


Fig. 1.2 Priming or activating MSCs with a stimulatory molecule increases their cytokine release and can enhance paracrine activities.

More studies are emerging showing the effects of activating TLRs in both mouse and human MSCs. Mastri et al. compared TLR3-activated MSCs (using poly I:C) to unprimed MSCs in cardiomyopathic hamsters[48]. They found that TLR3-activated MSCs stimulated regeneration of the heart and decreased inflammatory cells and cytokines. Contrary to several studies, Liotta et al.[49] showed that TLR3 and TLR4 activation inhibited MSCs ability to decrease T cell proliferation and concluded that activation of these TLRs suppress immune modulatory properties. Disagreement in the literature may be due to different cell types (mouse vs. human), in vivo vs. in vitro models and length of time cells are primed. Continued research is necessary

to clarify the function of primed MSCs and to study potential mechanisms in order to fully elucidate therapeutic potential.

Prostaglandin E2

Many different cell types and cytokines are involved in classifying immune responses as pro-inflammatory or anti-inflammatory. Several researchers have looked for specific cytokines responsible for both primed and unprimed MSCs immune modulating effects. One of the cytokines mentioned frequently in the literature is prostaglandin E2 (PGE2). PGE2 has been shown to promote acute local inflammation, while minimizing harmful, later stages of inflammation[50]. Ways in which PGE2 affects the immune response include suppressing cytolytic effector functions of NK cells[51,52], inhibiting T cell production of IL-2[53], and minimizing the Th1-type response (characterized by inflammation/cytotoxicity) through inhibition of IFNγ[54] and IL-12 production in monocytes[55]. Besides inhibiting pro-inflammatory responses, PGE2 can promote anti-inflammatory actions such as aiding development of Tregs[56], and stimulating production of anti-inflammatory IL-10 in tissue macrophages[57,58].

Researchers have found that inhibiting PGE2 synthesis minimizes MSCs anti-inflammatory effects[59,60]. Ylostalo et al.[59] showed that reducing PGE2 production (by inhibiting COX2, an essential enzyme for PGE2 synthesis), MSCs were unable to reduce TNFα and unable to increase IL-10 production by stimulated macrophages. Adding synthetic PGE2 to the system containing stimulated macrophages reproduced the effects that were lost by blocking PGE2. Ryan et al.[60] cultured peripheral blood mononuclear cells (PBMCs) from mismatched donors, which led to increased proliferation due to an allo-response to foreign antigen. However, when MSCs were

co-cultured with these cells, proliferation significantly decreased suggesting immunosuppression. When PGE2 was reduced (by inhibiting COX activity), T cell proliferation increased again demonstrating a key role for PGE2 in MSCs immune modulating capabilities. Based on these studies and the overall function of PGE2, the evidence suggests that PGE2 is an integral marker to examine during healing when using allogeneic MSCs.

Study Goals

The overall goal of this study is to investigate how ligament healing is enhanced using allogeneic mesenchymal stem cells (MSCs). MSCs are able to modulate the immune and inflammatory response and therefore we are specifically interested in characterizing these changes in early ligament healing. We will compare healing outcomes using different doses of MSCs as well as compare outcomes using unprimed and primed MSCs. We will establish our own priming protocol for rat MSCs using polyinosinic acid and polycytidylic acid (poly(I:C) with the hope of creating a more anti-inflammatory, regenerative scenario. We will measure how this affects healing in regard to cytokine and cellular response, mechanical properties and extracellular matrix organization. Studying how allogeneic MSCs modulate inflammation, promote healing and minimize scar-like tissue will provide essential knowledge necessary for potential future use as a clinical therapeutic treatment. Our interest in the use of allogeneic cells versus autologous cells relates to clinical applications that benefit from therapeutics that are readily available, such as with acute traumatic injuries.

AIM #1 Determine a dose response upon mesenchymal stem cell application to an injured ligament.

MSCs have shown promise when applied to various orthopedic injuries. However, there are inconsistent results in the literature suggesting that further optimization is required to move cell therapy to the next level. Mesenchymal stem cells have many applications including 1) differentiation into a mature cell type to recapitulate the tissue of interest or 2) used in a more medicinal fashion for growth factor and cytokine release. We are interested in their medicinal properties and believe optimizing their use as a pharmacological agent has potential to improve healing. Just like drug testing, there is a need to maximize MSC efficacy by determining a dose response. Since MSCs are responsive to their environment, there is a belief that more cells is better due to the notion that cells will be cued and interact as needed. Therefore the overall goal of Aim 1 is to determine whether a higher dose ($4x10^6$ cells) or a lower dose ($1x10^6$ cells) of MSCs results in improved functional properties and effective healing. *We hypothesize that the higher dose of MSCs will result in improved healing demonstrated by increased failure strength and fewer inflammatory cells (macrophages) compared to the lower dose of cells.*

AIM #2 Examine how altering the early inflammatory cascade using primed MSCs affects functional ligament properties.

Successful therapeutics for ligament healing will ultimately lead to an increase in regenerative healing instead of scar tissue formation. Two metrics for regenerative healing in ligaments are improved mechanical properties and extracellular matrix organization. These properties contribute to re-injury prevention and better clinical outcomes. Therefore the overall goal of Aim 2 is to improve the later stage functional properties of healing ligaments after altering the early inflammatory phase. Since we intend to affect multiple cytokines using primed and unprimed MSCs, we will investigate how these changes affect ligament strength, stiffness, and

ECM organization at later stages. We also will quantify the cellular response and MSC localization at an early (day 4) and later time point (day14). *We hypothesize that poly(I:C)-primed MSCs will result in a less inflammatory environment leading to improved mechanical properties and ECM organization at later stages of healing.*

Aim #3 Probe for factors that improve healing by characterizing the inflammatory cascade via cytokine expression in early phases of ligament healing.

Previous research has shown improved ligament/tendon healing using MSCs, but the mechanism is unknown[41,42]. Therefore the first goal of Aim 3 is to better understand how MSCs modulate inflammation during the early phases of ligament healing by measuring cytokine expression. We will compare healing environments of poly(I:C)-primed MSCs, unprimed MSCs, and control (no MSCs) ligaments by measuring cytokine levels during healing. We believe the balance of pro-inflammatory and anti-inflammatory cytokines are predictive of regenerative healing. *For the first goal we hypothesize that ligaments receiving poly(I:C)-primed MSCs will have the lowest amount of pro-inflammatory cytokines, such as IL1 and TNFα, followed by unprimed MSCs. We also hypothesize that ligaments receiving poly(I:C)-primed MSCs will produce greater levels of anti-inflammatory cytokines, such as IL-1Ra, IL-10, and IL-4.* The second goal of Aim 3 is to gain insight into whether MSCs applied locally to an injured ligament has systemic effects. *We hypothesize that primed and unprimed MSCs will have systemic effects demonstrated by changes in cytokine levels in the blood serum.*

Chapter 2: Mesenchymal Stem Cell Dosage Effects on Ligament Healing

Introduction

Ligament injuries are common musculoskeletal injuries that affect all age groups and often require an intervention for healing, depending on the location and healing environment. The healing process fills the void, yet the ligament portrays more scar-like extracellular matrix architecture and biology from the native tissue. This tissue can become problematic in ligaments because it consists of less organized, smaller collagen fibrils[3–5] that are associated with decreased tissue strength and compromised joint function. Subsequently, the normal healing cascade places the ligament at risk for re-injury. Thus, there is a growing interest in finding therapeutic interventions to minimize scar-like tissue and increase the tissue's regenerative potential.

Mesenchymal stem cell (MSC) research continues to expand due to its relatively untapped potential as a therapeutic agent. MSCs are generally used for two reasons: 1) the ability for MSCs to differentiate into several different connective tissues such as cartilage, bone, muscle and fat[21–23], and 2) the capacity for MSCs to modulate immune and inflammatory responses that affect various healing environments[24,25]. This paradigm shift from differentiation to immune modulation is studied in different areas of the body[26]. Several reports suggest MSCs decrease inflammation by reducing pro-inflammatory cytokines and changing the macrophage phenotype from type 1 (M1, classically-activated) to type 2 (M2, alternatively-activated)[27–29]. The M1 phenotype is classified as pro-inflammatory, while the M2 phenotype is more reparative and anti-inflammatory[31,32,42]. A shift in macrophage phenotype is thought to be associated with improved healing (more regeneration of native tissue with less fibrotic scarring). Many of these

studies have been performed in vitro but there is a lack of inquiry regarding the modulatory effects occurring within injured ligament. MSCs can behave differently depending on the tissue and healing environment they are exposed to, which leads to our specific interest in MSC's immune modulatory effects in healing ligaments.

Several researchers have demonstrated improved mechanics in tendons and ligaments using MSCs[41,42,61]. Kanaya et al.[42] found that MSC injections into a rat knee joint improved both histological scores and ultimate failure loads 4 weeks post- anterior cruciate ligament partial tear. Chong et al.[41] reported that administration of MSCs via fibrin glue in a rabbit Achilles injury model led to improved collagen organization and mechanical properties during early stage healing. These functional improvements show great promise for the way MSCs can modulate the healing cascade. However, not all studies utilizing MSCs have been successful[43]. A better understanding of MSC regenerative mechanisms is necessary and could elucidate more effective regeneration strategies.

In the current study, we examined the specific influence MSCs had on an injured ligament by measuring spatial and temporal cellular responses. Second, we explored if there was a dose response, ultimately determining an optimal healing effect depending on the amount of MSCs administered. Finally we examined production of common pro-inflammatory and anti-inflammatory cytokines in relation to treatment groups and normal healing ligaments. *We hypothesized that both doses of MSCs would result in a less inflammatory environment leading to improved mechanical properties, with the higher dose of MSCs yielding more optimal results.*

Materials and Methods

Experimental Design

A medial collateral ligament (MCL) injury model was used since the normal healing cascade in this model is well characterized[6] and provides an appropriate comparison when perturbing the healing cascade. MCLs were transected and MSCs were injected at the time of injury in the transected region without the use of a scaffold. Healing was analyzed at day 5 to examine a time point when macrophages peak[6], and day 14 to adequately assess mechanical properties. Two doses of MSCs were used: a low dose consisting of $1x10^6$ cells and a high dose of $4x10^6$ cells. Doses were selected based on animal model size and highest number of cells soluble in 50µl of fluid without becoming too viscous. Forty-seven adult male Wistar rats (275-299g) underwent bilateral MCL transections (right MCL=treatment, left MCL=control) with 15 rats (n=3 each dose and time point, 3 extras) used for immunohistochemistry, immunofluorescence, and hematoxylin and eosin (H&E) staining, 20 rats (n=5 each dose and time point) used for cytokine analysis, and 12 rats (n=6 each dose, day 14 only) used for mechanical testing.

Surgical Procedure

All procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee. Rats were anesthetized using isoflurane and prepared for surgery using sterile technique (day 0). A 1 cm longitudinal skin incision was made at the femoral-tibial junction. The subcutaneous tissue and gracilis muscle were dissected in order to expose the MCL. A scalpel blade was used to create a complete, uniform transection of the MCL just distal to the joint line. MCLs were transected instead of torn in order to improve reproducibility. Each rat underwent bilateral MCL transections with the right MCL being administered MSCs and the left

serving as a control by receiving Hanks Balanced Saline Solution (HBSS; Hyclone Laboratories Inc, Logan UT). Transected ligaments were not repaired with suture. Once cells or HBSS were injected, the 3 tissue layers were closed using a 5-0 vicryl suture. The animals were allowed unrestricted cage mobility post-operatively and were euthanized at days 5 and 14 for ligament analysis.

Mesenchymal Stem Cell Culture

Rat mesenchymal stem cells were purchased (Trevigen, Gaithersburg, MD) and expanded to passages 7 through 10. Cells were seeded in T175 flasks and administered Cultrex Qualified RMSC medium containing 10% fetal bovine serum (Trevigen, Gaithersburg, MD) and 1% antibiotic-antimycotic (Cellgro, Manassas, VA). Cells were incubated at 37° C and 5% CO_2 and passaged at 70% confluency. Media was changed every 3-4 days and cell morphology was monitored throughout expansion to ensure MSCs maintained a spindle-like appearance.

MSCs were collected the morning of surgery using Trypsin EDTA (Cellgro, Manassas, VA). Cells were fluorescently-labeled with Celltracker CM-DiI (Life Technologies, Grand Island, NY) in order to visualize their spatial distribution in the healing ligaments. Once fluorescently tagged, either $1x10^6$ or $4x10^6$ MSCs were suspended in 50µl of HBSS.

Immunohistochemistry/Immunofluorescence

At day 5 and day 14 post-injury, ligaments were collected, frozen in optimal cutting temperature (OCT), and longitudinally sectioned (5µm). Sections were mounted on Colorfrost Plus microscope slides and stored at -80° C. Mouse and rabbit monoclonal antibodies were used to

detect the cell type of interest. Standard procedure for staining consisted of acetone fixation followed by 3% hydrogen peroxide to prevent endogenous peroxidase activity. The samples were then treated with Background Buster (Innovex Biosciences, Richmond, CA) to protect against non-specific antibody-protein interactions. Primary antibody was applied (2 hours) followed by a biotin-linked secondary (10 minutes) and streptavidin conjugated to horseradish peroxidase tertiary antibody (10 minutes) using a Stat Q staining kit (Innovex Biosciences, Richmond, CA). Diaminobenzidine (DAB) was used to detect the antibody-antigen complex of interest. Light microscopy allowed for spatial localization and cell counting of all IHC stains.

Mouse monoclonal antibodies were utilized to detect type 1 macrophages (CD68; 1:100; AbDSertoc, Raleigh, NC), type 2 macrophages (CD163; 1:100; AbDSertoc, Raleigh, NC), endothelial cells (CD31; 1:100; AbDSertoc, Raleigh, NC), proliferating cells (Ki-67; 1:25; Dako, Carpinteria, CA), procollagen I (straight; SP1.D8; Developmental Hybridoma, Iowa City, Iowa) and collagen III (1:8000; Sigma-Aldrich, St. Louis, MO). A rabbit monoclonal antibody was used to detect T cells (CD3; 1:100, Abcam, Cambridge, MA). 4',6-diamidino-2-phenylindole (DAPI) and Celltracker CM-DiI (Life Technologies, Grand Island, NY) were used for fluorescent detection of total cells and MSCs, respectively.

Histology

Hematoxylin and Eosin (H&E) staining allowed healing region size measurements and morphological observation of the ligament. The healing region area was measured using Image J (National Institutes of Health, Bethesda, MD) and expressed as a percentage of total ligament area within a sagittal section.

Cell Quantification

Images were taken using a camera-assisted microscope (Nikon Eclipse microscope, model E6000 with Olympus camera, model DP79). Five areas of the healing ligament were imaged at 400x in order to measure spatial distribution of the various cell types. The areas included the healing region, distal healing region edge, proximal healing region edge, distal ligament and proximal ligament. All 5 areas were combined for analysis of the entire MCL providing a 6th measure for comparison (Fig. 2.1). Two or three sections from each ligament were counted and averaged for comparison. Cells were manually counted for each stain. Density of collagen III was measured using Image J (National Institutes of Health, Bethesda, MD).

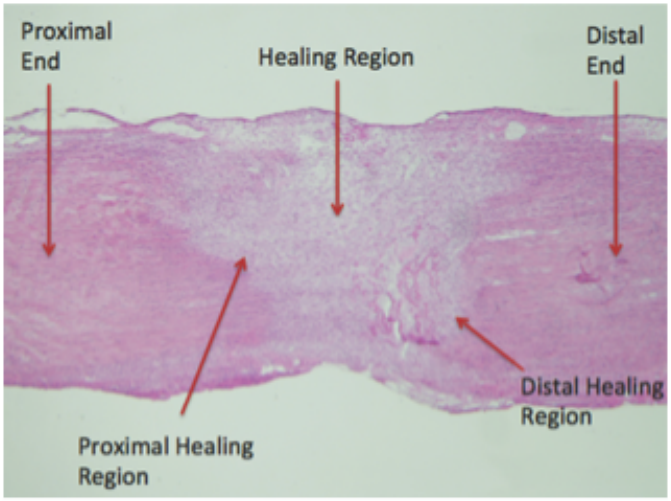


Fig. 2.1 Cells were quantified in five areas of the ligament including the healing region, proximal healing region, distal healing region, proximal end, and distal end. All five areas were added together to obtain an overall MCL sum.

Mechanical Testing

At day 14, rats were euthanized and frozen (-80°C) until dissections could take place. Right and left MCLs were carefully dissected by removing all surrounding tissue. The MCL tibial and

femoral insertions were kept intact for mechanical testing. Once dissected, phosphate buffered saline (PBS) was applied to maintain ligament hydration.

Testing was performed in a custom-designed load frame that held the tibia and femur in the anatomical position for uniform loading. MCLs started in a slack position (without tension) and were not preconditioned to avoid damaging the healing region prior to failure testing. Pulling at a rate of 4.0 mm/sec, each ligament was stretched until it failed. Load and displacement data were collected to determine maximum load before failure, along with stiffness in the most linear region of the load-displacement curve.

Cytokine Analysis

Ligaments were collected at day 5 and day 14 post-operatively to assess the influence of MSC dosage on cytokine expression. Five MCLs were collected and pooled for each time point and group. MCLs were washed in Cell Wash Buffer (Bio-Rad, Hercules, CA) placed in Navy Bead Lysis Kit tubes (Next Advance, Averill Park, NY) containing Lysing Solution (Bio-Rad, Hercules, CA). A Bullet Blender (Next Advance, Averill Park, NY) was used to homogenize the MCLs and separate soluble from insoluble proteins. Supernatant was collected and frozen for total protein measurement (Pierce BCA Protein Assay, Rockford, IL) and cytokine multiplex analysis.

A rat cytokine 10-plex kit (Life Technologies, Grand Island, NY) was used to measure 10 pro-inflammatory and anti-inflammatory proteins. The proteins measured included GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 and TNFα. Samples were run in triplicate and

incubated with primary antibodies overnight at 4° C on a plate shaker. The following day samples were treated with a biotinylated secondary antibody and streptavidin-RPE tertiary antibody for detection. Serial dilutions of standards, along with spleen (positive control) and lysis solution (negative control), were used for accurate and repeatable measurements. Samples were read using a Luminex Magpix (Life Technologies, Grand Island, NY) system. A standard curve was established and verified to ensure 80-120% recovery and detection a minimum of 2 standard deviations above background. Protein concentrations were normalized to total protein measurements and expressed as a percentage for analysis.

Statistics

A 2-tailed, paired student t-test was used to detect differences between MSC treated ligaments compared to controls since the comparison is based on ligaments within the same animal. A 2-tailed, unpaired student t-test was used to make comparisons between high and low dose MSC groups since the doses were administered in different animals. A p-value less than .05 was considered significant.

Results

Immunohistochemistry

Comparisons were made between each MSC dose and animal matched controls along with comparisons between the 2 doses (Table 2.1). No significant changes in cellular distribution between HBSS control ligaments and low dose MSC ($1x10^6$) ligaments were noted at day 5 post-injury. However, treatment with the high dose ($4x10^6$) of cells demonstrated significant changes throughout the ligament in type 2 macrophages (M2s). Fewer M2s ($p=.049$) were present in the

distal and proximal healing region of the high dose MSC group (Fig. 2.2, A-C) compared to control ligaments. Comparing different doses, procollagen I (precursor to collagen I) was decreased (p=.042) throughout MCLs that received the low dose of MSCs compared to the high dose group at day 5 (Fig. 2.2, D-F). Endothelial cell quantification showed that the low dose MSC group had more endothelial cells (p=.021) and lumen (p=.008) starting to form in the healing region compared to the high dose group (Fig. 2.2, G-I).

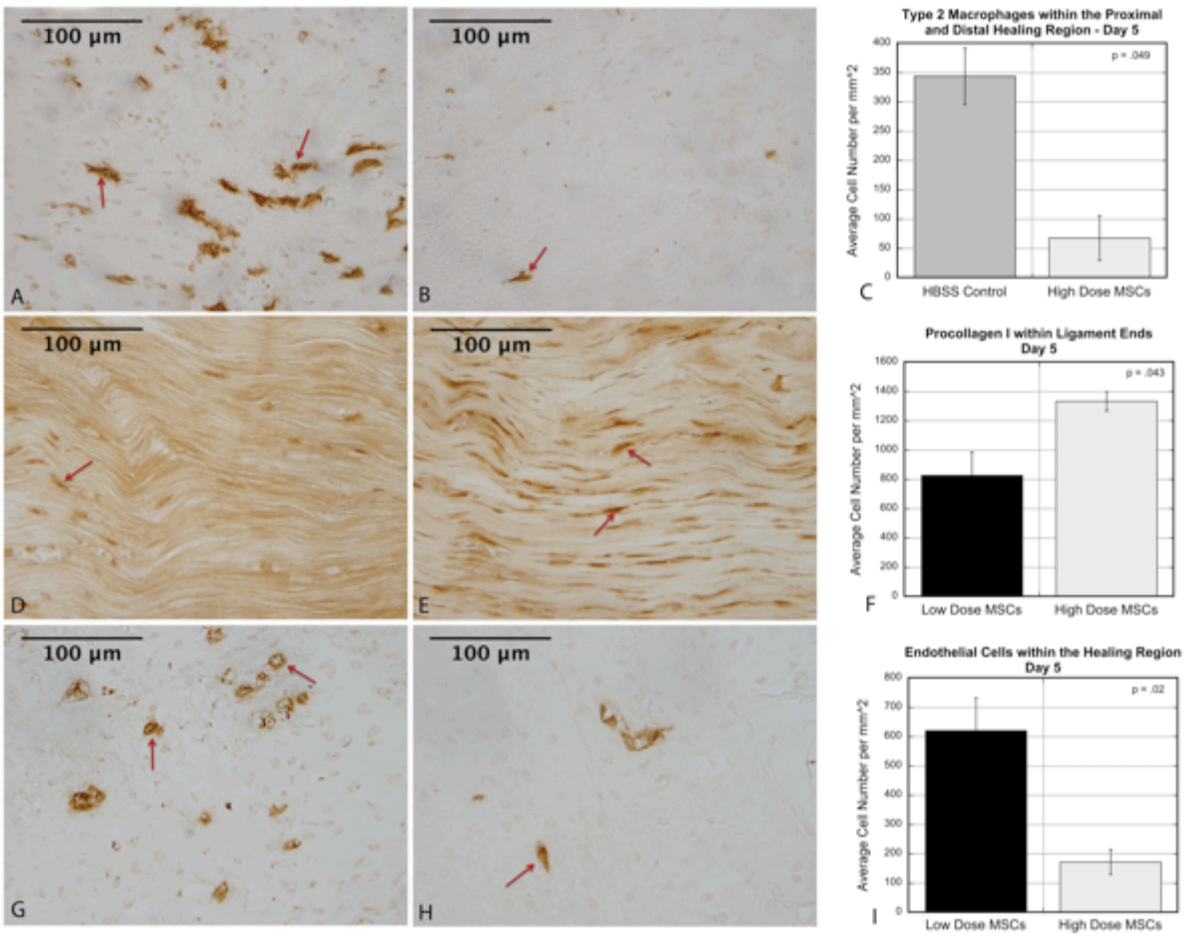


Fig. 2.2 A-C: At day 5 healing, there were decreased type 2 macrophages in the proximal and distal healing region of MCLs that received a high dose of MSCs compared to control ligaments (controls 344.7±48.2, high dose 67.7±38.0, p=.049). A: Representative image of IHC in control ligament. B: Representative image of IHC in high dose ligament. C: Graph comparing average cell number for each condition. D-F: At day 5 healing, there was decreased procollagen I in the ligament ends of the low dose MSC group compared to the high dose group (low dose 827.6±158.6, high dose 1333.3±66.8, p=.043). D: Representative image of IHC in low dose ligament. E: Representative image of IHC in high dose ligament. F: Graph comparing average cell number for each dose. G-I: At day 5 healing there were increased endothelial cells in the healing region of the low dose group compared to the high dose group (low dose 620.7±111.1, high dose 172.4±42.7, p=.02). G: Representative image of IHC in low dose ligament. H: Representative image of IHC in high dose ligament. I: Graph comparing average cell number for each dose. Values are expressed as mean cell numbers ± S.E.M.

In contrast to day 5 healing, there were significant changes in both the low dose and high dose groups at day 14 compared to controls, along with significant changes between doses. Fewer type 1 macrophages (M1s) were found in the ends (p=.010) and throughout the MCL (p=.043) in the low dose ligaments compared to controls. Comparing doses showed a similar pattern with fewer M1s in low dose ligament ends (p=.002) and throughout the MCL (p=.005) compared to the high dose group (Fig. 2.3, A-C). Similar to day 5, M2s were decreased in the proximal and distal ends (p=.049) in high dose ligaments compared to controls (Fig. 2.3, D-F). Cellular proliferation was altered in the low dose MSC group marked by fewer proliferating cells in the healing region (p=.003) compared to the controls (Fig. 2.3, G-I).

When comparing endothelial cells and lumen formation, there were fewer endothelial cells (p=.026) and blood vessel lumen (p=.044) in the ligament ends of the low dose MSC group compared to control ligaments. Significantly fewer lumen were observed throughout the low dose MCLs (p=.002) as a whole compared to controls. When comparing doses of MSCs at day 14, there were less endothelial cells in the distal and proximal healing region (p=.026) and throughout the MCL (p=.039) in the low dose group compared to the high dose group (Fig. 2.3, J-L).

T-lymphocytes were analyzed at both time points due to the extensive research showing that MSCs modulate T lymphocyte proliferation and function[24,25,34–36]. Regardless of treatment or time, there were few T cells found within the ligaments (data not shown), and therefore it was difficult to make meaningful comparisons based on such small numbers. Collagen III was analyzed at day 5 and yielded no difference between groups (data not shown).

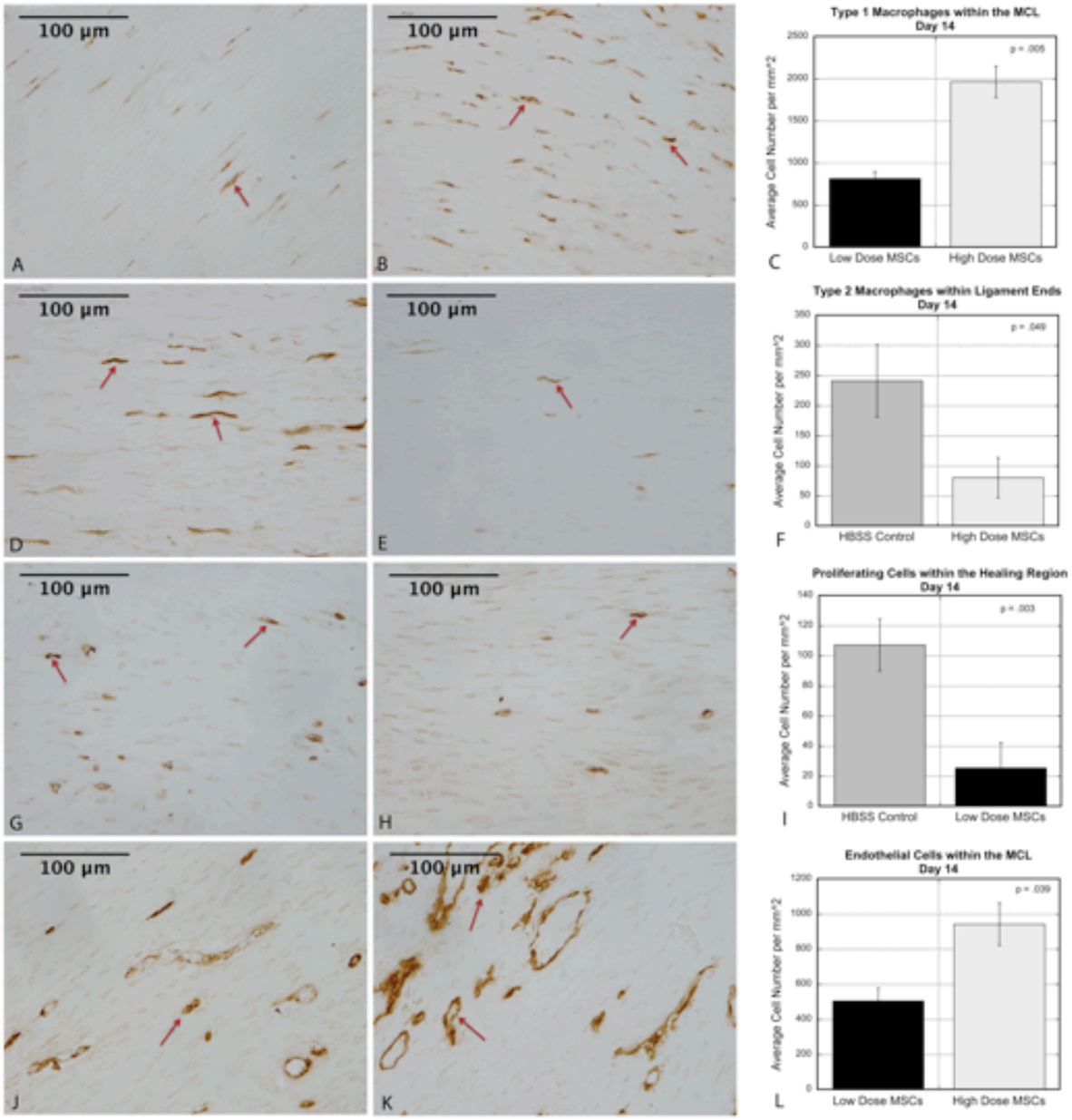


Fig. 2.3 A-C: At day 14 healing, there were fewer type 1 macrophages throughout the MCL in the low dose group compared to the high dose group (low dose 816.1±83.0, high dose 1965.5±186.0, p=.005). A: Representative image of IHC in low dose ligament. B: Representative image of IHC in high dose ligament. C: Graph comparing average cell number for each dose. D-F: At day 14 healing, there were fewer type 2 macrophages in the ligament ends of high dose ligaments compared to controls (controls 241.4±60.2, high dose 80.5±33.4, p=.049). D: Representative image of IHC in control ligament. E: Representative image of IHC in high dose ligament. F: Graph comparing average cell number for each condition. G-I: At day 14 healing, there were fewer proliferating cells in the healing region of low dose MCLs compared to controls (controls 107.2±17.3, low dose 25.5±16.9, p=.003). G: Representative image of IHC in control ligament. H: Representative image of IHC in low dose ligament. I: Graph comparing average cell number for each condition. J-L: At day 14 healing, there was decreased endothelialization throughout the MCL in the low dose group compared to the high dose group (low dose 505.7±73.4, high dose 942.5±121.0, p=.039). J: Representative image of IHC in low dose ligament. K: Representative image of IHC in high dose ligament. L: Graph comparing average cell number for each dose. Values are expressed as mean cell numbers ± S.E.M.

MSC Localization and Morphological Measurements

MSCs were detected in the healing region and healing region edges at days 5 and 14 in both dose groups using fluorescence microscopy (Fig. 2.4, A).

At day 14, a noticeable decrease was noted in the length of the healing region in the low dose MSC group compared to the controls. The average length of the healing region in the MSC group was 1.4 ± .61mm compared to 2.5 ± .18mm ($p=.003$) in the control group. There was also narrowing in the healing region of control ligaments whereas the low dose MSC group's granulation tissue looked congruent with the distal and proximal ligament ends (Fig. 2.4, B).

H&E staining was performed to measure the area of the healing region for comparison. By calculating percentage of the healing region size, the low dose MSC group demonstrated a significantly smaller healing region ($p=.049$) when compared to the controls (Fig. 2.4, C). This MSC group's average healing region size was 8.4±1.2% of the total area of the ligament and the control averaged 10.4± .72%.

Mechanical Data

Ligament failure loads and stiffness were compared at day 14 of healing to assess functional improvements. Larger differences were seen in the low dose group compared to the control when analyzing these two properties. The low dose MSC group demonstrated increased strength with an average failure load of 26.41± 1.95N compared to 20.88± 2.64N in the control group ($p=.029$). Ligaments receiving the low dose of MSCs also showed increased stiffness with an

average of 12.24± .95 N/mm compared to 10.01± 1.02 N/mm (p=.011) in the control ligaments (Fig. 2.5).

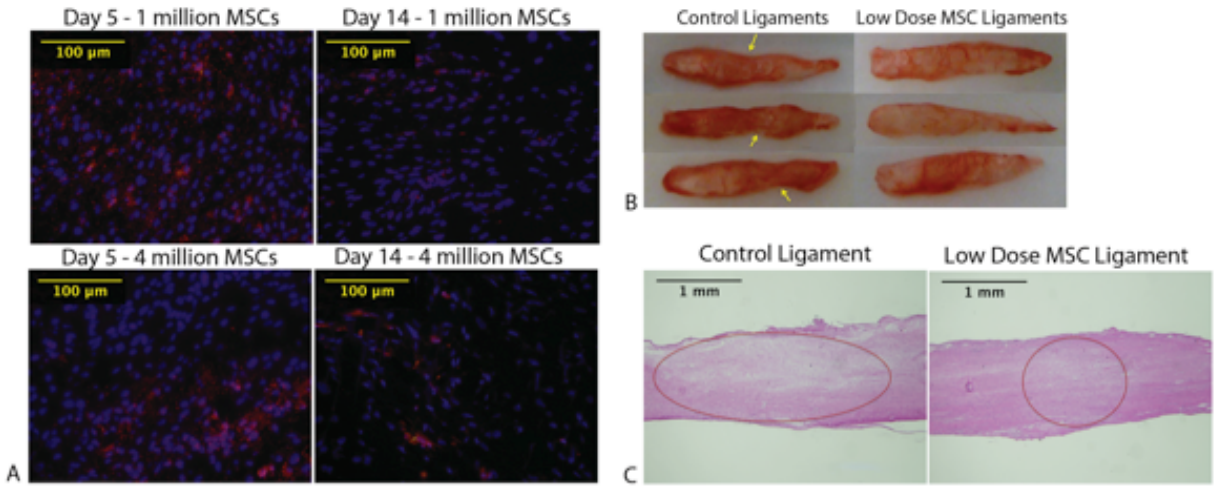


Fig. 2.4 A: MSCs were detected at day 5 and day 14 in the healing region in both dose groups. MSCs were stained red using Celltracker CM-DiI. Dapi was used as a nuclear stain for total cells (blue). B: At day 14, there was increased narrowing in the healing region of control ligaments (indicated by arrows) compared to low dose ligaments. C: H+E stains at day 14 healing show a larger healing region in control ligaments compared to the low dose group (controls 10.4± .7%, low dose 8.4±1.2%, p=.049). Healing region measurements were taken using Image J and values expressed as mean percentage of total ligament area ± S.E.M.

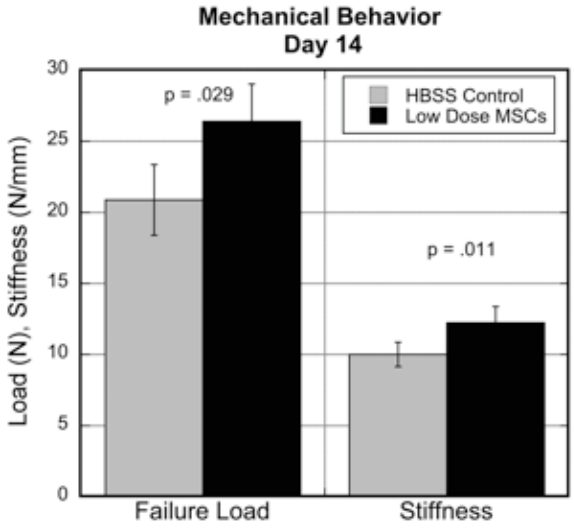
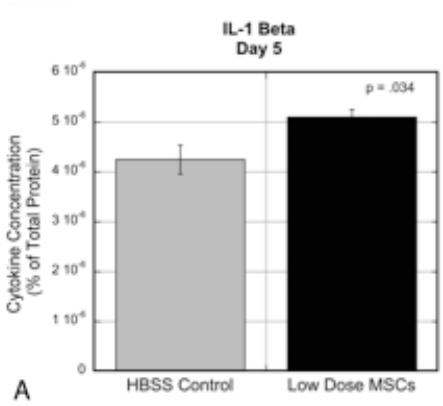
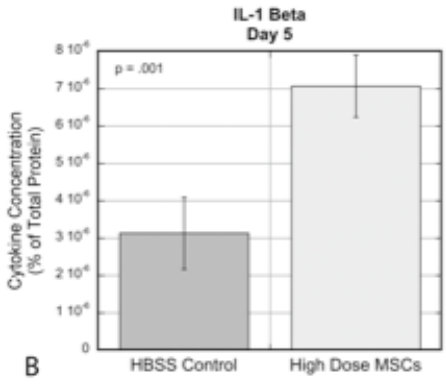


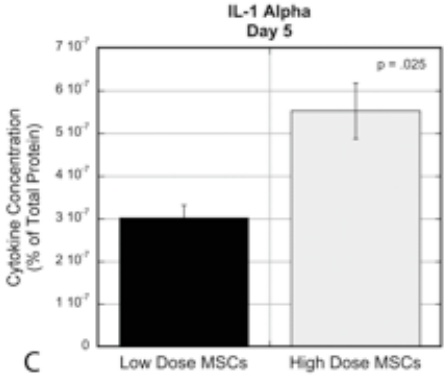
Fig. 2.5 Day 14 comparison of mechanical properties showed low dose ligaments exhibiting increased failure load (controls 20.88± 2.64N, low dose 26.41± 1.95N, p=.029) and stiffness measurements (controls 10.01± 1.02 N/mm, low dose 12.24± .95 N/mm, p=.011). Values are expressed as mean ± S.E.M.

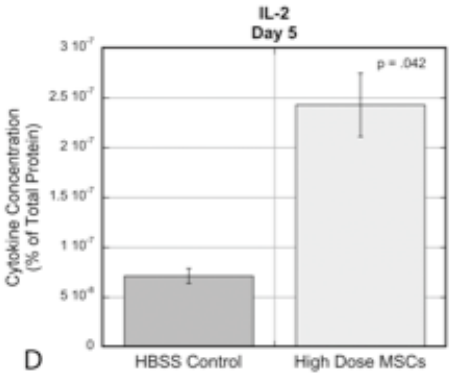
Cytokine Analysis

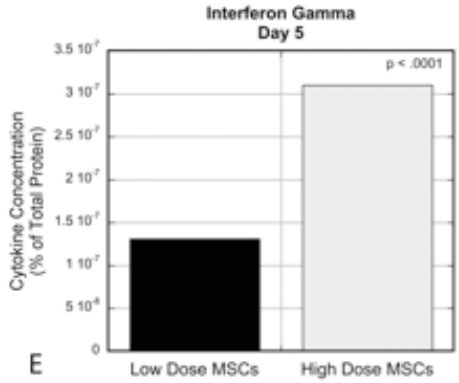
Significant changes in protein production were detected in 5 out of the 10 cytokines tested. Cytokine levels are expressed as a percentage of total protein within the ligament (Table 2.2). At day 5, IL-1β (Fig. 2.6, A) was increased in the low dose group compared to the control (controls $4.25x10^{-06}\pm 2.90x10^{-07}$% vs. low dose $5.10x10^{-06}\pm 1.52x10^{-07}$ %, p=.034). The same pattern was seen in the high dose group compared to controls (Fig. 2.6, B) but demonstrated a larger magnitude of change (controls $3.13x10^{-06}\pm 9.62x10^{-07}$% vs. high dose $7.07x10^{-06}\pm 8.35x10^{-07}$%, p=.001). IL-1α (Fig. 2.6, C) was decreased in both MSC dose groups at day 5 compared to controls along with the low dose group expressing significantly less cytokine compared to the high dose (controls $1.99x10^{-06}\pm 8.95x10^{-08}$% vs. low dose $3.02x10^{-07}\pm 3.03x10^{-08}$%, p=.004; controls $8.52x10^{-07}\pm 3.27x10^{-08}$% vs. high dose $5.53x10^{-07}\pm 6.51x10^{-08}$%, p=.020, high dose vs. low dose, p=.025). IL-2 (Fig. 2.6, D) was increased in the high dose group compared to controls at day 5 (controls $7.13x10^{-08}\pm 7.50x10^{-09}$% vs. high dose $2.43x10^{-07}\pm 3.18x10^{-08}$%, p=.042). IFN-γ (Fig. 2.6, E) was also increased in the high dose group compared to controls, and when compared to the low dose group (controls below detection level = 0 vs. high dose $3.10x10^{-07}$%, p<.0001; high dose vs. low dose $1.31x10^{-07}$%, p<.0001). At day 14, the high dose had increased IL-12 (Fig. 2.6, F) production compared to controls and compared to the low dose group (controls $1.00x10^{-06}\pm 2.88x10^{-08}$% vs. high dose $2.39x10^{-06}\pm 2.00x10^{-08}$%, p=.0008; high dose vs. low dose $1.53x10^{-06}\pm 5.95x10^{-08}$%, p=.0002). GM-CSF, IL-4, IL-6, IL-10, and TNFα were below detectable levels in all groups at both time points.


IL-1 Beta
Day 5
p = .034
Cytokine Concentration
(% of Total Protein)
HBSS Control
Low Dose MSCs
A


IL-1 Beta
Day 5
p = .001
Cytokine Concentration
(% of Total Protein)
HBSS Control
High Dose MSCs
B


IL-1 Alpha
Day 5
p = .025
Cytokine Concentration
(% of Total Protein)
Low Dose MSCs
High Dose MSCs
C


IL-2
Day 5
p = .042
Cytokine Concentration
(% of Total Protein)
HBSS Control
High Dose MSCs
D


Interferon Gamma
Day 5
p < .0001
Cytokine Concentration
(% of Total Protein)
Low Dose MSCs
High Dose MSCs
E

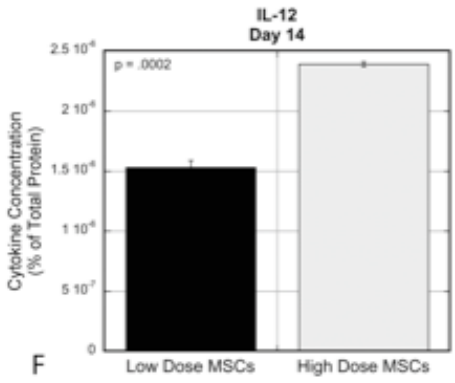

IL-12
Day 14
p = .0002
Cytokine Concentration
(% of Total Protein)
Low Dose MSCs
High Dose MSCs
F

Fig. 2.6 Cytokine analysis at day 5 and day 14 showed significant changes in 5 cytokines: IL-1β, IL-1α, IL-2, IFNγ, and IL-12. No significant changes were found in levels of TNFα, GM-CSF, IL-4, IL-6, and IL-10. A: At day 5, IL-1β was increased in the low dose group compared to controls (controls $4.25x10^{-06}$± $2.90x10^{-07}$%, low dose $5.10x10^{-06}$± $1.52x10^{-07}$ % , p=.034). B: At day 5, IL-1β was also increased in the high dose group compared to controls (controls $3.13x10^{-06}$± $9.62x10^{-07}$%, high dose $7.07x10^{-06}$± $8.35x10^{-07}$%, p=.001). C: At day 5, IL-1α was decreased in both dose groups compared to controls, with the low dose having significantly less IL-1α compared to the high dose (low dose $3.02x10^{-07}$± $3.03x10^{-08}$%, high dose $5.53x10^{-07}$± $6.51x10^{-08}$%, p=.025). D: At day 5, IL-2 was increased in the high dose group compared to controls (controls $7.13x10^{-08}$± $7.50x10^{-09}$%, high dose $2.43x10^{-07}$± $3.18x10^{-08}$%, p=.042). E: At day 5, IFNγ was increased in both dose groups compared to controls, along with the high dose having significantly increased expression compared to the low dose (low dose $1.31x10^{-07}$%, high dose $3.10x10^{-07}$%, p<.0001). F: At day 14, there was increased expression of IL-12 in the high dose group compared to the low dose group (low dose $1.53x10^{-06}$± $5.95x10^{-08}$%, high dose $2.39x10^{-06}$± $2.00x10^{-08}$%, p=.0002).

Discussion

The inflammatory response to injury is a complicated cascade of interactions that vary temporally, spatially and in magnitude. It is a well-conserved process that ultimately results in more scar-like versus native tissue in ligaments. A better understanding of this process is necessary to identify therapeutic interventions that minimize scar formation and stimulate regeneration of native tissue.

MSCs demonstrated a positive healing effect when applied at an appropriate dose. This was shown by a smaller wound size and improved mechanical properties at day 14. Interestingly, the lower dose of $1x10^6$ cells proved more successful than the higher dose of $4x10^6$ cells at day 14, indicating the importance of dosage in cell therapy. This is in contrast to our hypothesis where we expected the high dose to be more optimal. Cytokine production and cellular composition at the times examined portray the higher dose of MSCs as promoting inflammation. This is evidenced by increased production of pro-inflammatory cytokines (IL-1β, IL-2, IFNγ) and decreased anti-inflammatory M2 macrophages in the high dose group compared to controls. The reason for this response is unknown, however, evidence is emerging showing that allogeneic MSCs can trigger an immune response in the host[62–66]. Zangi et al.[63] showed that allogeneic

MSC survival upon transplantation was significantly shortened compared to syngeneic MSCs. Schu[64] and colleagues found that rats receiving an intravenous injection of allogeneic MSCs formed alloantibodies leading to complement-mediated lysis. These studies exploring the effects of allogeneic MSCs along with a review paper by Gebler et al.[67] detail circumstances where MSCs have pro-inflammatory effects under specific conditions. In our study, the low dose of MSCs expressing a certain amount of foreign antigen may have been insufficient to trigger an immune response and the MSCs modulated healing in a beneficial manner. The high dose of MSCs may have triggered an inflammatory reaction that negated the improved healing seen in the low dose group. These findings suggest that when using allogeneic MSCs for cell therapy, using an appropriate number of MSCs is essential to minimize host immune detection yet still be able to positively modulate the healing environment.

Past research performed in our laboratory on ligament healing showed that the wound size continues to expand with time due to remodeling[6]. Remodeling that creeps beyond the injury site and progresses into the healthy section of the ligament is thought to be one of the factors that contribute to inferior mechanical properties after injury. Therefore, our results in the low dose MSC group demonstrating a smaller wound size and less creeping holds promise for stronger ligaments. Inflammatory cytokines, such as IL-1β, are known to activate matrix metalloproteinases (MMPs) in tendon and ligament leading to remodeling activity[68,69]. The increased pro-inflammatory cytokines at day 5, including IL-1β, seen in the high dose MSC ligaments correlated with a larger healing region and active tissue seen later during healing. At day 14, more pro-inflammatory M1 macrophages were present throughout the MCL in the high dose group compared to control ligaments and the low dose group. This was consistent with

increased IL-12, a cytokine characteristic of M1s[31,32] found in the high dose group. A prolonged M1 macrophage response can be indicative of chronic inflammation, which represents increased cellular activity and results in more scar tissue. Collectively, these cytokine and macrophage profiles, along with increased proliferating cells in the healing region, suggest that the healing response in the high dose group was still active, whereas the low dose ligaments were becoming more quiescent.

Previous research has reported the ability of MSCs to alter macrophage phenotype to be more anti-inflammatory[26–29]. Our day 5 cellular and cytokine data did not show this same trend. We hypothesized there would be increased M2s and anti-inflammatory cytokines in the MSC treatment groups, with the higher dose of MSCs having higher levels compared to the low dose. Instead we found that the high dose of MSCs had significantly fewer M2s and increased pro-inflammatory cytokines both at day 5 and day 14. The differing results between our findings and previous reports may be due to in vivo versus in vitro experimental models and a potential immune reaction to larger amounts of foreign antigen present in the higher dose of MSCs.

Collagen I and collagen III are the predominant extracellular matrix proteins that make up ligaments and tendons. Healthy ligaments and tendons consist mainly of collagen I, whereas injured structures have increased collagen III[70,71]. Increased procollagen I at day 5 in the high dose group appeared to predict that these ligaments were on the path to regenerative healing. Surprisingly, this increased production did not lead to better healing at day 14 based on mechanical properties. Blood vessel formation is another marker used to analyze healing in ligaments. Some level of vessel formation is necessary to promote healing, however, excessive

formation can negatively impact mechanical properties[72,73]. At day 14, there were fewer endothelial cells and blood vessel lumen in the low dose MSC ligaments compared to the controls. This decrease in lumen formation correlated with improved mechanical properties in the low dose group and may have contributed to these outcomes.

Consistent with other reports, this study supports the potential therapeutic value of MSCs to enhance ligament healing. However, the precise mechanisms remain unclear. Inflammatory cytokines were not all down-regulated with either of our MSC therapy groups. Our study emphasizes that more MSCs is not necessarily better when using allogeneic MSCs and therefore dosage should be closely examined for each application. The high MSC dose had a measureable impact on early healing (day 5) in cellular and cytokine changes, which altered the course of healing and led to poorer outcomes. The low dose had fewer detectable changes during early healing, but resulted in improved functional mechanical outcomes (day 14). Dosage needs to be considered in each injury model since MSCs have the ability to alter the progression and final outcomes both positively and negatively during healing. There will likely be unique contributions from MSCs depending on the source of MSCs (including auto- versus allo-MSCs), amount of MSCs, animal model and tissue of interest. In summary, dose can affect the cellular response and cytokine expression during healing when used as a therapeutic intervention for ligament tears. Optimizing the regenerative response to accelerate healing and minimize re-injuries may prolong independence and mobility for people who experience ligament tears in a cost-effective manner.

Table 2.1 Summary of IHC results at day 5 and day 14 post-injury.

	Day 5			Day 14		
	Low dose MSCs vs. Control	*High Dose MSCs vs. Control*	*Low dose vs. High Dose MSCs*	*Low Dose MSCs vs. Control*	*High Dose MSCs vs. Control*	*Low dose vs. High Dose MSCs*
M1 macrophages	-----	-----	-----	↓ (LD) Ends, MCL	-----	↓ (LD) Ends, MCL
M2 macrophages	-----	↓ (HD) D/P HR	-----	-----	↓ (HD) Ends	-----
Endothelial cells	-----	-----	↑ (LD) HR	↓ (LD) Ends	-----	↓ (LD) D/P HR, MCL
Blood vessel lumen	-----	-----	↑ (LD) HR	↓ (LD) Ends, MCL	-----	-----
Proliferating cells	-----	-----	-----	↓ (LD) HR	-----	-----
Procollagen I	-----	-----	↓ (LD) MCL	-----	-----	-----
T lymphocytes	-----	-----	-----	-----	-----	-----
Collagen III	-----	-----	-----	NT	NT	NT

Abbreviations: ↓ - decreased, ↑ - increased, HD – high dose MSC group, LD – low dose MSC group, HR – healing region, D/P HR – distal and proximal healing region edges, Ends – distal and proximal ligament ends, MCL – medial collateral ligament (Ends+D/P HR+HR), ----- no significance, NT – not tested.

Table 2.2 Summary of cytokine results day 5 and day 14 post-injury.

	Day 5			Day 14		
	Low dose MSCs vs. Control	*High Dose MSCs vs. Control*	*Low dose vs. High Dose MSCs*	*Low Dose MSCs vs. Control*	*High Dose MSCs vs. Control*	*Low dose vs. High Dose MSCs*
IL-1α	↓ (LD)	↓ (HD)	↑ (HD)	-----	-----	-----
IL-1β	↑ (LD)	↑ (HD)	-----	-----	-----	-----
IL-2	-----	↑ (HD)	-----	-----	-----	-----
IFNγ	-----	↑ (HD)	↑ (HD)	-----	-----	-----
IL-12	-----	-----	-----	-----	↑ (HD)	↑ (HD)

Abbreviations: ↓ - decreased, ↑ - increased, HD – high dose MSC group, LD – low dose MSC group, ----- no significance.

Chapter 3: Enhanced Ligament Healing Using Primed Mesenchymal Stem Cells

Introduction

Musculoskeletal injuries account for 33 million of the injuries in the US annually with 50% involving soft tissue such as ligament and tendon[2]. The high prevalence of ligament injuries combined with poor healing makes this an important area of study. Ligaments can be repaired surgically or left to heal independently depending on the location of the injury. Both approaches have successes and failures resulting in a weaker tissue. Ligament repair after surgery and/or injury is a slow process that lingers over an extensive period of time. Research has shown that ligaments continue to remodel beyond 1 year post-injury and may continue as long as 2.5 years after the injury[74]. The resultant tissue is often less organized and made up of smaller collagen fibrils[3–5], and exhibits decreased mechanical strength[4,7]. These factors put the ligament at risk for re-injury or chronic symptoms associated with poor healing. Therefore there is a need for therapeutic treatments with the potential to improve the rate and quality of ligament healing.

Mesenchymal stem cells (MSCs) have been studied in many pathological conditions including but not limited to neurological diseases, diabetes and graft versus host disease (GVHD) as well orthopedic injuries. The primary focus in many of these studies is exploiting MSCs paracrine effects with less of a focus on differentiation potential. MSCs have been shown to have several key paracrine effects that alter tissue healing and disease states in a beneficial manner. A review by Meirelles et al.[75] compiled data and divided the therapeutic benefits into 6 categories: immunomodulation, anti-apoptosis, angiogenesis, support of stem/progenitor cells, anti-scarring,

and chemoattraction (Fig. 3.1). There is overlap and complex interplay among these categories which illustrates the difficulty in trying to isolate the fundamental mechanisms for improved healing.

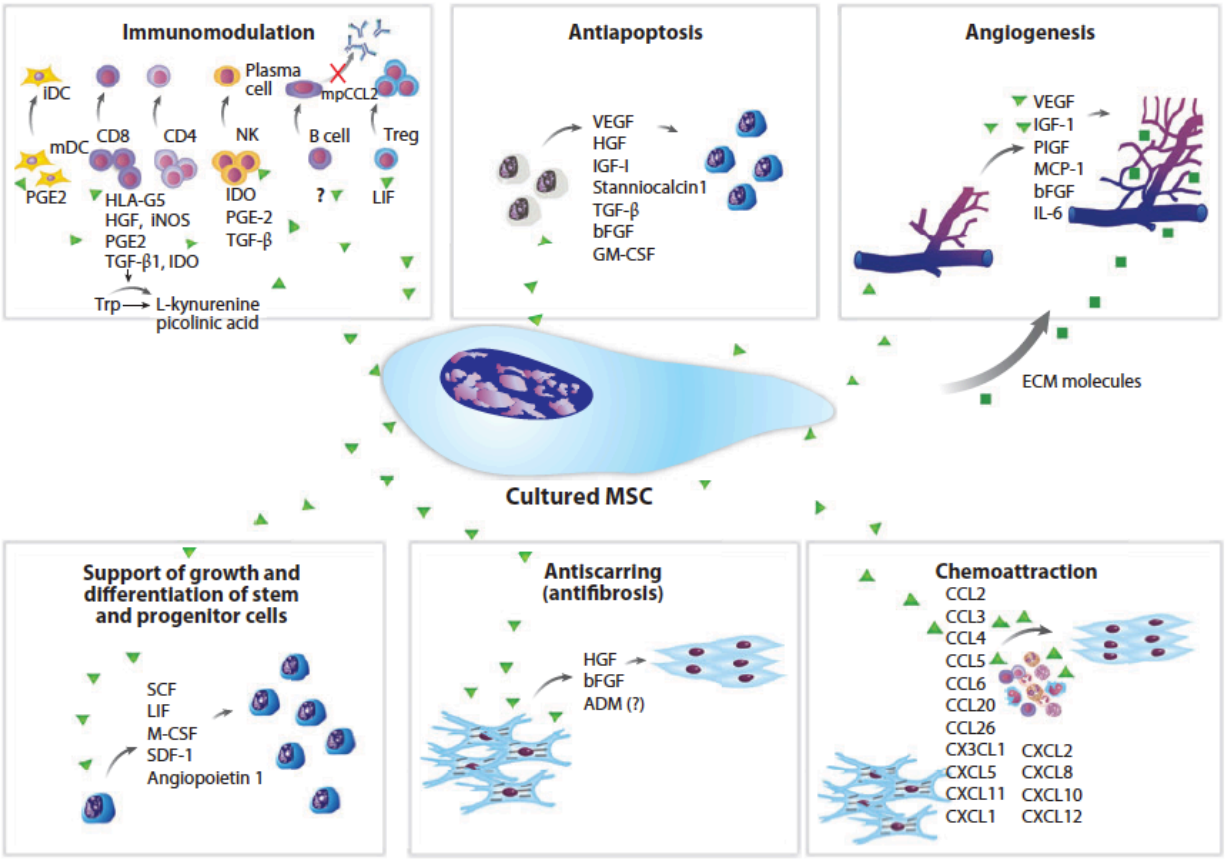


Fig. 3.1 (Reprinted with permission) Bioactive molecules released by mesenchymal stem cells that result in paracrine effects. MSCs paracrine mechanisms can be divided into 6 categories: immunomodulation, anti-apoptosis, angiogenesis, support of the growth and differentiation of local stem and progenitor cells, anti-scarring and chemoattraction.

One category of particular interest is immunomodulation due to many disease processes and injuries involving immune and inflammatory cells. We previously examined MSCs immune modulating capabilities during ligament healing and found significant changes that correlated with improved healing[76]. Interestingly the best results were found in ligaments that were

administered a lower dose ($1x10^6$ cells) vs a higher dose ($4x10^6$ cells) of cells. Due to improved healing, economical benefits, and ease of application associated with fewer cells, we chose to optimize this result further. The goal of the current study is to prime MSCs with a stimulus prior to putting them into an injured environment with the idea being once in the injured environment, the primed cells will have a more robust paracrine response leading to optimal healing.

There is a body of literature demonstrating the requirement of some form of stimulation or 'licensing'[44] for MSCs to exert their paracrine effects. For in vivo studies, an injured or inflammatory environment can provide activating stimuli. For in vitro studies, a stimulus needs to be added to the system. Several researchers have looked at activating MSCs via inflammatory cytokines, such as IL1-α/β, IFNγ, and TNFα, and reported that this exposure was necessary to stimulate MSCs immunosuppressive abilities[45,46]. Others have looked at activating MSCs by treating them with molecules that activate specific toll-like receptors (TLRs), which recognize danger signals. While some studies have shown improved anti-inflammatory effects with primed MSCs[47,48], others report the opposite[49]. Disagreement in the literature may be due to different cell types (mouse vs. human), in vivo vs. in vitro models and length of time cells are primed. Priming cells holds promise but the concept requires further research in injury specific models.

We designed a study to examine rat medial collateral ligament healing using MSCs and primed MSCs. Polyinosinic and polycytidylic acid (poly(I:C)) was used as a primer due to it's specificity to toll-like receptor 3 (TLR3) and previous research demonstrating an anti-inflammatory phenotype[47,48]. Since our previous study showed improved healing using $1x10^6$ cells, we used this same number of cells and aimed to increase efficacy with the priming.

Discovering methods to maximize MSCs anti-inflammatory phenotype by priming prior to implantation could yield beneficial outcomes for translational applications. *We hypothesized that the primed MSCs would result in a less inflammatory environment leading to improved ligament healing demonstrated by increased ligament strength and allowing for a faster rate and higher quality of healing.*

Materials and Methods

Experimental Design

The healing model used for this study examines extra-articular ligament healing. The rat medial collateral ligament (MCL) serves as an appropriate tissue of study in this category and has been well characterized by our lab[6]. Rats underwent bilateral MCL transection using a scalpel blade to ensure consistency between imposed injuries. Treatment was administered at the time of injury and consisted of 3 groups: 1) control group receiving carrier solution only (Hanks Balanced Saline Solution, (HBSS; Hyclone Laboratories Inc, Logan UT) 2) unprimed MSC group ($1x10^6$ cells) and 3) primed MSC group ($1x10^6$ cells). The cell number used in this study was chosen due to dose optimization performed in a previous study[76]. Forty-two adult male Wistar rats (275-299g) underwent bilateral ligament surgery (14 per group) and healing was analyzed at days 4 and 14 post-injury.

Surgical Procedure

All procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee. Sterile technique was used while preparing and performing all rat surgeries. Animals were anesthetized using isoflurane for the duration of surgery and monitored daily for 7

days post-op to ensure animal welfare. A medial skin incision was made longitudinally and superficial to the MCL. Another incision was made in the subcutaneous tissue and gracilis muscle in order to expose the MCL. Each MCL was horizontally transected distal to the medial knee joint line. A stitch was then placed in the muscle to create a pocket for cell/HBSS administration. For the unprimed and primed MSC groups, $1x10^6$ cells were suspended in 25ul of HBSS and administered using a sterile pipette at the location of the ligament transection. The control group received 25ul of HBSS without cells in the same location. The same treatment was administered to bilateral knees in each animal in order to avoid confounding results due to any systemic effects. MCLs were not sutured. The gracilis muscle and skin were closed using 5-0 vicryl suture and animals were allowed full cage mobility without knee motion restrictions post-op. Animals were euthanized at days 4 and 14 and MCLs were used for immunohistochemistry (IHC) and mechanical testing.

Mesenchymal Stem Cell Culture and Priming

Rat mesenchymal stem cells were purchased (Cyagen Biosciences Inc, Santa Clara, CA) at passage 5 and expanded using Mesenchymal Stem Cell Growth Medium (Cyagen Biosciences Inc, Santa Clara, CA) consisting of MSC basal medium, MSC-qualified fetal bovine serum, penicillin-streptomycin, and glutamine. Cells were originally obtained from Fisher 344 rat bone marrow and cultured in monolayer. The vendor transfected the cells using an EmGFP-expressing lentiviral construct and selected with hygromycin. Cells were then analyzed for specific cell marker expression using flow cytometry. The guidelines for selection: positive for CD44 and CD90 (>70%), as well as negative for CD34, CD11b, CD45 (<5%). Along with expression analysis of specific markers, tri-lineage differentiation (osteogenic, chondrogenic, and

adipogenic) were performed by the vendor. Once we received the cells, they were seeded in flasks and maintained in an incubator at 37° C and 5% CO_2. The media was changed every 3-4 days and cells were passaged upon reaching 70% confluency. Cell morphology was monitored to confirm a spindle-like appearance throughout passaging. All cells used for surgeries were at passage 8 through 10. Several flasks of cells were allocated for the priming group and were administered media that contained polyinosinic acid and polycytidylic acid (Poly (I:C), Sigma Aldrich) at a concentration of 1μg/ml. Cells were maintained in the primed media for 48 hours and collected for surgery at the end of this time point. All cells were removed from flasks using Trypsin EDTA (Cellgro, Manassas, VA) and counted using Tali® Image-Based Cytometer (Life Technologies, Grand Island, NY). In order to track cell number and localization in vivo, cells were fluorescently labeled with Celltracker CM-DiI (Life Technologies, Grand Island, NY) before being administered to the injured ligament.

Immunohistochemistry/Immunofluorescence

Ligaments designated for IHC were dissected and frozen in optimal cutting temperature (OCT) at days 4 and 14 post-injury. Ligaments were longitudinally sectioned (5μm), mounted on Colorfrost Plus microscope slides and stored at -80° C. Mouse and rabbit monoclonal antibodies were selected to measure the protein of interest. The staining protocol began with acetone fixation followed by 3% hydrogen peroxide to prevent endogenous peroxidase activity. Background Buster (Innovex Biosciences, Richmond, CA) or Rodent block R (Biocare Medical, Concord, CA) was applied to each slide to minimize non-specific antibody-protein interactions. Selected primary antibodies were applied for 2 hours at room temperature in a humidified slide chamber. A biotin-linked secondary antibody and streptavidin conjugated to horseradish

peroxidase tertiary antibody were applied for 10 minutes each using a Stat Q staining kit (Innovex Biosciences, Richmond, CA) or a rabbit-on-rodent HRP polymer (Biocare Medical, Concord, CA). The antibody-antigen complex was processed and detected using Diaminobenzidine (DAB). For staining that required a fluorescent secondary antibody, Alexa Fluor® 488 (Life Technologies, Grand Island, NY) was used for detection.

Mouse monoclonal antibodies were applied to measure procollagen I (straight; SP1.D8; Developmental Hybridoma, Iowa City, Iowa), endothelial cells (CD31; 1:100; AbDSertoc, Raleigh, NC), type 2 macrophages (CD163; 1:100; AbDSertoc, Raleigh, NC), type 1 macrophages (CD68; 1:100; AbDSertoc, Raleigh, NC), vascular endothelial growth factor (VEGF; 1:100; Abcam; Cambridge, MA), and proliferating cells (Ki-67; 1:25; Dako, Carpinteria, CA). A rabbit monoclonal or polycolonal antibody was used to detect transforming growth factor beta (TGFβ; 1:100; Abcam; Cambridge, MA), prostaglandin E2 (PGE2; 1:100, Abcam; Cambridge, MA), and apoptosis (Cleaved Caspase 3; 1:50; Cell Signaling Technology, Danvers, MA). Celltracker CM-DiI (Life Technologies, Grand Island, NY) was used for fluorescent detection of MSCs and 4',6-diamidino-2-phenylindole (DAPI) used for total cell detection.

Collagen Organization

Ligaments were stained with Picroserius Red (Polysciences Inc, Warrington, PA) and imaged using polarized light microscopy to visualize matrix organization. Images were taken of the healing region and converted to gray scale. Two automated techniques were used to quantify collagen fiber organization: Fractal dimension analysis (FA) and fast Fourier transformation

(FFT). Both methods examined linearity of the matrix and assigned a number to each image through an existing Matlab routine[77].

Immunohistochemistry Quantification

In order to measure the spatial distribution of cells and protein, 5 areas are the ligament were imaged at 400x using a camera-assisted microscope (Nikon Eclipse microscope, model E6000 with Olympus camera, model DP79). The 5 areas represented were the healing region, distal healing region edge, proximal healing region edge, distal ligament and proximal ligament. A macro was created for each stain using Image J (National Institutes of Health, Bethesda, MD). Two or three sections from each ligament were measured by calculating the percent area positively stained and then averaged for comparison. The healing region, healing region edges, ligament ends, and total MCL were examined for any changes upon treatment.

Mechanical Testing

Ligament failure strength and stiffness measurements were performed at day 14 post-injury. Rats were euthanized and frozen (-80°C) until dissections could take place. The MCL surrounding tissue was removed and the ligament tibial and femoral insertions were kept intact. The femur and tibia were cut in order to achieve a tight press fit into the mechanical tester. Phosphate buffered saline (PBS) was applied to maintain ligament hydration throughout the testing process.

Uniformly distributed axial loading was applied by placing the femur and tibia in the anatomical position in a custom-designed load frame. Ligaments were placed in a position of slack and

were not preconditioned prior to mechanical testing in order to avoid damaging the healing region. Each ligament was pulled at a rate of 4.0 mm/sec until it failed (tore). Load and displacement values were recorded to calculate maximum load before failure. Stiffness was measured in the most linear region of the load-displacement curve for comparison between groups.

Statistics

Differences between the control group, unprimed MSC group and primed MSC groups were analyzed using a one-way analysis of variance (ANOVA). If the overall p-value for the F-test in ANOVA was <.10, post-hoc comparisons were performed using Fisher's least significant difference (LSD). Experimental data is presented as the least squares means ± standard error of the means (SEM) of replicates. All p-values reported are two sided. Kaleidagraph, version 4.03, was used for all computations.

Results

Immunohistochemistry

Procollagen 1α, the precursor to type 1 collagen, was measured throughout the healing ligaments due to type 1 collagen being the most abundant matrix protein. At day 4, the ligaments that received cells primed with poly (I:C) and control ligaments had increased procollagen 1α in the healing region compared to unprimed MSCs (PMSC vs. MSC p=.0045, HBSS vs. MSC p=.0077, Fig. 3.2, A-D). At day 14, the primed MSC group continued to have increased procollagen 1α along the healing region edges compared to the MSC and control groups (PMSC vs. MSC p=.0273, PMSC vs. HBSS p=.0551, Fig. 3.2, E-H).

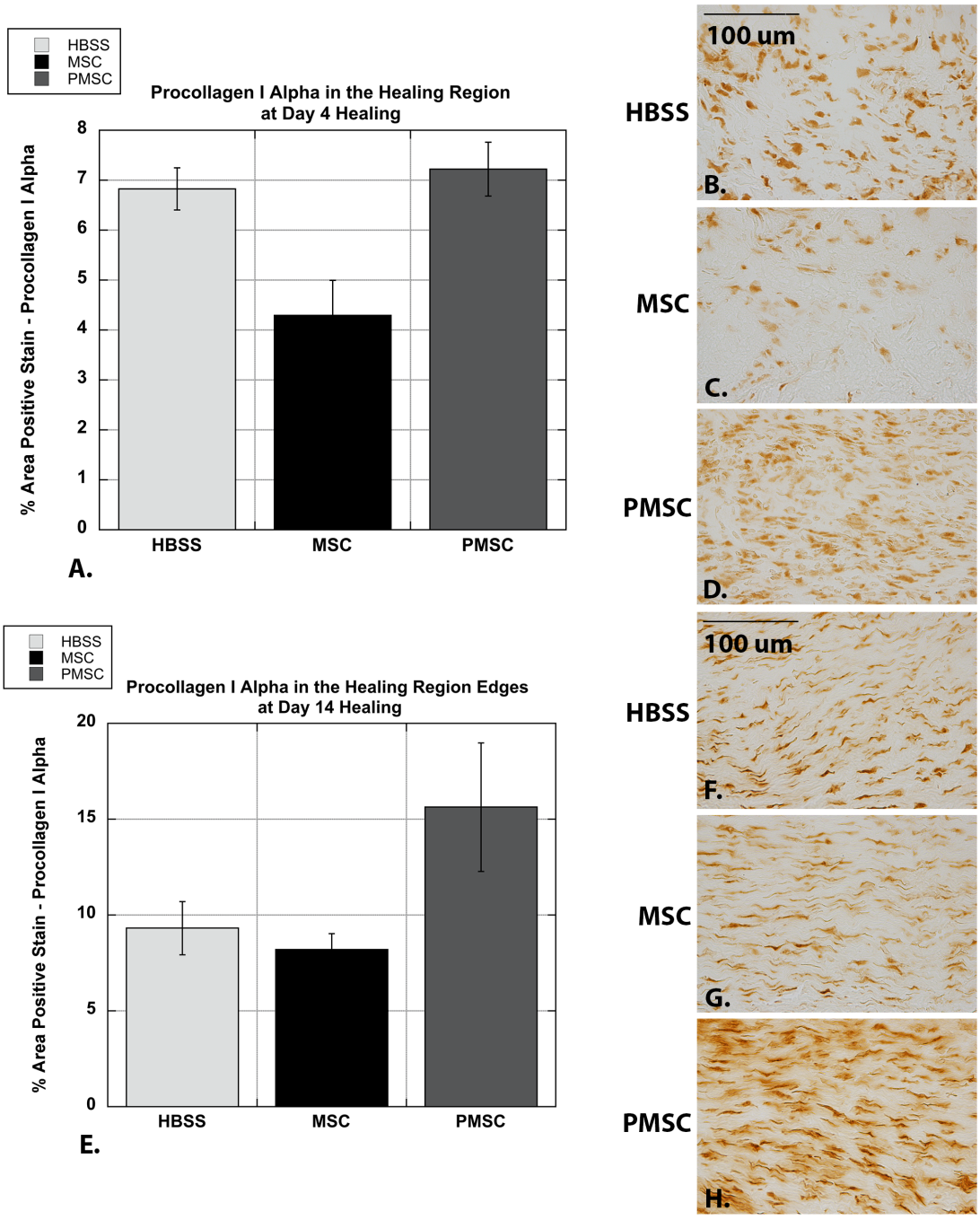


Figure 3.2 A-D: At day 4 healing, there was increased procollagen 1α in the healing region and healing region edges in the primed MSC group (p=.004) and the HBSS controls (p=.008) compared to the MSC group (PMSC 7.219±.538%, MSC 4.294±.699%, HBSS 6.826±.423%). A: Graph comparing average percentage area stain for each condition. B: Representative image of IHC in control ligament. C: Representative image of IHC in MSC group. D: Representative image of IHC in PMSC group. E-H: At day 14 healing, procollagen 1a was increased in the healing region edges in the primed MSC group compared to the MSC group (p=.027) and HBSS controls (p=.055) (PMSC 15.627±3.349%, MSC 8.207±.813%, HBSS 9.317±1.390%). E: Graph comparing average percentage area stain for each condition.F: Representative image of IHC in control ligament. G: Representative image of IHC in MSC group. H: Representative image of IHC in PMSC group. Values are expressed as mean area stain ± S.E.M.

Apoptosis, cellular proliferation, and overall cellularity were measured at days 4 and 14 to determine whether either treatment had an effect on these general functions in a healing environment. When examining apoptosis, no significant differences were detected at day 4 even though apoptosis appeared higher in the primed MSC group (Fig. 3.3, A-D). At day 14, the primed MSC group had less apoptosis in the healing region compared to both the MSC and control groups (PMSC vs. MSC p=.0081, PMSC vs. HBSS p=.0034, Fig 3.3, E-H). A significant increase in proliferation was detected throughout the MCL at day 4 in the primed MSC group compared to the unprimed MSC (p=.0475) and control group (p=.0194) (Fig. 3.4, A-D). However, at day 14 there was very little proliferation in any of the groups and no significant differences between groups at this time point (Fig. 3.4, E-H). No significant differences were detected in overall cellularity between groups at day 4 or day 14 (data not shown).

Endothelialization was examined to determine treatment effects on blood vessel formation in the injured ligaments. At day 4, the primed MSC and MSC treated ligaments had increased endothelial cells in the healing region and healing region edges compared to the control group (PMSC vs. HBSS p=.0029, MSC vs. HBSS p=.0341, Fig. 3.5, A-D). The primed MSC group had more endothelialization compared to the MSC group as well, but not to a level of statistical significance (PMSC vs. MSC p=.1575). At day 14, the pattern reversed and the primed MSC group had the least amount of endothelial cells in the healing region and healing region edges compared to the MSC and control groups (PMSC vs. MSC p=.0157, PMSC vs. HBSS p=.0592, Fig 3.5, E-H).

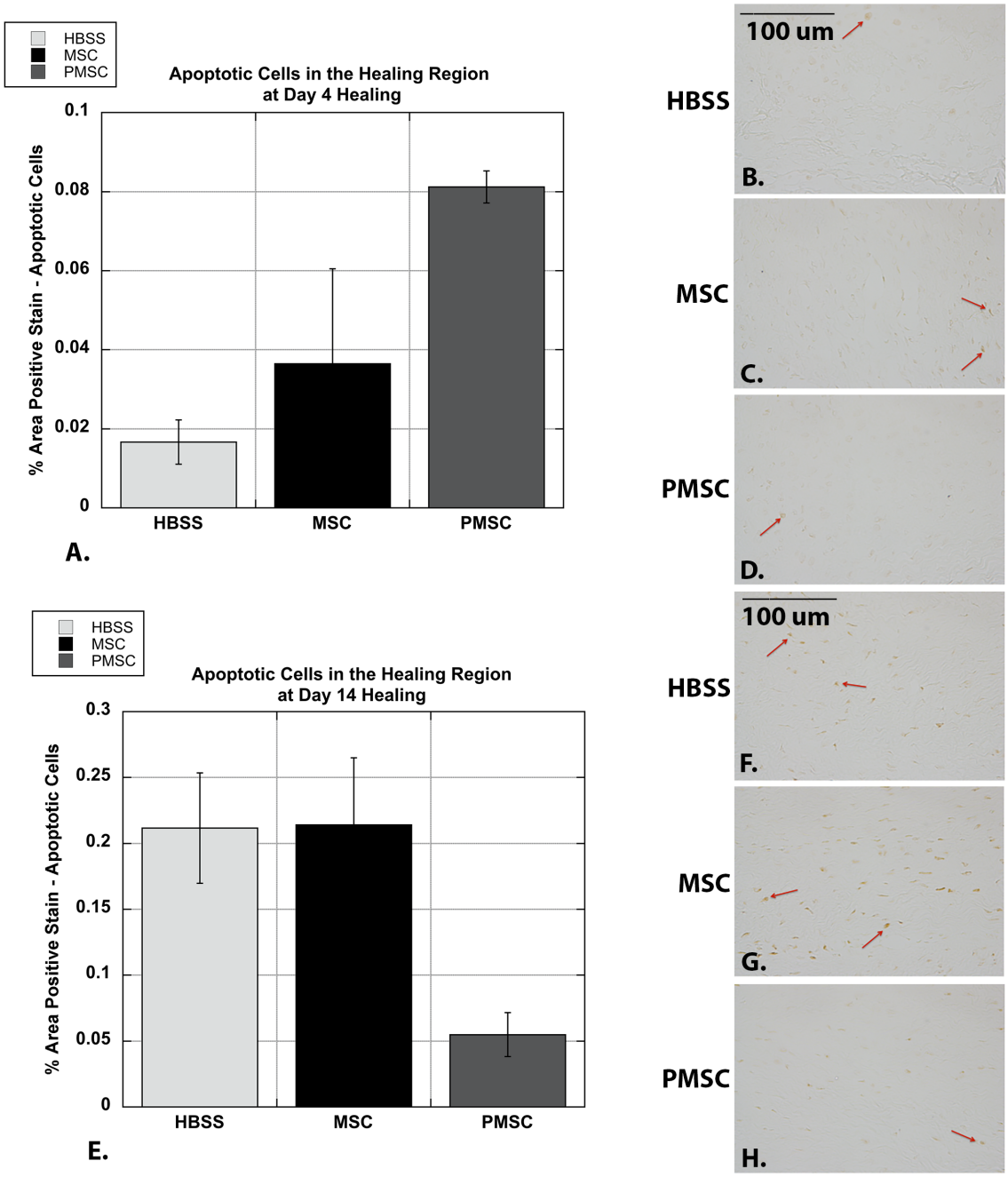


Fig. 3.3 A-D: At day 4 healing, there were no significant differences between groups in apoptotic cells in the healing region. (PMSC .0812±.0594%, MSC .0365±.0240%, HBSS .0167±.0056%). A: Graph comparing average percentage area stain for each condition. B: Representative image of IHC in control ligament. C: Representative image of IHC in MSC group. D: Representative image of IHC in PMSC group. E-H: At day14 healing, the primed MSC group had fewer apoptotic cells compared to the MSC group (.008) and the HBSS controls (.003) (PMSC .055±.017%, MSC .214±.051%, HBSS .212±.042%) E: Graph comparing average percentage area stain for each condition. F: Representative image of IHC in control ligament. G: Representative image of IHC in MSC group. H: Representative image of IHC in PMSC group. Values are expressed as mean area stain ± S.E.M.

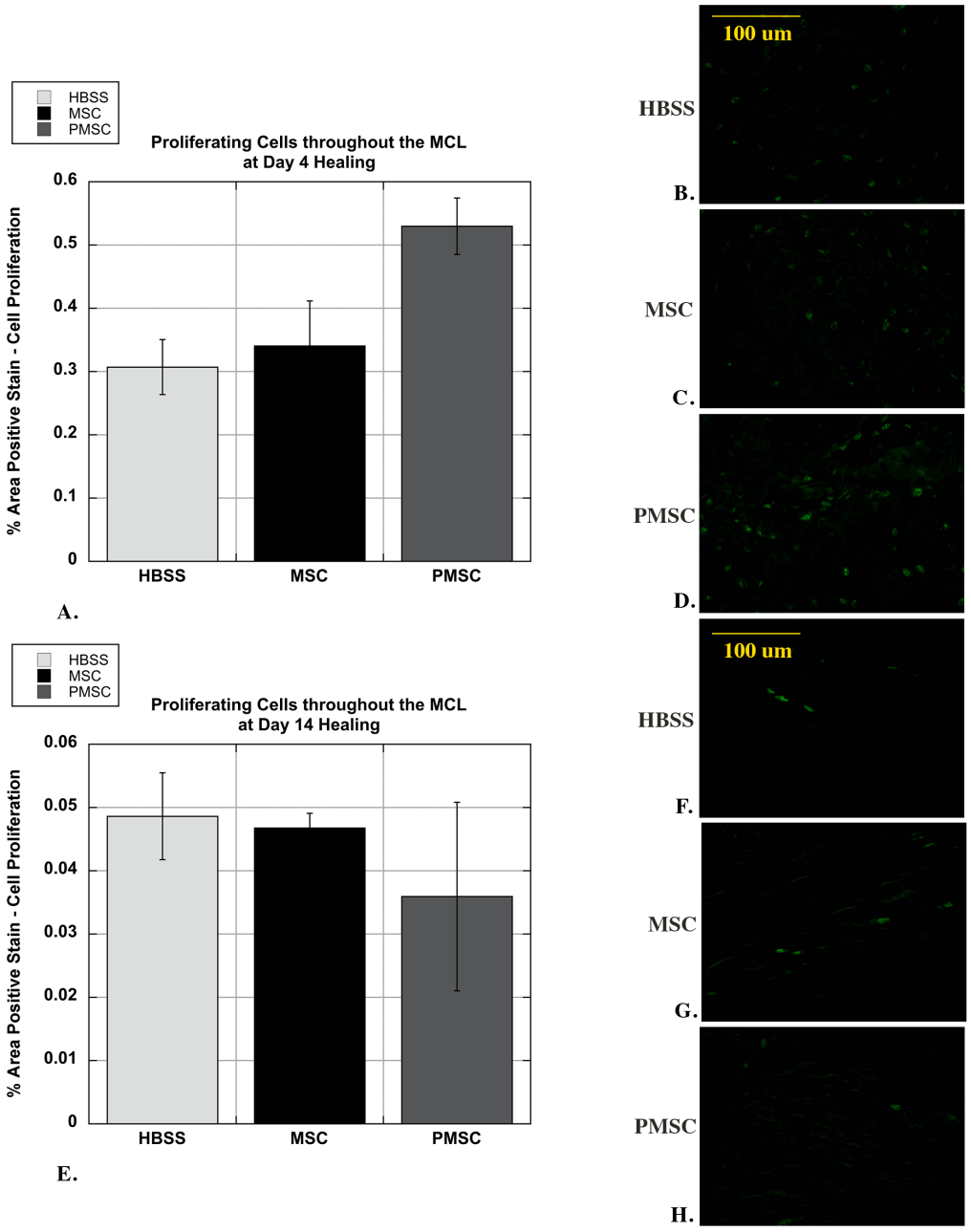


Fig. 3.4 A-D: At day 4 of healing, the primed MSC group had increased cellular proliferation throughout the MCL compared to both the MSC group (p=.0475) and control group (.0194) (PMSC .5296±.0446%, MSC .3405±.0712%, HBSS .3071±.0436). A: Graph comparing average percentage area stain for each condition.B: Representative image of IHC in control ligament. C: Representative image of IHC in MSC group. D: Representative image of IHC in PMSC group. E-H: At day 14 of healing, there was very little cellular proliferation in any of the groups and no significant differences between groups (PMSC .0359±.0149, MSC .0468±.0023, HBSS .0486±.0069). E: Graph comparing average percentage area stain for each condition. F: Representative image of IHC in control ligament. G: Representative image of IHC in MSC group. H: Representative image of IHC in PMSC group. Values are expressed as mean area stain ± S.E.M.

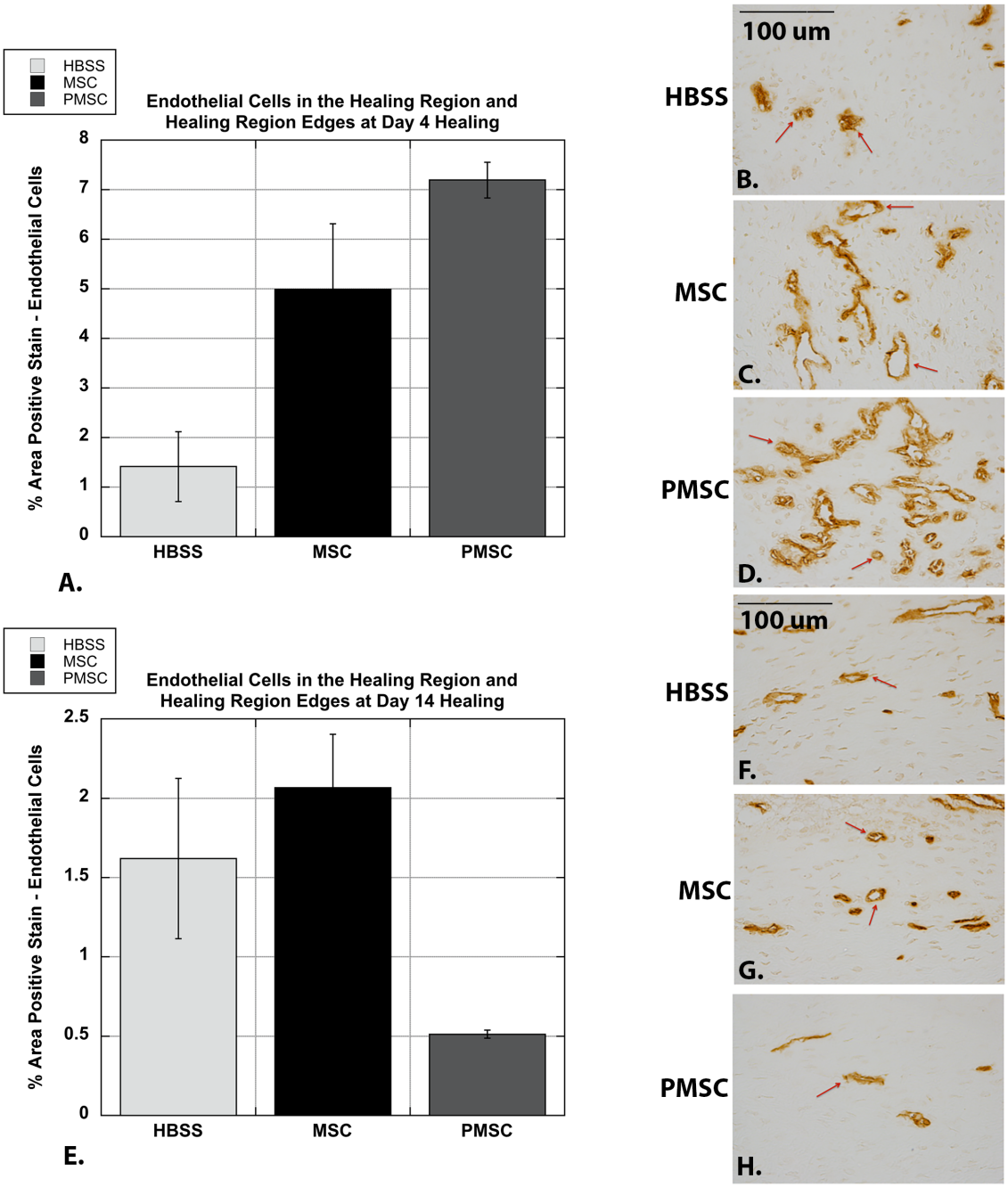


Fig. 3.5 A-D: At day 4 healing, the primed MSC group (p=.003) and MSC group (p=.034) had increased endothelial cells in the healing region and healing region edges compared to the HBSS controls. (PMSC 7.193±.908%, MSC 4.986±1.324%, HBSS 1.416±.704). A: Graph comparing average percentage area stain for each condition. B: Representative image of IHC in control ligament. C: Representative image of IHC in MSC group. D: Representative image of IHC in PMSC group. E-H: At day 14 healing, the primed MSC group had fewer endothelial cells in the healing region and healing region edges compared to the MSC group (p=.016) and HBSS controls (p=.059). (PMSC .512±.111%, MSC 2.067±.337%, HBSS 1.620±.505%). E: Graph comparing average percentage area stain for each condition. F: Representative image of IHC in control ligament. G: Representative image of IHC in MSC group. H: Representative image of IHC in PMSC group. Values are expressed as mean area stain ± S.E.M.

Next we analyzed factors involved in the inflammatory response including type 1 macrophages (M1) and type 2 macrophages (M2). At D4, M2s were increased in the healing region and healing region edges in the primed MSC group compared to the MSC and control groups (PMSC vs. MSC p=.0562, PMSC vs. HBSS p=.0172, Fig. 3.6, A-D). These same findings remained consistent when the MCL was examined as a whole (PMSC vs. MSC p=.0285, PMSC vs. HBSS p=.0098, data not shown). However, at day 14, the MSC group had increased M2s throughout the MCL compared to both the primed MSC and control groups (MSC vs. PMSC p=.0562, MSC vs HBSS p=.0365, Fig. 3.6, E-H). We then analyzed the ratio of M2s to M1s to determine the balance of macrophage phenotypes since this may be a better representation of inflammation in the healing environment. At day 4, the primed MSC group had a higher ratio of type 2 macrophages to type 1 macrophages throughout the MCL compared to the MSC and control groups (PMSC vs. MSC p=.0346, PMSC vs. HBSS p=.054, Fig.3.7, A). At day 14, even though the MSC group had significantly more M2s throughout the MCL compared to both groups, the ratio of M2s to M1s was not increased (Fig. 3.7, B). On the contrary, the MSC group had the lowest ratio, however, no level of significance was reached (p=.6624).

Along with looking at various cellular responses using unprimed and primed MSCs, we measured a few cytokines known to play an essential role in healing. First, we analyzed transforming growth factor beta (TGFβ) at day 4 of healing. There were no significant differences detected between groups when looking at TGFβ in the healing region and healing region edges (p=.62477) or when comparing TGFβ throughout the MCL (p=.35256). Even though a level of significance was not reached, a pattern was detected throughout the MCL with

the unprimed MSC group having more TGFβ in its matrix compared to the primed MSC and HBSS control groups which displayed similar levels of TGFβ (Fig. 3.8, A-D).

Next we examined vascular endothelial growth factor (VEGF) levels at day 4 due to its role in angiogenesis. No significant differences were detected between groups at this healing time point (p=.38781 HR sum, p=.55075 MCL) however, the primed MSC group had greater amounts of VEGF throughout the healing region and healing region edges followed by the unprimed MSC group and the HBSS controls having the lowest amount of VEGF (Fig. 3.8, E-H). This same pattern was seen when measuring the MCL as a whole.

Prostaglandin E2 (PGE2) levels were measured at day 4 due to its potential influence on macrophage phenotype. There were significant differences in PGE2 expression detected throughout the healing region and healing region edges as well as in the ligament ends. In the healing region area, the unprimed MSC group had significantly more PGE2 than the control group, and more PGE2 than the primed MSC group but not to a level of significance (MSC vs. HBSS p=.0197, MSC vs. PMSC p=.1679, PMSC vs HBSS p=.2563, data not shown). In the ligament ends, the unprimed MSC group had increased levels of PGE2 compared to both the primed MSC group and HBSS group (MSC vs HBSS p=.0161, MSC vs PMSC p=.0123, PMSC vs HBSS p=.7533, Fig. 3.9).

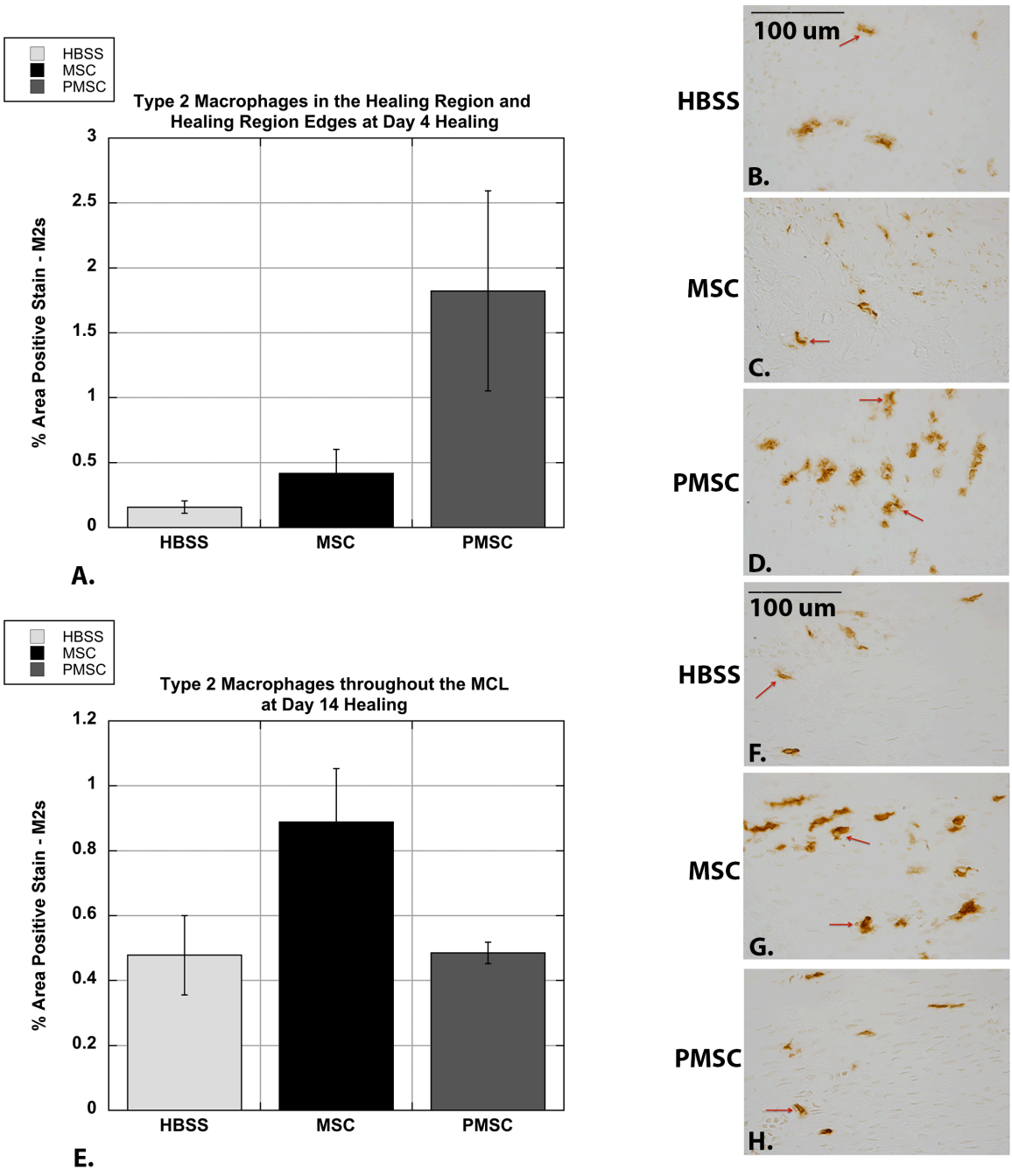


Fig. 3.6 A-D: At day 4 healing, the primed MSC group had more M2s in the healing region and healing region edges compared to the MSC group (p=.046) and the HBSS group (p=.017) (PMSC 1.823±.772%, MSC .418±.183%, HBSS .157±.048). A: Graph comparing average percentage area stain for each condition. B: Representative image of IHC in control ligament. C: Representative image of IHC in MSC group. D: Representative image of IHC in PMSC group. E-H: At day 14 healing, the primed MSC group (p=.056) and HBSS control group (p=.037) had fewer M2s throughout the MCL compared to the MSC group. (PMSC .485±.033%, MSC .888±.165%, HBSS .478±.122%). E: Graph comparing average percentage area stain for each condition. F: Representative image of IHC in control ligament. G: Representative image of IHC in MSC group. H: Representative image of IHC in PMSC group. Values are expressed as mean area stain ± S.E.M.

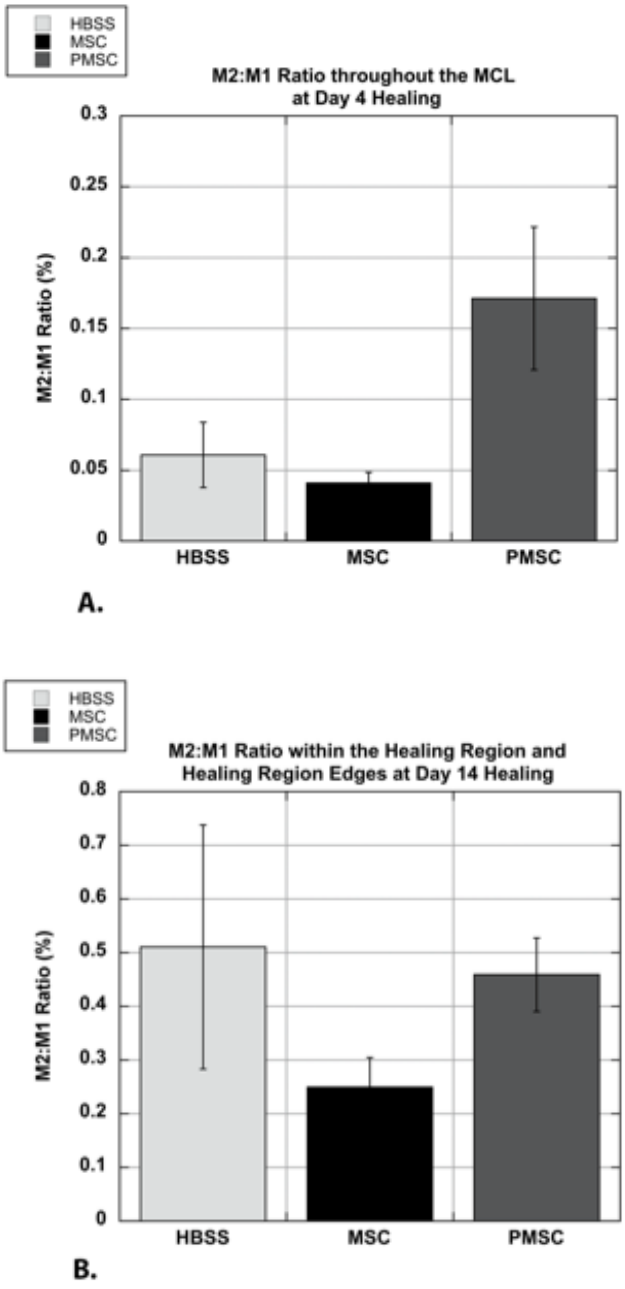


Fig. 3.7 A: At day 4, the primed MSC group had an increased M2:M1 ratio throughout the MCL compared to the MSC group (p=.035) and HBSS control group (p=.054) (PMSC .171±.050, MSC .041±.008, HBSS .061±.036). B: At day 14, the were no significant differences between groups (Healing region/healing region edges: PMSC .459±.068, MSC .250±.056, HBSS .510±.227) Values are expressed as mean area stain ± S.E.M.

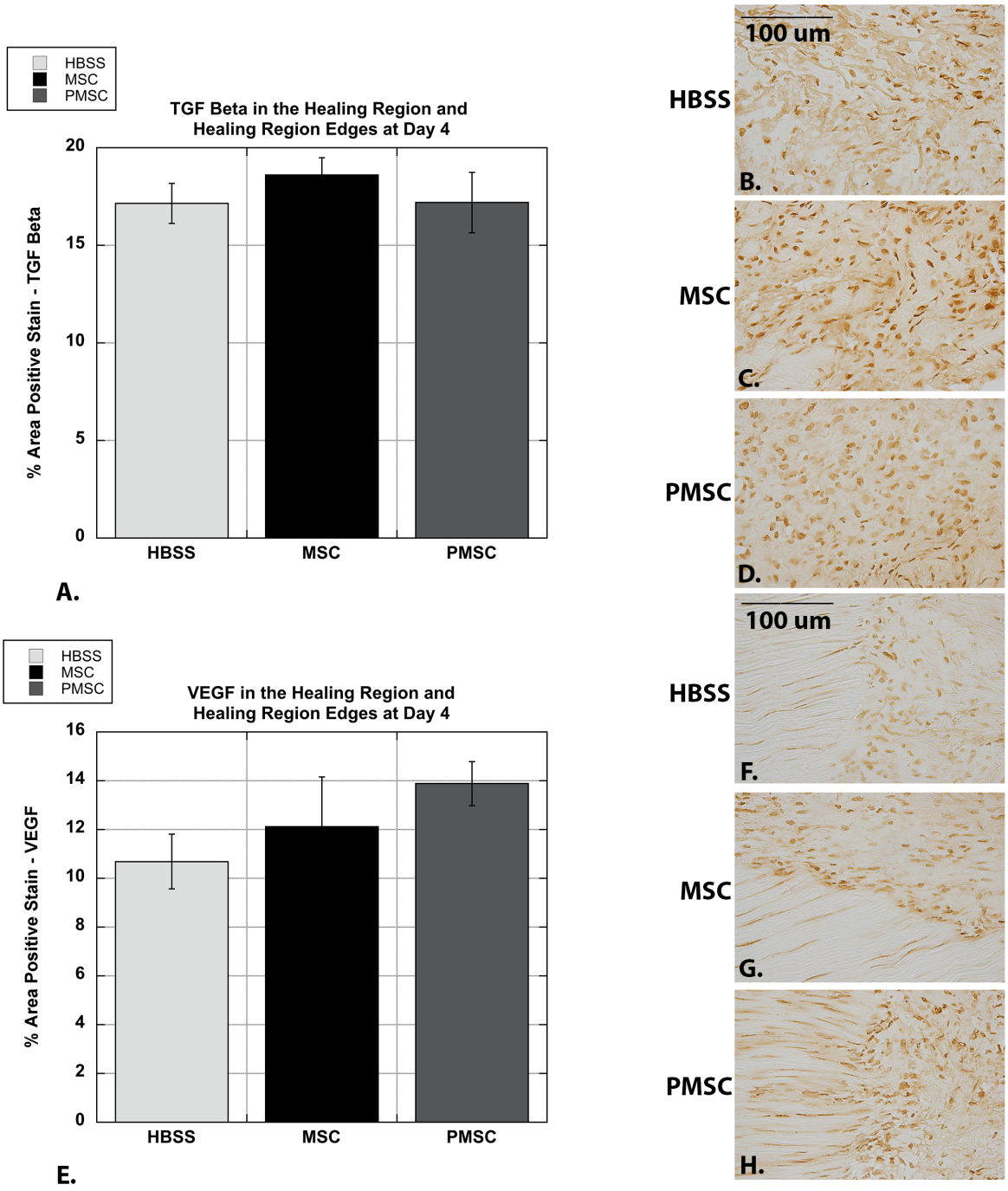


Fig. 3.8 A-D: At day 4 healing, there were no significant changes in TGFβ levels between groups. However both the primed MSC group (PMSC 17.191±1.539%) and HBSS group (HBSS 17.155±1.025%) had less TGFβ within the healing region and healing region edges compared to the MSC group (MSC 18.613±.875%) A: Graph comparing average percentage area stain for each condition. B: Representative image of IHC in control ligament. C: Representative image of IHC in MSC group. D: Representative image of IHC in PMSC group. E-H: At day 4 healing, there were no significant differences in VEGF levels between groups. However there was a pattern with the PMSC group having the most VEGF in the healing region and healing region edges (PMSC 13.882±.908%), followed by the MSC group (MSC 12.120±2.029%), and the control group having the least amount (HBSS 10.690±1.122). E: Graph comparing average percentage area stain for each condition. F: Representative image of IHC in control ligament. G: Representative image of IHC in MSC group. H: Representative image of IHC in PMSC group. Values are expressed as mean area stain ± S.E.M.

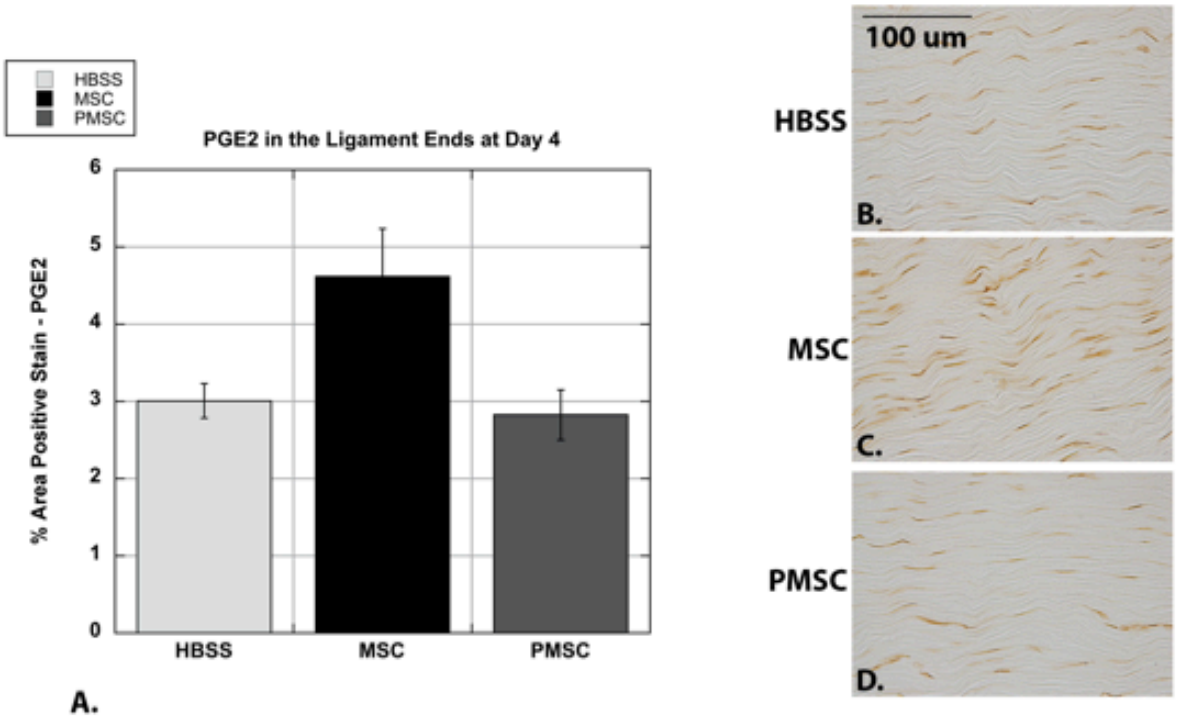


Fig. 3.9 A-D: At day 4 healing, the MSC group had more PGE2 throughout the ligament ends compared to the primed MSC group (p=.0123) and control group (p=.0161). (PMSC 2.826±.325%, MSC 4.617±.621%, HBSS 3.006±.224). A: Graph comparing average percentage area stain for each condition. B: Representative image of IHC in control ligament. C: Representative image of IHC in MSC group. D: Representative image of IHC in PMSC group. Values are expressed as mean area stain ± S.E.M.

Mechanical Properties and Matrix Organization

Functional mechanical properties were measured at day 14 of healing. The ligaments that received primed MSCs demonstrated superior failure strength (13.699 N ± .850) compared to ligaments receiving unprimed MSCs (9.988 N ± 1.221, p=.0219). The primed MSC group had greater failure strength than the control group (11.585 ± 1.107), but not to a level of significance (p=.1758, Fig. 3.10, A). This same pattern was demonstrated with ligament stiffness measures. The primed MSC group had significantly increased stiffness (7.2774 N/mm ± .4236) compared to the unprimed MSC group (5.7711 N/mm ± .5835, p=.0325), and increased stiffness compared

to the control group (6.7465 N/mm ± .3755), but not to a level of significance (p=.4317, Fig. 3.10, B).

Matrix organization was analyzed at day 14 to determine whether this may be contributing to the improved mechanical properties found in the primed MSC group. Neither quantitative technique (FA and FFT) measured any differences between groups (Fig. 3.10, C-E, Table 3.1).

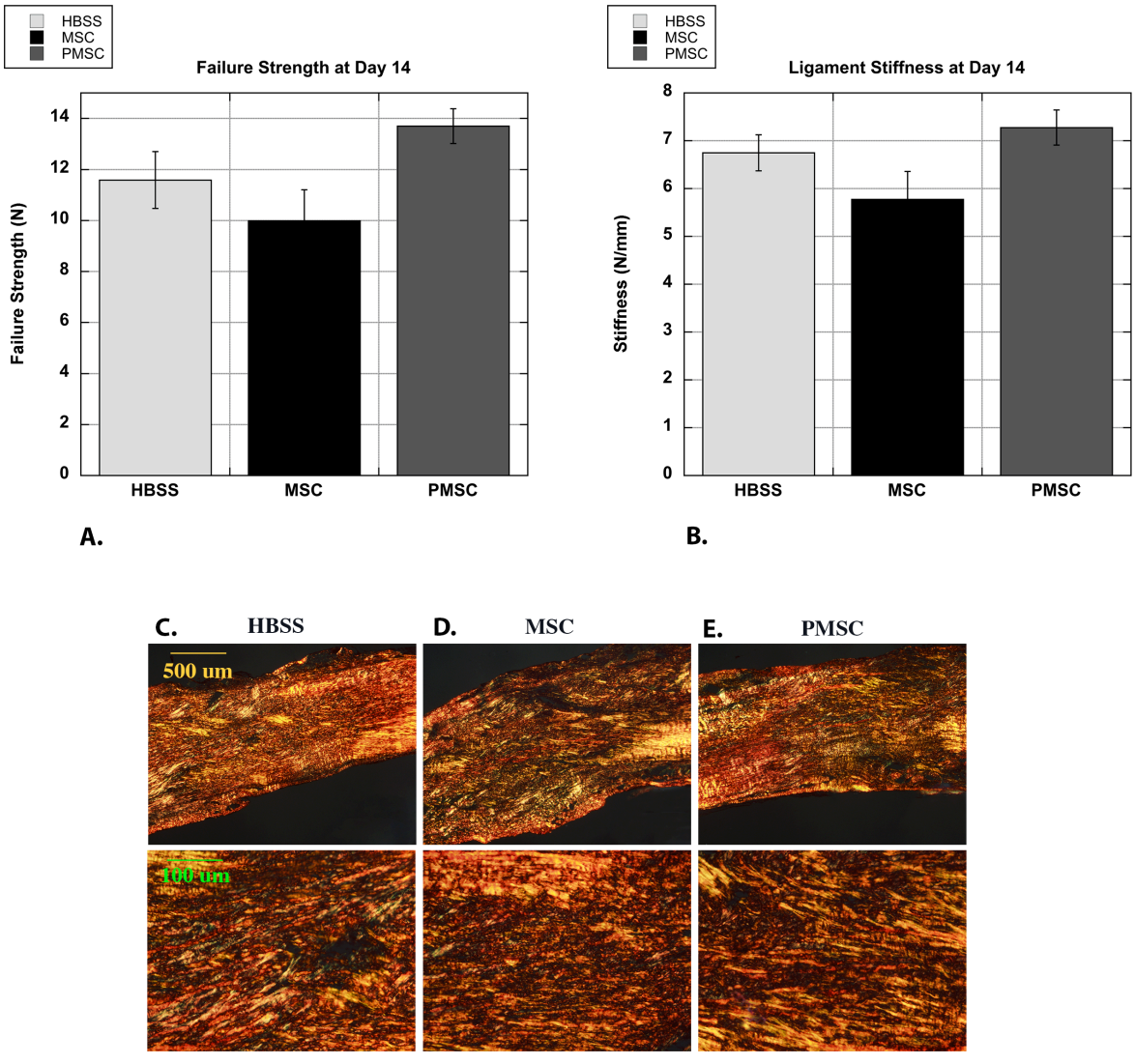


Fig. 3.10 A: At day 14, the primed MSC group had increased failure strength compared to the MSC group (p=.022) (PMSC 13.699±.850N, MSC 9.988±1.222N, HBSS 11.586±1.107N). B: At day 14, the primed MSC group had increased stiffness compared to the MSC group (p=.033) (PMSC 7.277±.424 N/mm, MSC 5.771±.584 N/mm, HBSS 6.747±.376 N/mm) Values are expressed as mean ± S.E.M. C-E: Ligaments were stained with Picrosirius Red and imaged using polarized light microscopy. Fractal dimension analysis (FA) and fast Fourier transformation (FFT) were used to quantify collagen linearity. There were no significant differences detected between groups at day 14 healing. C: Representative image of injured control ligament. D: Representative image of MSC group. E: Representative image of PMSC group.

Table 3.1 Numerical Values for Collagen Organization

	HBSS	MSC	PMSC
Fractal Dimension Analysis (FA)	1.766 ± .273	1.773 ± .274	1.772 ± .274
Fast Fourier Transformation (FFT)	1.398 ± .041	1.494 ± .052	1.474 ± .042

FA p= .8762 FFT p= .3149

MSC Localization and Function

MSCs in both the primed and unprimed groups localized to the healing region and healing region edges. At day 4, there was no difference in the number of MSCs in the healing region when comparing the primed and unprimed MSC groups (p=.8058, Fig. 3.11, A-D). At day 14, there were more DiI + MSCs in the unprimed group compared to the primed group (p=.06488) within the healing region (Fig. 3.11, E-H). Due to this change seen at day 14 in the number of MSCs, we performed a co-stain incorporating a proliferation marker (Ki67, data not shown). Upon observation of the healing region, there didn't appear to be a difference in proliferation of unprimed or primed MSCs, even though total cellular proliferation was increased throughout the MCL in the primed MSC group at day 4 (reported earlier).

As we examined MSC localization more closely, we noticed that they congregated around blood vessel lumen. In order to look more closely at cell fate, we co-stained for an endothelial cell marker (CD31) and a pericyte marker (CD146). Upon close examination, neither the primed or

unprimed MSCs were co-expressing these markers. Rather MSCs appeared to be interacting with both endothelial cells and pericytes via paracrine action and cell-to-cell contact (Fig. 3.12).

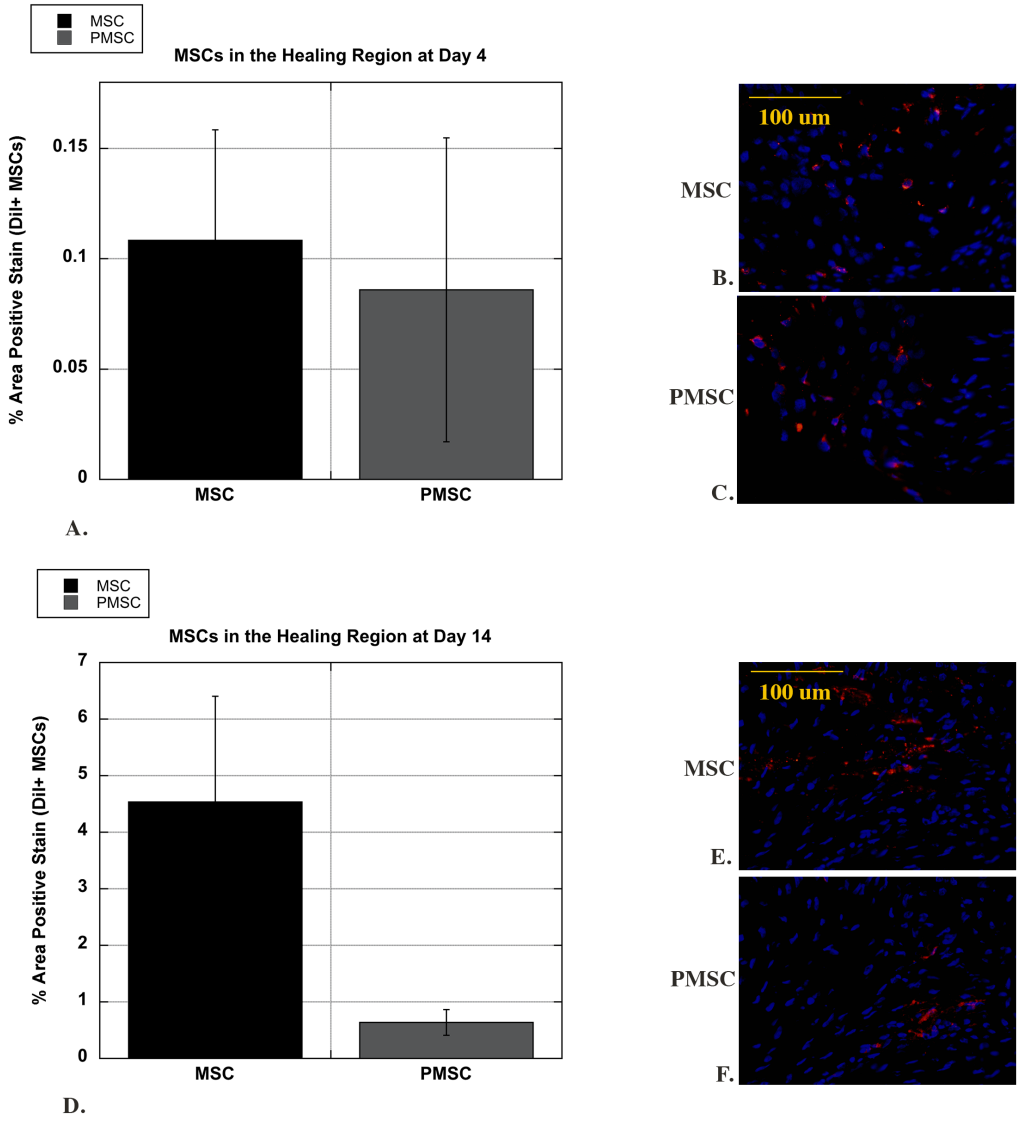


Fig. 3.11 A-C: At day 4 healing, there were no significant differences in the number of MSCs in the healing region between the primed and unprimed groups (PMSC .0860±.0689%, MSC .1083±.0501%). A: Graph comparing average percentage area stain for each condition. B: Representative image of IHC in MSC group. C: Representative image of IHC in PMSC group. D-F: At day 14, more MSCs remained in the healing region and healing region edges in the MSC group compared to the primed MSC group. (PMSC .637±.230%, MSC 4.535±1.866%) D: Graph comparing average percentage area stain for each condition. E: Representative image of IHC in MSC group. F: Representative image of IHC in the PMSC group. Values are expressed as mean area stain ± S.E.M.

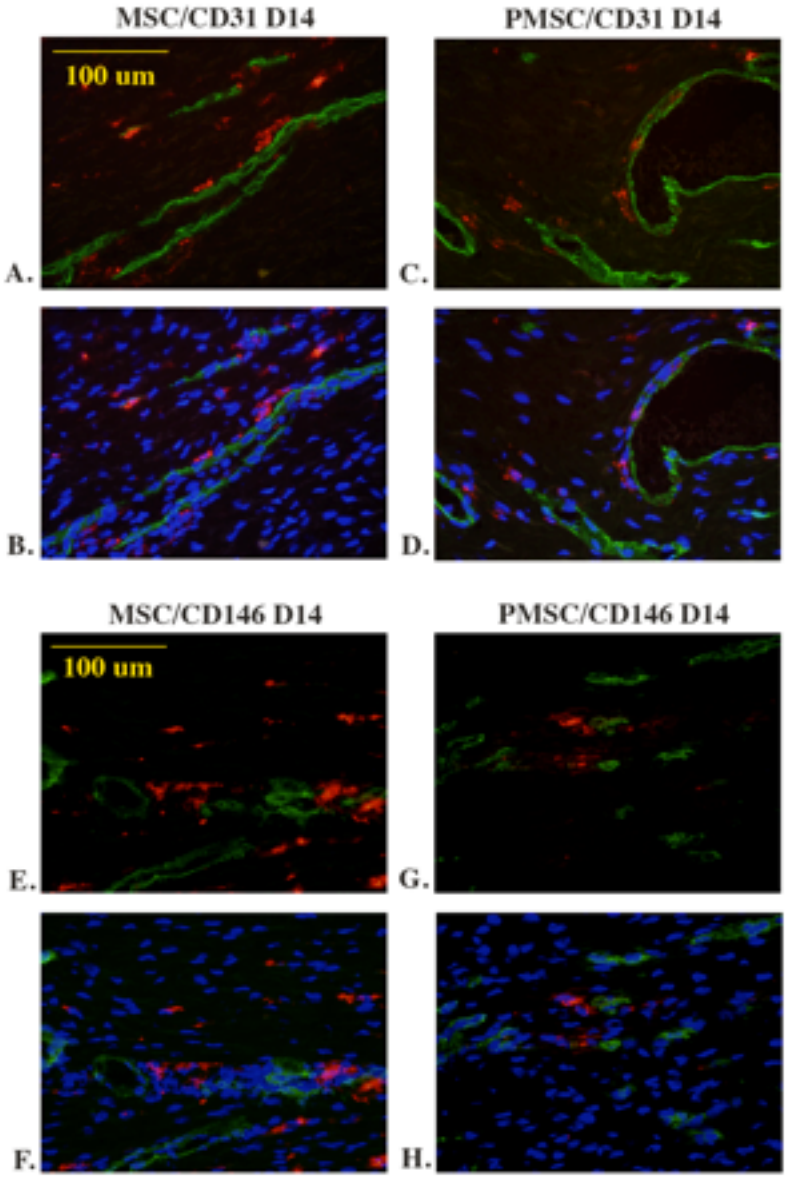


Fig. 3.12 A-D: At day 14 healing, we observed the localization of both primed and unprimed MSCs around lumen in the healing region of ligaments. A: Unprimed MSCs (red) co-localizing with endothelial cells (green) at day 14. B: Primed MSCs (red) co-localizing with endothelial cells (green) at day 14. C: Unprimed MSCs (red), endothelial cells (green), total cells (blue) at day 14. D: Primed MSCs (red), endothelial cells (green), total cells (blue) at day 14. E-H: At day 14, unprimed MSCs (E) and primed MSCs (G) did not express the common pericyte marker (CD146) and instead showed a similar co-localization as with endothelial cells (MSC = red, Pericyte = green, Total cells = blue).

Discussion

Altered Healing as it Relates to Priming

Closely examining differences between the primed and unprimed MSC groups allows us to outline any beneficial healing effects produced by priming MSCs prior to implantation. In our current study, the increased mechanical properties (failure strength and stiffness) in the primed MSC group is indicative of improved healing. Increasing the strength of ligaments allows for more aggressive rehabilitation protocols and has the potential to result in stronger ligaments once the remodeling phase of healing is complete. However, further studies testing later time points are needed to confirm this. It also appeared that the rate of healing was increased in the primed MSC group due to the early M2 infiltration. The primed MSC group had more M2s present in the healing region and an increased percentage of M2s to M1s during early healing (day 4). We believe this to be positive based on the role of M2s. M2s are more reparative and anti-inflammatory whereas M1s are more pro-inflammatory[31–33]. M1s generally enter the healing region early and kick off the inflammatory cascade by releasing pro-inflammatory cytokines and phagocytosing cellular and matrix debris. M2s are associated more with the resolution of healing due to a release of anti-inflammatory factors and matrix building proteins. It's important to note that both cell types are essential for healing and significantly decreasing macrophage number has proven to be detrimental to ligament healing[78]. However, tipping the balance of these cells by initiating an earlier M2 response was beneficial in our healing model due to their matrix building properties and ability to minimize the effects of an excessive inflammatory response. The exact mechanism employed by primed MSCs to influence the M2 response is

unknown but may be related to a less inflammatory healing environment, which we will discuss in greater detail in the next chapter.

Along with increased M2s in the healing region, there was increased procollagen 1α in the healing region at day 4 and in the healing region edges at day 14 in ligaments that received primed MSCs. As mentioned previously, procollagen 1α is the precursor to type 1 collagen which is the most abundant matrix component in ligaments. Therefore these increases during early and later healing may be contributing to the improved mechanical strength at day 14.

TGFβ was not significantly changed at day 4 between our treatment groups, but is worth discussing due to its important role during healing and the pattern of expression that occurred in our study. We saw an increase in TGFβ in the unprimed MSC group which is consistent with other published studies[75]. This molecule has been shown to be involved with MSCs anti-apoptotic effects[75] and immunomodulating capacity[75,79,80]. Specifically looking at healing, TGFβ is known to be produced by most cells in the healing environment[81] and promote collagen deposition[82]. TGFβ, along with TNFα, induce prostaglandin production by fibroblasts[83,84] which is another key molecule during healing. It is released by macrophages upon ingestion of apoptotic neutrophils[85] and with phagocytosis of tissue debris. Taken together, it would seem that increasing TGFβ would benefit healing ligament healing in several ways. Although not significant, we saw a dampening of TGFβ in the healing matrix that received primed cells. This is consistent with in vitro results that showed stimulating hMSCs with TLR3 ligand repressed TGFβ1,3 expression[47] in a similar fashion. These counterintuitive findings are representative of some of the challenges with elucidating a mechanism. The lack of an increase in TGFβ

expression in the primed group still resulted in better healing. Future studies that either up regulate or down regulate TGFβ in MSCs may help further elucidate its role.

The slight dampening of TGFβ seen throughout the primed group's matrix correlates with the improved M2:M1 ratio seen at day 4. An increased M2:M1 ratio suggests a decreased presence of phagocytic macrophages and therefore less phagocytosis, which would minimize TGFβ expression. Another study found that TLR3 activated MSCs prolong the life of neutrophils in vitro[86]. We did not look at neutrophil numbers, but if this is occurring in our model, a decrease in ingestion of apoptotic neutrophils could explain decreased TGFβ as well. The dampened TGFβ levels also coincide with decreased PGE2 detected in the primed MSC group. TGFβ is partly responsible for the induction of PGE2 and therefore the lower levels of TGFβ, though not significant, may be resulting in the lower levels of PGE2, which were significant.

This decrease seen in PGE2 was somewhat unexpected. PGE2 is extensively mentioned throughout the literature as a main mechanism for MSC's anti-inflammatory properties[59,60,87] and therefore we thought that priming with poly (I:C) would increase this anti-inflammatory cytokine as it did with an in vitro hMSC study[47]. However, we found the opposite with priming rat MSCs in vitro (data not shown) and now we found this same decrease in vivo. Our different findings may be explained by the length of time the cells were exposed to poly (I:C) and the use of rat MSCs vs human. PGE2, along with TGFβ, TNFα, and IFNγ, are difficult molecules to categorize into specific roles during healing because they shift functions throughout healing and can be anti-inflammatory as well as pro-inflammatory[85].

Finally, to explain the lack of an increase in TGFβ and increased procollagen 1α in the primed MSC group at day 4 is complicated. One would expect an increase in TGFβ production to match the increased procollagen 1α due to its collagen promoting activities. However, PGE2 suppresses collagen synthesis[88–90] and therefore the dampened levels of PGE2 may have minimized this inhibitory action, allowing deposition. As Nathan and Ding[91] so eloquently stated: "Thus it's not just the extinction or expression of mediators that is critical, but the orchestration of their succession – their tuning and timing."

Blood vessel formation is essential for healing, however, excessive formation later in healing is thought to be detrimental to healing ligaments due to mechanical deficits[72,73]. Due to the nature of vessel formation essentially burrowing holes into tissue, it is thought that the holes disrupt matrix integrity and can ultimately affect mechanical strength. In the current study, there was an early increase in endothelial cells in both the primed and unprimed groups compared to control ligaments, with the primed MSC group displaying the greatest amount. This early increase in endothelial cells is beneficial due to the ability to allow nutrient infiltration into the injured areas and minimizing the hypoxic effect on native ligament cells. After injured tissue receives the blood flow necessary for healing, it's important for the vessels to stop sprouting or even retract so as not to affect mechanical properties. At day 14, there were fewer endothelial cells in the primed MSC group compared to the unprimed group, which may have contributed to strength gains measured.

VEGF was not significantly altered at day 4, but a pattern emerged where the primed MSC group had the greatest levels of VEGF followed by the unprimed MSC group and the HBSS controls

having the lowest amounts. This is worth discussing due to the fact that this correlates with significant changes in endothelial cells at day 4. VEGF is a potent inducer of angiogenesis and is most active during the proliferative and remodeling phases of healing[92]. However, it also plays a role in cell proliferation and migration[92]. Increased VEGF correlates with ingrowth of vasculature in tendons which provides extrinsic cells, nutrients, and growth factors to the healing region[73]. Therefore kicking this cascade off early during injury has the potential to improve healing, as was shown in our study. Increased VEGF production by MSCs is thought to be a key activity contributing to their anti-apoptotic and angiogenic properties[75,93].

The increased M2s and procollagen 1α, early increase and later decrease in endothelial cells, combined with decreased apoptosis later in healing suggests that priming MSCs prior to implantation may be improving the rate of healing resulting in stronger ligaments compared to unprimed MSCs at day 14. The MSC group had the lowest levels of procollagen 1α matrix deposition of all 3 groups at day 4 healing and there wasn't a rebound or significant increase noted at day 14. Although not significant, it's also important to note that the MSC group had the lowest ratio of M2:M1s at day 4 and day 14. These factors may be contributing to this group's tissue strength deficits at day 14. The current findings suggest that the level of matrix deposition early is important in determining ligament functional properties later in healing.

Engraftment as it Relates to Priming

One of the questions with cell therapy is whether the cells engraft and what their functional role is within the healing environment. Previous studies, including our MSC dose study, have shown limited MSC engraftment indicating that engraftment may not be necessary for beneficial

effects[76,94,95]. An interesting observation to note in the current study is that there were fewer DiI+ MSCs remaining in the healing environment for the primed group compared to the unprimed group. Yet the primed group demonstrated improved healing based on several measures. This strengthens the argument that cell engraftment is not essential. It also suggests that the priming had an effect on cellular fate. Priming may have affected engraftment by altering several cellular characteristics. One reasonable explanation may be that priming changed the cells homing, chemotactic, and/or adhesion properties. There are several studies that have examined this but no consensus has been reached. An in vitro study showed decreased migration with TLR3 priming for 24 hours using our same concentration of poly(I:C) in human MSCs, whereas priming for shorter periods of time led to enhanced migration in vitro[47]. Another study treated hMSCs for an even shorter amount of time (4 hours) with poly(I:C) in vitro and reported increased cell migration[96]. A group of researchers used porcine MSCs and applied a higher concentration of poly(I:C) (4ug/ml). They reported no change in migration in vitro upon exposure to poly(I:C) for 24 hours[48]. Finally, others examined the expression of 2 key molecules involved in cell migration (CXCR4, CXCR7) upon exposing hMSCs to poly(I:C) (10ug/ml) for 6 hours and found that these molecules were significantly down-regulated[97]. These findings represent the variability found in the literature and are most likely due to different species of MSCs, length of time cells are primed, concentration and identity of the priming agent, in vivo versus in vitro, animal model, along with the injury/disease model studied in the animal model. A general observation regarding the aforementioned studies suggests that higher concentrations or longer exposures to poly(I:C) can decrease migratory capabilities in vitro. Further studies are required to determine any conclusive patterns related to time and concentration, and more information is needed to describe this phenomenon in vivo. Although not proven, it's possible

that the longer time frame of 48 hours used in our current study may have altered these migratory properties during rat ligament healing.

Another explanation revolves around the limitation of our fluorescent membrane stain on MSCs. As MSCs divide and proliferate, the membrane stain will become lighter. Therefore if MSCs in the primed MSC group proliferated at a faster rate, it's possible that they would be harder to detect due to decreased fluorescent intensity. Our proliferation data suggest that this probably is not the reason for fewer MSCs in the primed group. Although proliferation was increased throughout the matrix in the primed MSC group, the MSCs themselves did not appear to be proliferating extensively at the time points measured. Again, there is a lack of consensus in the literature due to different experimental methods regarding proliferation. A couple studies showed either decreased or no change in proliferation in vitro with TLR3 activation of MSCs[48,98].

Lastly, another potential reason for the differences found in MSC number is that there was increased apoptosis in the primed MSC group leading to fewer cells in the matrix at day 14. Our apoptotic data at day 4 and 14 suggest that this is probably not the case. Although apoptosis was greater in the primed group at day 4, it was not significantly greater than the MSC group. At day 14, there was significantly less apoptosis in the healing region (where MSCs localized) in the primed group compared to both the unprimed and HBSS groups. Some published data suggests that priming MSCs with TLR4 ligands can promote their survival and decrease the rate of apoptosis, however, it is unclear as to whether TLR3 ligands have the same effect[99,100]. If there was increased cell death prior to engraftment in the primed MSC group, they may have been

cleared from the environment via the circulatory system before we could accurately measure through IHC.

Fate of MSCs/Co-localization

Cells injected into the healing region of ligaments may serve multiple functions and contribute to healing in several ways. One of the prominent roles the cells display in the current study is an interaction with endothelial cells and pericytes. Both primed MSCs and unprimed MSCs localize to regions of blood vessel formation at day 14 of healing. It was also shown that MSCs localize near pericytes during healing when measured at day 14. This suggests that the MSCs may be playing a supportive role for endothelialization via paracrine action and cell-to-cell contact with both endothelial cells and pericytes. An extensive study by Crisan et al.[101] demonstrated a perivascular origin of mesenchymal stem cells throughout various human organs both in fetal and adult tissue. However, there is still a debate as to whether MSCs are identical to a pericyte and it's thought that pericytes may demonstrate a greater level of plasticity[102]. Feng et al.[103] reported a dual origin of MSCs during tissue repair with some MSCs of pericyte origin whereas others were not of pericyte origin. Although our focus has been more on the paracrine effects of MSCs, it's also interesting to note potential cell fate. In this study, it appears that at least one major role of these cells is supporting endothelial cells and pericyte function either through cytokine release such as increased VEGF production or cell-to-cell interactions. Our data suggests that the MSCs in this study are not pericytes based on the lack of expression of a common pericyte marker (CD146). However, other pericyte markers exist and therefore further examination is necessary to make conclusive statements.

This study highlights the potential benefits of priming cells prior to application into injured ligaments. MSC therapy for ligaments and tendons have varying results with some studies demonstrating enhanced healing, while others showing no improvement. This is one of the challenges when working with MSCs and relates to the fact that cells may differ in quality based on collection and manufacturing methods. Priming is a method to activate cells prior to implantation and may increase their efficacy. Priming may also be a way to test a cell's quality and trophic factor producing ability. A way to improve priming in the future will involve sorting the cells prior to application so that the most active and responsive cells are administered and the less reactive cells can be eliminated. Future studies should focus on optimizing the priming of MSCS to exploit the early matrix building properties seen in this study. Improving the efficacy and efficiency of MSC therapy through priming has the potential to increase the rate of healing and result in stronger ligaments. Quicker strength gains post-injury will minimize the time needed to rehabilitate these injuries and could decrease the risk for re-injury.

Chapter 4: Local and Systemic Cytokine Changes Resulting from Primed and Unprimed Mesenchymal Stem Cell Administration

Introduction

In the previous chapter we discussed improved mechanical properties seen in the primed MSC group compared to the unprimed MSC group. We observed changes in cellular response and matrix deposition and hypothesized that these may be influencing mechanical properties. To get a better understanding of how the various cell types involved in healing are functioning via cytokine regulation and to be able to describe the microenvironment in more detail, we measured 13 cytokines known to have varying effects on inflammation during healing. Local changes within the healing ligament along with systemic changes in blood serum levels were analyzed.

Modulating the immune/inflammatory response is believed to be a therapeutic approach to improve healing and has been attempted in a few different ways. This idea holds promise due to several of the 1st cellular responders to injury being inflammatory cells that kick off the healing cascade[6]. Therein lies an opportunity to tweak the initial response. It's also been shown that weaker ligaments post-injury can be the result of an excessive inflammatory response leading to overzealous remodeling and becoming a ligament made up of scar-like tissue. A few of the common methods used to knockdown the response include anti-inflammatory drugs, macrophage depletion and cytokine modulation such as addition of anti-inflammatory cytokines IL-1Ra and IL-4. Our lab has studied several of these methods to try to improve ligament healing with moderate success. Using IL-1Ra injections, we were able to increase procollagen type 1α, however, this did not lead to an increase in mechanical properties[104]. Interestingly, a single

injection of IL-1Ra was more beneficial based on healing modulation measures compared to multiple injections suggesting that some level of inflammation is useful[104]. Another study looking at a single IL-1Ra injection showed a decrease in several pro-inflammatory cytokines, along with an increase in IL-10 (anti-inflammatory), although again there were no significant changes in mechanical properties[105]. A different study used IL-4 injections and found similar changes to that of IL-1Ra injections which involved increased procollagen 1α. However, continued treatment with IL-4 had the opposite effect and actually slowed healing [106]. The limitation with injections of a single cytokine is that often times the effects are transient and the action is streamlined rather than being multifactorial. It also appears that when trying to bypass the transient effect with multiple injections, the result worsens. This suggests that immune/inflammatory modulation of a single factor may not be the most effective approach to ligament healing and choosing a method that is responsive to cues in the environment for more complex modulation, such as cells, may lead to better outcomes. This makes cell therapy a potential target therapy and in need of development.

MSCs are no longer just used for their differentiation capacity, but rather used for paracrine effects, release of trophic factors and immunomodulatory functions[25]. The cells are capable of releasing a number of biological factors that affect the injured environment. Some of the aspects of healing that MSCs modulate include the immune response, angiogenesis, apoptosis, scarring, support and growth of other cells, and chemoattraction[75]. All these processes are intertwined and coordinated for proper healing. Due to our belief that controlling the inflammatory response is a key to improved ligament healing and decreased scar tissue formation, we measured 13 common cytokines used to define environments as pro-inflammatory or anti-inflammatory. This is a

simplistic view due cytokines having dual actions and complex interactions. However, we believe this is a good start to surveying the environment. Due to the fact that we found improved healing using primed MSCs from a mechanical and cellular perspective, by completing this study we were able to define patterns of cytokine expression that are associated with the improved mechanical and cellular outcomes.

The purpose of injecting the primed and unprimed cells into an injured ligament is to use these cells as a drug and compare effects. Like other pharmacological agents, these cells not only affect the area where they are applied, but also have systemic effects. A drawback of pharmaceuticals is that they are generally designed as a single agent with a single purpose. MSCs are attractive since they can release multiple factors and be environmentally responsive[87]. This allows for a more complex healing paradigm, which seems necessary for optimal outcomes. Focusing on immunomodulation, some key cytokines outlined in a review by Murphy et al.[87] include PGE2, TGFβ1, HGF, SDF-1, NO, IDO, IL-4, IL-6, IL-10, IL-1Ra and soluble TNFαR. In the previous chapter, we analyzed PGE2 and TGFβ as they relate to matrix deposition. Here we discuss their pro-inflammatory and anti-inflammatory roles as well as look at IL-1Ra expression in response to cell therapy. We will also measure 10 common cytokines involved in healing: GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 and TNFα.

First, examined changes in cytokine levels locally within the injured ligament. Next, we monitored changes in serum cytokine levels to determine whether there were any systemic effects resulting from a local MSC injection. Measuring serum levels also gave us the opportunity to measure multiple time points to better understand the temporal fluctuations

occurring with injury and MSC therapy. For both local and systemic measures, we focused on early time points of healing to capture any changes that potentially alter the progression of the remaining healing cascade. The body is remarkable at compensating to maintain a prototypical healing response and therefore the goal was to capture early fluctuations before the body could self correct. For local analysis, we measured cytokine levels at day 4 of healing. For systemic measures, we examined days 1, 2, 3, and 4 post injury and normalized the levels to pre-injury baseline measures. *We hypothesized that the primed MSC group would be more effective with decreasing pro-inflammatory cytokines as well as increasing anti-inflammatory cytokines compared to both the MSC group and the HBSS controls.*

Materials and Methods

Experimental Design

The normal healing cascade of the rat medial collateral ligament has been well characterized[6] and therefore we chose to use this injury model in order to have a comparison to analyze how MSCs modulate healing. This model focuses on extra-articular ligament healing. Bilateral MCLs were transected and MSCs were administered to the injured region at the time of surgical transection. 3 groups were compared: unprimed MSCs ($1x10^6$ cells), primed MSCs ($1x10^6$ cells), and a control group (no cells). The number of cells were determined from our previous study showing $1x10^6$ cells to be an adequate dose to improve ligament healing[76]. Blood was collected (200 ul) from the tail artery prior to surgery (D0) and at days 1, 2, 3, 4 (D1-4) post surgery in order to capture the early changes in serum cytokine levels upon primed and unprimed MSC treatment. Fifteen adult male Wistar rats (275-299g) underwent bilateral MCL surgery (5

per group). Animals were euthanized at day 4 and MCLs were processed for cytokine analysis and immunohistochemistry.

Surgical Procedure

All procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee. Rats were prepared for surgery using sterile technique and anesthetized using isoflurane. A longitudinal skin incision was made medially at the femoral-tibial junction. The MCL was exposed by creating an incision through the subcutaneous tissue and gracilis muscle. A uniform, complete transection of the MCL was created just distal to the joint line. A scalpel blade was used to transect MCLs in order to improve reproducibility. Bilateral MCL transections were performed on each animal and both MCLs received the same treatment in order to avoid confounding variables due to systemic effects. MCLs were not repaired with suture. For the treatment groups, cells suspended in 25 ul of Hanks Balanced Saline Solution (HBSS; Hyclone Laboratories Inc, Logan UT) were injected in the transected region while the control group received 25 ul of HBSS (no cells). After cell/HBSS administration, the gracilis muscle and skin were closed using 5-0 vicryl suture. The animals were not immobilized post-surgery and were allowed unrestricted cage mobility. Animals were euthanized at day 4 post-operatively and the right MCL was used for IHC while the left MCL was homogenized and used for multiplex cytokine analysis.

Serum Collection

Blood was collected from the tail artery of each animal starting D0 (day of surgery, prior to any procedures) and collected daily through D4 (day 4 post-op, prior to euthanization). A 25 gauge

needle was used to draw 200 ul of blood. Blood was transferred to a Capiject Micro Collection Tube (Terumo Medical Corporation, Somerset, NJ), allowed to clot for 30 minutes, and then centrifuged at 5,000 rpms for 5 minutes. The serum was collected from the tubes and transferred to a microcentrifuge tube and frozen until analysis could take place.

Mesenchymal Stem Cell Culture and Priming

Rat mesenchymal stem cells were obtained from a commercial vendor (Cyagen Biosciences Inc, Santa Clara, CA) at passage 5. Cells originated from the bone marrow of Fisher 344 rats and cultured as a monolayer. Prior to receiving the cells, they were transfected by the vendor using an EmGFP-expressing lentiviral construct and selected with hygromycin. Flow cytometry was used to select specific cell markers. Cells were positive for CD44 and CD90 (>70%), as well as negative for CD34, CD11b, CD45 (<5%). Tri-lineage differentiation potential was confirmed with osteogenic, chondrogenic, and adipogenic assays. Once the cells were received from the vendor, they were seeded in flasks and expanded using Mesenchymal Stem Cell Growth Medium (Cyagen Biosciences Inc, Santa Clara, CA) consisting of MSC basal medium, MSC-qualified fetal bovine serum, penicillin-streptomycin, and glutamine. Media was changed every 3-4 days and flasks were maintained in an incubator at 37° C and 5% CO_2. Cells were passaged upon reaching 70% confluency and morphology was monitored throughout passaging to ensure a spindle-like appearance. Cells selected for the priming group received media that contained polyinosinic acid and polycytidylic acid (Poly (I:C), Sigma Aldrich) at a concentration of 1 µg/ml. The cells were exposed to this priming agent for 48 hours prior to final collection. All cells were collected at passages 8 through 10 for injection into the injured ligaments.

Just prior to surgery, Trypsin EDTA (Cellgro, Manassas, VA) was used to remove MSCs from flasks. Cells were fluorescently labeled with Celltracker CM-DiI (Life Technologies, Grand Island, NY) so spatial distribution of the original MSCs could be analyzed in vivo. After fluorescent label application, cells were counted using the Tali® Image-Based Cytometer (Life Technologies, Grand Island, NY) and suspended in HBSS at a concentration of $1x10^6$ cells/25ul HBSS.

Immunohistochemistry

At day 4 post-injury, ligaments designated for IHC were frozen in optimal cutting temperature (OCT), longitudinally sectioned (5μm), mounted on Colorfrost Plus microscope slides and stored at -80° C. Rabbit monoclonal or polyclonal primary antibodies were used to measure selected cytokines. Upon antibody application, slides were thawed, fixed with acetone and then treated with 3% hydrogen peroxide to prevent endogenous peroxidase activity. Non-specific antibody-protein interactions were blocked by applying Background Buster (Innovex Biosciences, Richmond, CA) or Rodent block R (Biocare Medical, Concord, CA) to each slide for 30 minutes. Ligament sections on slides were exposed to primary antibodies at optimized concentrations for 2 hours. Following primary antibody application, a biotin-linked secondary antibody and streptavidin conjugated to horseradish peroxidase tertiary antibody were applied for 10 minutes each using a Stat Q staining kit (Innovex Biosciences, Richmond, CA) or a rabbit-on-rodent HRP polymer (Biocare Medical, Concord, CA). Diaminobenzidine (DAB) was used as the chromagen to detect the antibody-antigen complex.

Rabbit monoclonal or polycolonal antibodies were used to detect transforming growth factor beta (TGFβ; 1:100; Abcam; Cambridge, MA), prostaglandin E2 (PGE2; 1:100, Abcam; Cambridge, MA), and interleukin-1 receptor antagonist (IL-1RA, 1:200, Abcam; Cambridge, MA).

Cytokine Analysis

Serum cytokine levels were analyzed from D0 (prior to injury) through D4. Serum was pooled (n=5 per group) and analyzed using a rat cytokine 10-plex kit (Life Technologies, Grand Island, NY). The proteins measured included GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 and TNFα. Serum samples were run in triplicate and read using a Luminex Magpix (Life Technologies, Grand Island, NY) system. A standard curve was created for each cytokine and verified to ensure 80-120% recovery and a detection baseline of 2 standard deviations above background. Day 1, 2, 3, and 4 cytokine levels were normalized to day 0 to account for any variability between groups of rats. A 96 well plate was prepared with each sample and incubated overnight with primary antibodies at 4° C on a plate shaker. A biotinylated secondary antibody and streptavidin-RPE tertiary antibody were applied the following day to allow for antibody detection. In order to obtain accurate and repeatable measurements, serial dilutions of standards, along with a positive control (spleen) and negative control (lysis solution) were included in each well plate.

The same 10-plex rat cytokine kit was use to measure cytokine levels in rat MCLs at day 4 of healing. Ligaments were collected, homogenized and pooled (n=5 per group) at day 4 post-injury. Like the serum samples, each group of ligaments was run in triplicate using the Luminex Magpix system. MCLs were washed in Cell Wash Buffer (Bio-Rad, Hercules, CA) and exposed

to Lysing Solution (Bio-Rad, Hercules, CA) upon being placed in Navy Bead Lysis Kit tubes (Next Advance, Averill Park, NY). Ligaments were homogenized using a Bullet Blender (Next Advance, Averill Park, NY) and centrifuged to separate soluble and insoluble proteins. Supernatant (soluble protein content) was collected and frozen for cytokine analysis and total protein measurement (Pierce BCA Protein Assay, Rockford, IL). Cytokine levels were normalized to total protein measures to account for any variability in rat ligament size.

Statistics

A one-way analysis of variance (ANOVA) was used to examine differences between the control group, unprimed MSC group and primed MSC group. If the overall p-value for the F-test in ANOVA was significant, post-hoc comparisons were performed using Fisher's least significant difference (LSD). Experimental data is presented as the least squares means ± standard error of the means (SEM) of replicates. All p-values reported are two sided and a value of $p < 0.10$ for the F-test in ANOVA was used as the criterion for statistical significance. Kaleidagraph, version 4.03, was used for all computations.

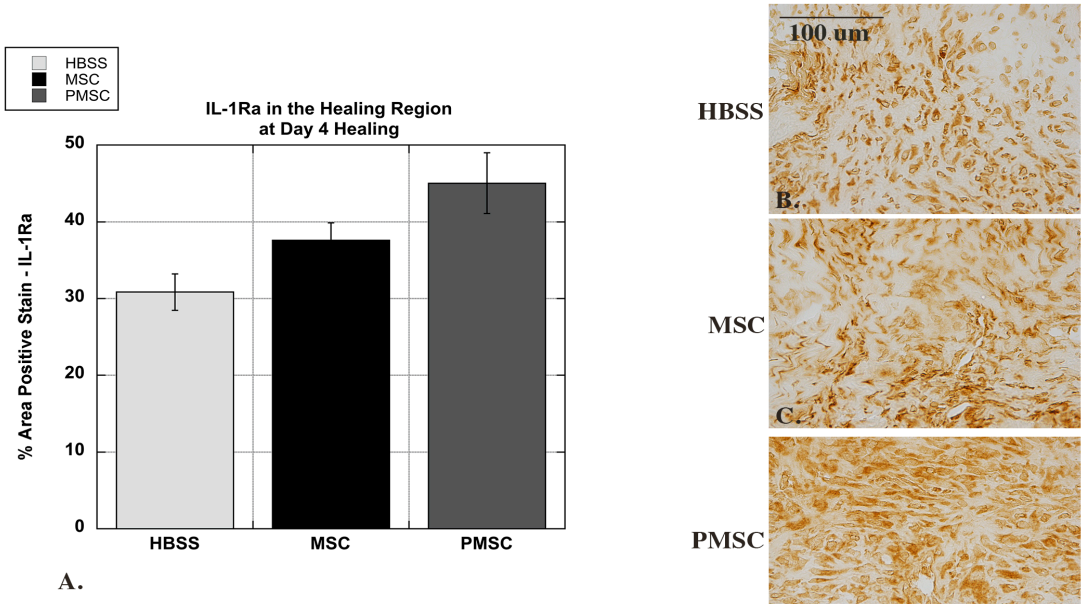
Results

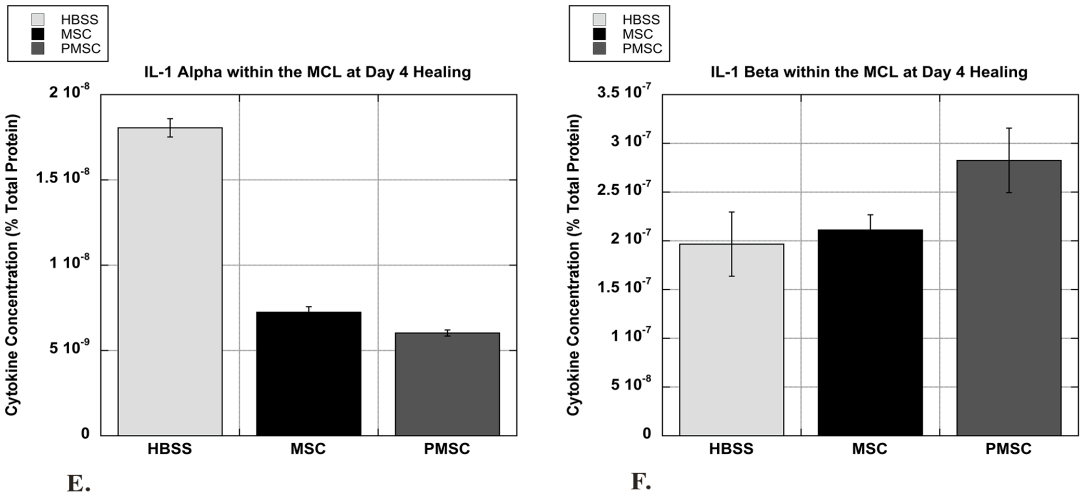
Ligament Cytokines

Of the 10 cytokines (rat multi-plex assay) measured locally within the ligament at day 4 post-injury, 4 were at detectable levels (IL-1β, IL-1α, IL-10, and IL-12) based on the standard curve created for each cytokine (Table 4.1). Of the 4 cytokines detected, IL-1α and IL-12 levels were significantly different between groups, whereas IL-10 and IL-1β did not reach a level of

significance. IHC was performed to look at IL-1Ra levels, and significant changes were detected at day 4 in the ligament.

IL-1Ra is an anti-inflammatory cytokine that competes with binding sites for pro-inflammatory IL-1β and IL-1α. At day 4, the primed MSC group had increased IL-1Ra within the healing region and healing region edges compared to the control group (PMSC vs. HBSS p=.0057). It was also increased compared to the MSC group, however, not to a level of significance (PMSC vs. MSC p=.1115, Fig. 4.1 A-D). Il-1α, a pro-inflammatory cytokine, was significantly higher in the control group compared to the MSC group (p<.0001) and primed MSC group (p<.0001). The primed MSC group had the lowest levels of IL-1α compared to both the MSC group (p=.0633) and control group (Fig. 4.1, E). Il-1β, a pro-inflammatory cytokine and key initiator of the inflammatory cascade, was not significantly different between groups at day 4 healing (p=.152, Fig. 4.1, F). However, the primed MSC group had the greatest levels, followed by the MSC group and then the HBSS controls. IL-10, an anti-inflammatory cytokine, was detected within the healing ligament but there were no significant changes between groups (p=.508, Fig. 4.2, A). IL-12, which is a pro-inflammatory cytokine, had the highest expression in the primed MSC group and lowest levels in the MSC group at day 4 healing (PMSC vs MSC p=.0008, PMSC vs HBSS p=.0615, MSC vs HBSS p=.0076, Fig. 4.2, B).





A-D: At day 4 healing, the primed MSC group had more IL-1Ra in the healing region and healing region edges compared to the HBSS group (p=.006) and the MSC group, although not signficant (p=.112). The MSC group had increased IL-1Ra compared to the HBSS group, but not to a level of significance (p=.127) (PMSC 45.021±3.962%, MSC 37.575±2.276, HBSS 30.832±2.384%). A: Graph comparing average percentage area stain for each condition. B: Representative image of IHC in control ligament. C: Representative image of IHC in MSC group. D: Representative image of IHC in PMSC group. E: At day 4 healing, the primed MSC group had decreased levels of IL-1α compared to the unprimed MSC group (p=.0633) and control ligaments (p<.0001). The MSC group also had significantly less IL-1α compared to the HBSS group (p<.0001) was lowest in the primed MSC group. F: There were no significant differences in IL-1β levels between groups at day 4 healing, although the primed MSC group expressed the highest levels (PMSC vs. HBSS p=.0766, PMSC vs MSC p=.1252, HBSS vs. MSC p=.7351). Values are expressed as mean area stain ± S.E.M.

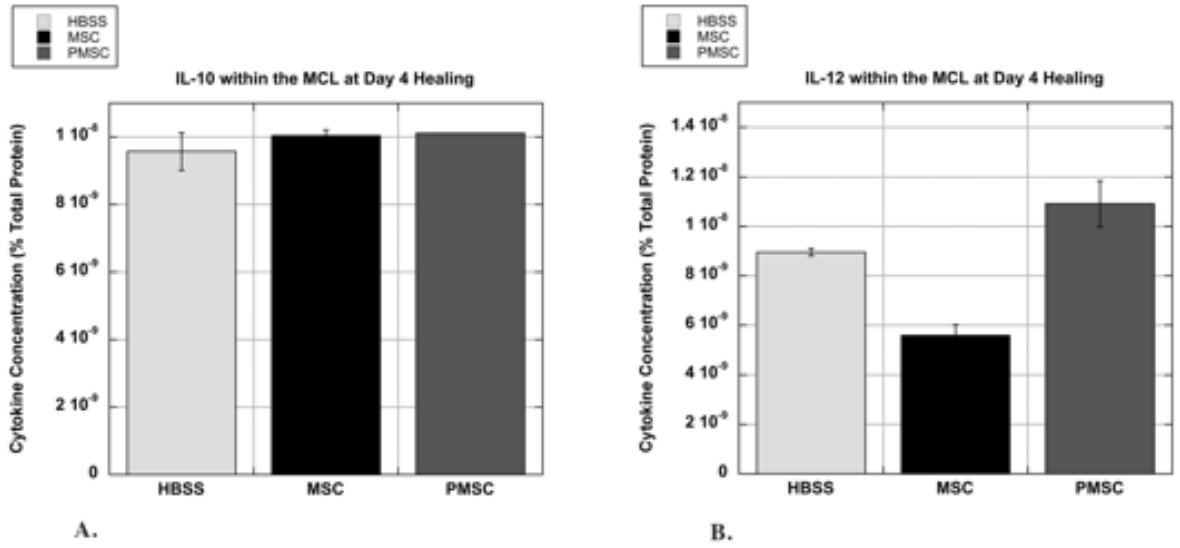


Fig. 4.2 A: There were no significant differences in IL-10 levels at day 4 between treatment groups (HBSS vs. MSC p=.3616, HBSS vs. PMSC p=.3002, PMSC vs. MSC p=.8886). B: At day 4 healing, the primed MSC group had increased levels of IL-12 compared to the unprimed MSC group (p=.0008) and the control group (p=.0615). The MSC group had less IL-12 compared to the control group (p=.0076).

Serum Cytokines

At days 1 through 4 post-injury, 9 of the 10 cytokines measured using the rat multi-plex assay were detected in blood serum (Table 4.2). GM-CSF, generally considered a pro-inflammatory cytokine, measured at significantly different levels between groups on days 1-3 post-surgery. At day 1, the primed MSC had the lowest level (below baseline), whereas the HBSS and MSC groups were above baseline, with the HBSS group having the highest level (PMSC vs HBSS p=.0002, MSC vs HBSS p=.0744, PMSC vs MSC p=.0014). This exact pattern was seen at day 2 as well (PMSC vs HBSS p<.0001, MSC vs HBSS p=.0004, PMSC vs MSC p=.0446) . At day 3, the primed MSC group still remained the lowest and was below baseline, whereas the HBSS and MSC groups were both above baseline, but not significantly different from each other (PMSC vs HBSS p=.0326, PMSC vs MSC p=.0158). Although not significant at day 4, the pattern of

cytokine expression changed from the previous 3 days with the MSC group have the lowest levels and the primed MSC group having the highest values (p=.2106, Fig. 4.3, A).

TNFα is another pro-inflammatory cytokine involved in healing. At day 1 of healing, both the primed and unprimed MSC groups had significantly higher levels of this cytokine compared to the HBSS group and both were above baseline (PMSC vs HBSS p=.0073, MSC vs HBSS p=.0037). At day 2, the MSC group remained high, while the PMSC substantially dropped below baseline and was significantly lower than both the MSC and HBSS groups (PMSC vs HBSS p=.0019, MSC vs HBSS p<.0001, PMSC vs MSC p<.0001). At day 3, the HBSS and PMSC groups were similarly below baseline and significantly less than the MSC group, which was above baseline (MSC vs HBSS p=.0100, PMSC vs MSC p=.0042). At day 4, the TNFα levels increased again in the primed MSC group above baseline and were significantly higher than the HBSS controls which remained below baseline (PMSC vs HBSS p=.0280, Fig. 4.3, B).

IL-6 is a dual action cytokine but is mostly considered pro-inflammatory in our healing model. At day 1, the IL-6 levels in the MSC group are well above baseline and significantly higher than the HBSS and primed MSC groups (MSC vs HBSS p=.0376, PMSC vs MSC p=.0210). At day 2, the HBSS and primed MSC groups drop below baseline, with the primed MSC group being significantly lower than the MSC group (PMSC vs MSC p=.0134). At day 3, a similar pattern is seen with the primed MSC group now significantly lower than both the HBSS and MSC groups. The primed MSC group is the only group below baseline at this time point (PMSC vs HBSS p=.0005, MSC vs HBSS p=.0091, PMSC vs MSC p<.0001). At day 4, all 3 groups are below

baseline levels. The primed MSC group still has the lowest level, although there are no longer significant differences between groups (Fig. 4.3, C).

IL-12, as mentioned earlier, is a pro-inflammatory cytokine and associated with type 1 macrophages. At day 1, all groups were below baseline with the MSC group being significantly less than the HBSS and primed MSC group (MSC vs HBSS p=.0009, PMSC vs MSC p=.0009). At day 2, both the MSC and primed MSC groups were below the HBSS group (PMSC vs HBSS p=.0022, MSC vs HBSS p=.0036). At day 3, the MSC group had the highest level of IL-12 followed by the HBSS group, while the primed MSC group had the lowest amount (PMSC vs HBSS p=.0003, MSC vs HBSS p=.0027, PMSC vs MSC p<.0001). By day 4, there were no significant differences between groups. Although there were significant changes between the groups throughout the first 3 days of healing, the levels all stayed below baseline and the changes were relatively minor compared to other cytokines (Fig. 4.3, D).

IL-2 is generally considered pro-inflammatory and related to T cell actions. At day 1 post-injury, the serum level dropped below baseline in the MSC group and was significantly lower than the HBSS and primed MSC groups (MSC vs HBSS p=.0560, PMSC vs MSC p=.0218). At day 2, the MSC and primed MSC groups were below baseline and significantly less than the HBSS group (PMSC vs HBSS p=.0463, MSC vs HBSS p=.0087). At day 3, the primed MSC group remained significantly lower than both the HBSS and MSC groups (PMSC vs HBSS p=.0106, PMSC vs MSC p=.0062), but by day 4 the groups were no longer statistically different (Fig. 4.4, A).

IL-4 is considered an anti-inflammatory cytokine and involved with conversion of the M1 to M2 phenotype. At day 1, all 3 groups were around baseline and not significantly different (p=.1697). At day 2, the MSC group dropped below baseline and had significantly less cytokine compared to the HBSS and primed MSC groups (MSC vs HBSS p=.0079, PMSC vs MSC p=.0062). However, at day 3 the MSC group rebounded and had significantly higher IL-4 levels compared to the primed MSC group and was slightly above baseline (PMSC vs MSC p=.0395). At day 4, the primed MSC group had significantly higher serum IL-4 levels compared to the MSC and HBSS groups. At this same time point, the HBSS group had higher levels compared to the MSC group and was just above baseline levels (PMSC vs HBSS p=.0073, MSC vs HBSS p=.0775, PMSC vs MSC p=.0009, Fig. 4.4, B).

IL-1α, as mentioned previously, is a cytokine associated with necrotic cells and is considered pro-inflammatory. At day 1, all 3 groups were below baseline levels and the primed MSC group had the highest level and the HBSS group had the lowest level (PMSC vs HBSS p=.0006, MSC vs HBSS p=.0247, PMSC vs MSC p=.0124). At day 2, there was a large spike in the IL1α levels in the MSC group well above baseline whereas the primed MSC and HBSS group levels remained below baseline. The HBSS group remained the lowest at day 2 (PMSC vs HBSS p=.0004, MSC vs HBSS p<.0001, PMSC vs MSC p<.0001). At day 3, all groups were well below baseline with the HBSS group being significantly lower than the MSC and primed MSC groups (PMSC vs HBSS p=.0034, MSC vs HBSS p=.0022). At day 4, the HBSS and MSC groups had similar levels of cytokine and the PMSC group was significantly higher. All 3 groups were well below baseline at this time point (PMSC vs HBSS p=.0001, PMSC vs MSC p<.0001, Fig. 4.4, C).

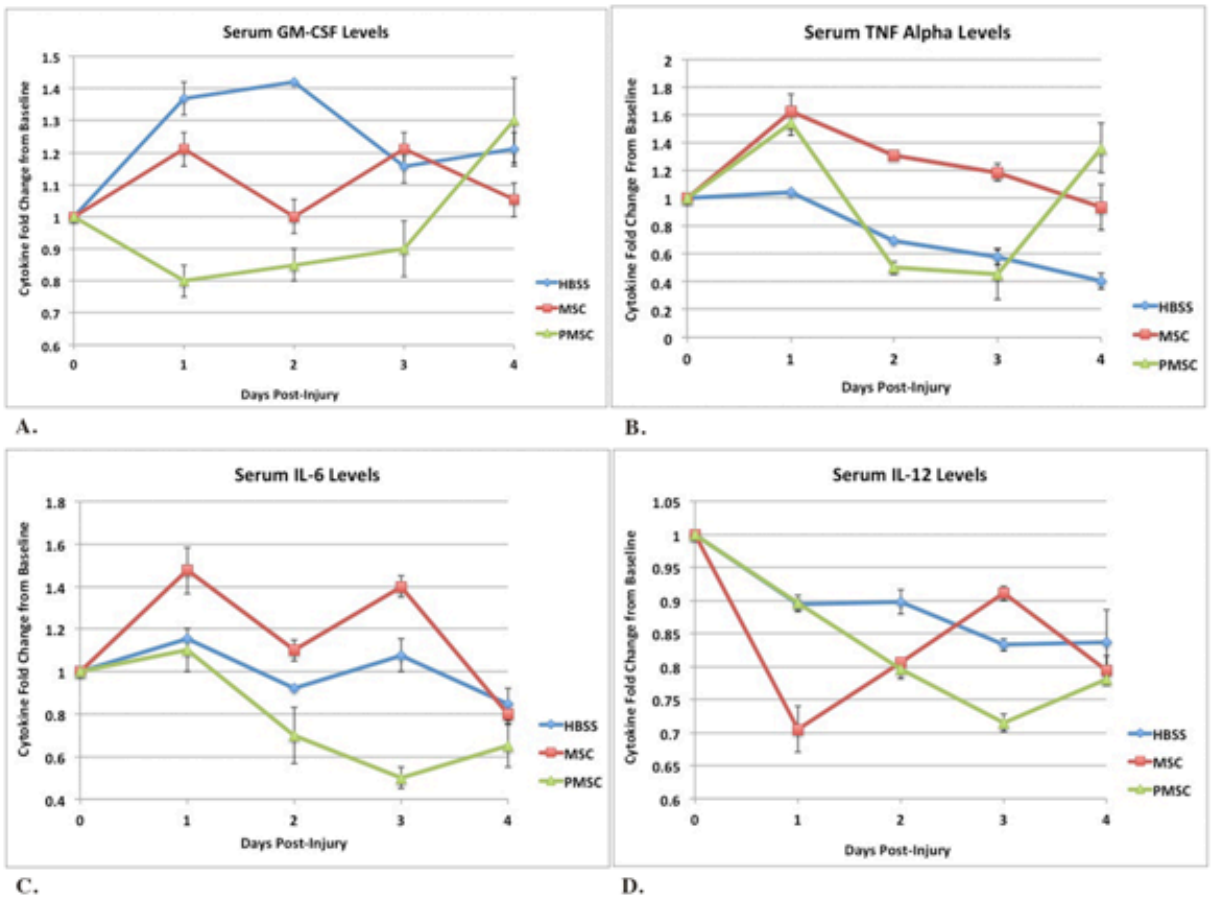


Fig. 4.3 A: GM-CSF was significantly decreased in the primed MSC group ($p<.05$) compared to the MSC group and HBSS group days 1-3 post-injury in the serum. B: After an initial increase in TNFα levels day 1 post injury, levels were significantly decreased in the primed MSC group compared to the unprimed MSC and control group ($p<.05$) 2 days post-injury and significantly decreased compared to the unprimed MSC group ($p<.05$) 3 days post injury. C: The primed MSC group had significantly lower levels of IL-6 days 1-3 post injury compared to the unprimed MSC group ($p<.05$) along with lower levels compared to the control group reaching a level of significance at day 3 post-injury ($p<.05$). D: There was a consistent decrease in IL-12 levels days 1-3 post-injury in the primed MSC group which were significantly lower than the unprimed MSC and control groups ($p<.05$) at day 3 post-injury.

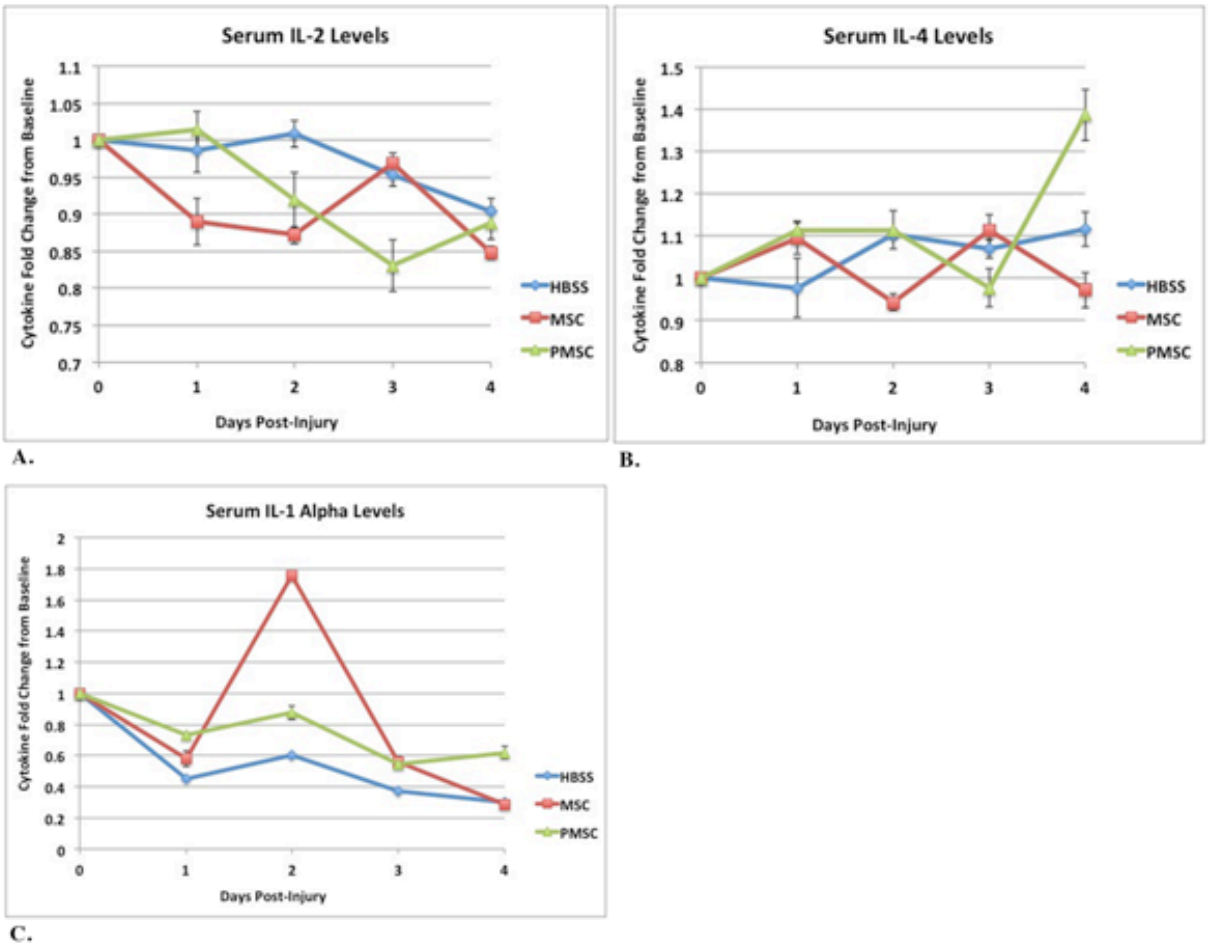


Fig. 4.4 A: The primed MSC group had significantly lower levels of IL-2 compared to the control group ($p<.05$) 2 days post-injury and significantly lower levels compared to the unprimed MSC and control groups ($p<.05$) 3 days post-injury. B: IL-4 levels were significantly increased in the primed MSC group compared to the unprimed MSC and control groups at 4 days post-injury ($p<.05$). C: IL-1α levels in the primed MSC group followed the same pattern of expression as the control group days 1-4 post-injury, but remained significantly elevated ($p<.05$) at all time points.

IL-10, an anti-inflammatory cytokine previously mentioned, is associated with M2 macrophages and ECM building activities. At day 1, all 3 groups were above baseline with the HBSS and MSC groups significantly higher than the PMSC group (PMSC vs HBSS p=.0044, PMSC vs MSC p=.0101). At day 2, the HBSS group had the highest level of IL-10 while the primed MSC group had the lowest level, which was just above baseline (PMSC vs HBSS p<.0001, MSC vs HBSS p=.0027, PMSC vs MSC p=.0007). At day 3, the primed MSC IL-10 levels remained low

and measured around baseline compared to the HBSS and MSC groups. At this time point, the MSC group had significantly higher cytokine levels compared to the HBSS group (PMSC vs HBSS p=.0045, MSC vs HBSS p=.0292, PMSC vs MSC p=.0003). At day 4, all 3 groups were above baseline and had similar levels of IL-10 (Fig. 4.5, A).

IFNγ is considered pro-inflammatory but also has anti-inflammatory and anti-fibrotic effects during healing. At day 1, the primed MSC group had increased IFNγ above baseline and was significantly higher than the MSC and HBSS groups (PMSC vs HBSS p=.0004, MSC vs HBSS p=.0682, PMSC vs MSC p=.0025). At day 2, this same pattern was seen with the primed MSC group having above baseline levels, the MSC group having baseline levels and the HBSS group having below baseline levels (PMSC vs HBSS p=.0005, MSC vs HBSS p=.0134, PMSC vs MSC p=.0136). At day 3, the HBSS cytokine levels remained below baseline and significantly lower than the MSC and primed MSC groups. The MSC group had the highest level of cytokine at day 3 (PMSC vs HBSS p=.0227, MSC vs HBSS p=.0002, PMSC vs MSC p=.0031). At day 4, the primed MSC group had the highest level of IFNγ and was above baseline. The HBSS group had significantly lower levels compared to the MSC and primed MSC group and were below baseline levels (PMSC vs HBSS p<.0001, MSC vs HBSS p=.0006, PMSC vs MSC p<.0001, Fig. 4.5 B).

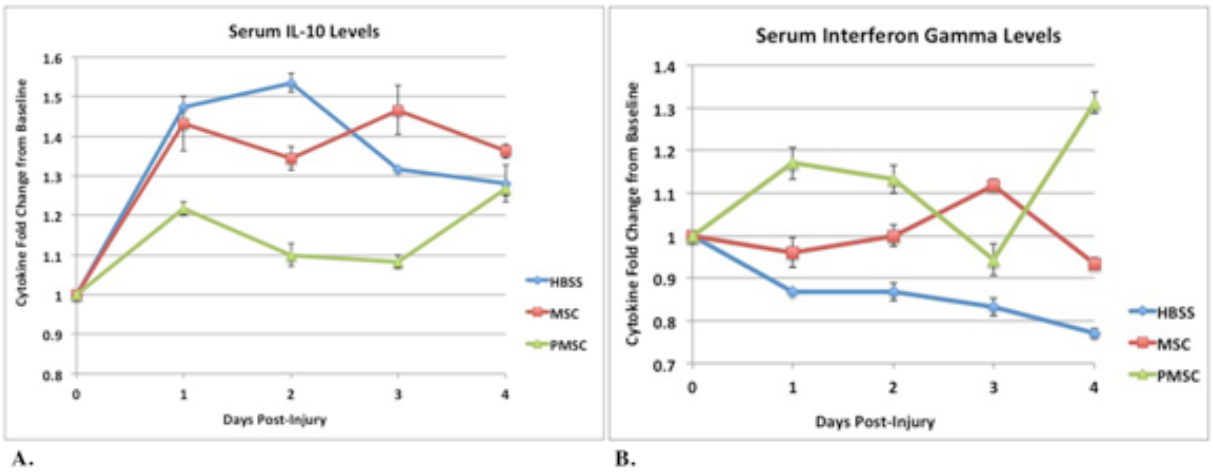


Fig. 4.5 A: IL-10 levels were significantly decreased in the primed MSC group days 1-3 post-injury compared to the unprimed MSC group and control group ($p<.05$). B: IFNγ levels were significantly increased in the primed MSC group compared to the unprimed MSC and control groups at days 1, 2, and 4 post-injury ($p<.05$).

Discussion

Ligament Cytokines

Il-1 cytokines (IL1β, IL1α, IL1Ra) play a large role in healing and have been a target for improving healing by our lab and other labs. Il-1 is a key initiator of the inflammatory process by signaling nearby cells and thus activating the clotting cascade followed by the release of downstream cytokines and growth factors[107]. Due to IL-1's role in the beginning of the inflammatory cascade, modulating this cytokine early suggests that this could impact and control later events with healing. Therefore the changes seen in this study in IL1 cytokines may be a contributing factor to the improved healing seen in the primed MSC group.

IL1β is produced by monocytes and macrophages[108,109], aids in the recruitment of neutrophils and monocytes[110] and can stimulate the release of other pro-inflammatory cytokines[111]. IL1α is associated with neutrophil[112] and other inflammatory cell recruitment when released by necrotic

cells during sterile injury[113,114]. Although IL1α is released by necrotic cells, the main producer of IL1β and IL1α during sterile inflammation are macrophages[109]. IL1β and IL1α both activate the same receptor (IL-1R1) which propagates the inflammatory response[108]. Il-1Ra also binds this same receptor but does not induce IL-1 signaling[108]. Rather it blocks the receptor so that IL1α and IL1β are unable to bind and therefore minimizes the inflammatory response acting as an antagonist. A study by Chen[113], found that IL1α may have a more essential role with the recruitment of inflammatory cells compared to that of IL1β. They found that antibodies to IL1α inhibited the neutrophil number (inflammatory response) whereas antibodies to IL1β did not. This study also reported that IL-1 is a potent neutrophil recruiter and appears less involved with the recruitment of monocytes. Therefore blocking the IL-1 pathway has therapeutic potential without altering monocyte infiltration which allows monocytes to function in host defense and tissue repair[113].

The interplay of IL1α, IL1β, and IL1Ra described above may explain the improved healing we saw in the primed MSC group indicating an anti-inflammatory effect. The primed MSC group, which resulted in the best functional ligament healing demonstrated by improved mechanical strength, had increased IL1Ra and decreased IL1α compared to the control group. The primed group also had significantly less IL1α compared to the unprimed MSC group. Since IL1α is pro-inflammatory and IL1Ra is anti-inflammatory, the respective decrease and increase of these molecules in the primed group appear to be altering the local ligament healing environment to be less inflammatory.

However, we cannot conclude that the healing environment is entirely anti-inflammatory based off the fact that IL-12, a pro-inflammatory cytokine was increased in the primed MSC group. IL-12 is associated with the pro-inflammatory type 1 macrophage phenotype[31,32]. IL-12 is known to induce lymphocyte release of IFNγ[85] which has pro and anti-inflammatory properties. Il-12 promotes differentiation of the Th1 phenotype which mediates host defense to viruses and intracellular pathogens, mainly by the production of IFNγ[115]. This cytokine may be playing a role in our model due to our priming methods with poly (I:C). Poly (I:C) is a synthetic version of viral double stranded RNA. Even though the cells were washed prior to injection into our ligaments, it's possible that some poly (I:C) entered the healing region triggering a host viral response. Although IFNγ was not at detectable levels within our homogenized ligaments, it was significantly increased in the animal's serum (discussed later). Even though IL-12 is mostly considered pro-inflammatory, it also has anti-fibrotic effects that could be beneficial in our healing model (anti-scar)[18].

Another molecule we were interested in was PGE2 due to previous research demonstrating an immune modulating role within MSCs[59,60]. PGE2 has can be classified as both pro-inflammatory and anti-inflammatory depending on timing and the surrounding environment[85]. Initially, PGE2 furthers acute inflammation however, due to a feedback loop, PGE2 can suppress later stages of inflammation[50,85]. One of PGE2's anti-inflammatory actions is dampening IL-12 production[27]. In our study, priming MSCs lead to a suppression of PGE2 expression both in vitro and in vivo. This dampening of PGE2 seen at day 4 may explain why the primed MSC group had an increase in IL-12 whereas the unprimed MSC group which had higher levels of PGE2, had lower levels of IL-12.

IL-10 is an anti-inflammatory cytokine that plays a key role in suppressing excessive inflammatory responses[116] and is associated with the anti-inflammatory macrophage phenotype[31–33]. Increases in IL-10 can also be the result of increased PGE2 levels[27]. Due to the increased number of M2s in the primed MSC group, there was the potential to see increased IL-10 levels. Another scenario could have been increased IL10 in the unprimed MSC group due to higher PGE2 expression. However, there were no significant differences between groups within homogenized ligaments at day 4 healing. This illustrates the complexity involved in the regulation of cytokines and the fact that predictions cannot be made off of single factors. Clearly there are many factors at play orchestrating the healing path for each treatment group.

TGFβ plays an active role throughout healing and is produced by most cells in the healing environment[81,92]. Like several other cytokines (PGE2, IFNγ, IL-2) it has both pro-inflammatory and anti-inflammatory properties which are time and context dependent[85]. TGFβ stimulates collagen deposition[82] which is important for ECM production and healing, however, too much TGFβ can lead to adhesion formation[117]. TGFβ aids in the transition of monocytes to macrophages which leads to granulation tissue formation[107] and is a profibrotic factor that enhances αSMA and collagen type I expression[118,119]. TGFβ is released from macrophages after they phagocytose neutrophils and helps initiate the tissue repair process[85]. This molecule is a potent suppressor of classical macrophage activation[120] and a critical mediator of tissue repair[91]. We analyzed TGFβ expression at day 4 of healing and did not find any significant differences between groups. This was unexpected since we measured an increase in procollagen 1α in the primed MSC group (chapter 3). Even though there was not significant differences in TGFβ

levels there was a pattern seen throughout the ligament with the unprimed MSC group expressing increased TGFβ and primed MSCs expressing similar levels of TGFβ as HBSS controls. Therefore, it is possible that priming MSCs changed their TGFβ expression, which has been shown by other researchers[47].

Serum cytokines

Some interesting findings emerged when analyzing serum cytokine levels demonstrating systemic implications for local application of primed and unprimed MSCs. One of the patterns that emerged upon analysis is that the primed group had a general dampening of pro-inflammatory cytokines GM-CSF, TNFα, IL-6, and IL-12 during early healing. This was followed by an increase detected in TNFα in the primed group at day 4.

GM-CSF immediately decreased in the primed MSC group at day 1 and remained low for the first 3 days. It rebounded at day 4 which may have been a compensatory correction by the immune system. Previous research has identified GM-CSF as a key cytokine during the inflammatory phase due to its ability to increase the number and function of neutrophils[121]. GM-CSF also has a role in up regulating IL-6[122], which is a cytokine with both pro and anti-inflammatory functions. In the current study, IL-6 gradually decreased at days 2 and 3 and then started to increase at day 4 in the primed MSC group. Therefore the dampening of GM-CSF and IL-6 correlated well systemically. IL6 (along with IL1 and TNFα) has proven to be an early cytokine that is up regulated during the inflammatory phase of wound healing[123,124] and plays a large role in kicking off the inflammatory cascade. It is produced by monocytes and neutrophils[107] and can influence the differentiation of monocytes to macrophages[125]. On the

other side of regulation, IL-6 also has a role in resolving acute inflammation by inducing neutrophil apoptosis[126]. We believe the dampening of this cytokine during the early stages of healing in the primed MSC group is beneficial due to its role as an early inflammatory initiator.

TNFα is another key cytokine involved in the early inflammatory phase thus kicking off the healing cascade (along with IL1 and IL6 mentioned above). At low levels, TNFα promotes healing indirectly by stimulating inflammation and growth factor release from macrophages[107]. However, high levels of TNFα become problematic due to its ability to increase MMP synthesis while inhibiting ECM protein synthesis and TIMP production[127–130]. IL1β leads to a similar scenario during healing and these 2 cytokines can perpetuate each other's activity[131]. Both IL1β and TNFα are high is chronic wounds along with high MMP activity[107]. TNFα and IL-1 are released by leukocytes (neutrophils and macrophages) after they remove cellular debris[109]. These 2 cytokines up regulate leukocyte adhesion molecules which recruits more immune cells and results in increased production of growth factors and proteases by macrophages[132]. Unfortunately our detection methods for IL-1β in the serum were unsuccessful so we can't comment on serum levels of this cytokine. In the current study, very little fluctuation was seen in TNFα levels in the control group. Levels remained at baseline at day 1 and then gradually declined days 2-4. The MSC group spikes at day 1 and gradually decreases to around baseline by day 4. The primed MSC group spiked at day 1, then dropped drastically for 2 days before increasing at day 4. IL-12, which was discussed in detail in the ligament cytokine section, is a pro-inflammatory cytokine that was also dampened during the first few days of healing in the primed MSC group. Based on the role of GM-CSF, IL-6, TNFα, and IL-12 during healing, the

decreased levels seen in the primed MSC group during the first days of healing indicates systemic anti-inflammatory action of these cells.

Even though the dampening of pro-inflammatory cytokines offers some mechanistic details about MSC therapy, all cytokines measured did not follow this pattern. Anti-inflammatory IL-10 and dual action IFNγ responded differently. IL-10 was decreased in the primed group whereas IFNγ was consistently increased in the primed group during the first several days of healing. It's difficult to determine what lead to a decrease in IL-10 and may be related to systemic monocyte and macrophage regulation. However, the increased IFNγ may be an outcome based on our priming methods using poly (I:C) (mentioned earlier). One of the main roles for interferons in the body is to exert anti viral effects[133]. Poly (I:C) is a synthetic version of viral double stranded RNA and therefore an IFNγ response may be appropriate. This could explain the consistent increase of IFNγ seen in the primed MSC group during the first few days of healing.

IFNγ is produced by T cells and NK cells[118]. This cytokine acts in a pro-inflammatory fashion by increasing the expression of intracellular adhesion molecule 1 and the adherence of lymphocytes to endothelial cells[134]. IFNγ is also known to act early to induce macrophage chemokine production, but later acts in more of an anti-inflammatory fashion by suppressing it[135]. IFNγ, along with TNF, TGFβ, and PGE2, can be classified as both pro and anti inflammatory depending on the timing of expression and healing context. Another characteristic of IFNγ is its ability to suppress collagen synthesis[136,137]. Since TGFβ and IFNγ employ opposite effects on collagen synthesis, the balance of these 2 cytokines in the healing environment are likely important mediators of tissue repair and homeostasis[118]. TNFα acts

synergistically with IFNγ to inhibit collagen synthesis[138] and therefore TNFα levels need to be factored in to the healing scenario. IFNγ levels in the control group remained consistently below baseline days 1-4 post-injury. The MSC group's IFNγ levels fluctuated around baseline whereas the primed MSC group's levels were consistently above baseline (with the exception of day 3) and reached its highest level at day 4.

Previous studies have shown other anti-inflammatory effects of IFNγ demonstrated by a decreased foreign body response to an implanted device upon local administration of IFNγ[139], and decreased acute inflammatory response (decreased PMNs) upon systemic administration of IFNγ[60]. This is a tricky molecule to analyze in our healing model due to its many effects. We believe the anti-inflammatory effects are beneficial to healing ligaments, however, molecules that favor collagen synthesis would also be beneficial compared to molecules that inhibit synthesis. This is due to the fact that the main ECM protein of ligaments is collagen I and therefore early deposition has the potential to increase the rate of healing. Since IFNγ has anti-inflammatory effects as well as inhibitory effects on collagen deposition, the desired response of this molecule is not clearly obvious. However it's important to note that although systemic levels of this molecule were elevated in the primed MSC group, it did not have an inhibitory effect on collagen synthesis locally (chapter 3).

By day 4 analysis of serum cytokines, 5 of the 9 cytokines detected in the serum (GM-CSF, IL-6, IL-12, IL-2, and IL-10) had leveled out and were no longer different between groups. All of these have been discussed previously with the exception of IL-2. Less of a clear pattern was established in the serum for IL-2 with each group displaying unique modulation. In the primed

MSC group, IL-2 decreased at days 2 and 3 and then started to increase at day 4. IL-2 is usually thought of as pro-inflammatory due to its role as a T cell growth factor[115], its proliferative effects on NK cells[140], and its ability to promote antibody production and proliferation of B cells[141]. However, IL-2 is context dependent and has roles during healing that can be considered anti-inflammatory. Il-2 is essential for regulatory T cell development and thus participates in immune homeostasis[142,143]. IL-2 has the ability to increase the responsiveness of cells to IL-4 (anti-inflammatory) and therefore promotes the Th2 arm of cellular differentiation which is considered more anti-inflammatory[144]. IL-2 is mainly produced by CD4+ T cells, some CD8+ T cells, NK T cells, activated DCs and mast cells[115]. Our healing studies in the MCL haven't elucidated a large role for T cells[6,76] at this point and therefore we haven't studied this cytokine extensively.

The 4 cytokines that remained elevated in the primed group at day 4 were IL-4, IFNγ, TNFα, and IL1α. Of these 4 cytokines, IL-4, IFNγ, and TNFα are known to have anti-inflammatory properties depending the timing and comparative expression levels of other cytokines. IL-4 is an anti-inflammatory cytokine and promotes the differentiation of Th2 cells which produce IL4, IL5, and IL13[115]. IL-4 skews the macrophage phenotype towards an M2 phenotype which is more anti-inflammatory and reparative[31–33]. It invokes macrophages to release TGFβ, PDGF, and arginase. One product of arginase (ornithine) is a substantial source of proline and hydroxyproline which account for almost 25% of the residues in collagen[91]. Therefore the presence of IL4 in a healing environment is crucial for matrix building. A lot of fluctuation was detected in IL-4 cytokine levels without a consistent pattern in any of the groups in the current study. The IL-4 level at day 4 in the primed MSC group was the highest detected in any of the

groups days 1 through 4 and correlated with the increased M2 levels found in the ligament matrix outlined in the previous chapter.

IL1α (discussed earlier) is a pro-inflammatory cytokine that remained high in the primed group at day 4. This is interesting because a very different pattern of IL-1α is measured locally in the ligament at day 4 of healing with the primed MSC group having the lowest level locally. Another notable observation in serum IL-1α is the large spike seen in the MSC group at day 2. It's difficult to determine the cause of these patterns systemically but it could be an indication of necrotic MSCs being removed from the local environment, since it has been shown that few cells remain in the healing region. Since the MSC and primed MSC groups had much lower IL-1α in the ligament itself, the cells that do remain there may be guarding against necrosis. The HBSS group, which did not receive any cells, remained the lowest or tied for the lowest serum levels throughout all 4 days of cytokine analysis.

Along with IL-1α, IL-12 and IL-10 levels in the serum did not match the patterns seen in the ligaments. This raises the question as to how systemic levels of cytokines affect local sites of injury and vice versa. More detailed studies examining more time points are necessary to start to answer this question. One explanation is a potential lag between systemic and local levels of cytokines due to the time it takes for cells to communicate with each other and react via cytokine production. Another explanation could be that different regulation is occurring throughout the body versus at the injury site. A limitation with drawing conclusions for this comparison is the amount of ligament tissue required for accurate analysis. In order to get enough soluble proteins for cytokine measurements, the entire ligament is homogenized which could dilute the effects

occurring in the more active healing region. Future studies using a larger animal model or more animal ligaments isolating the injured region may provide a more accurate comparison. This would increase the likelihood of measuring more cytokines within detectable limits for tissue homogenate allowing advanced conclusions.

Overall, this general dampening of pro-inflammatory cytokines combined with an increase in anti-inflammatory cytokines appears to be a key contributor to the improved healing and stronger ligaments in the primed MSC group at day 14. Regardless of outcomes, it is clear that local application of MSCs has both local and systemic implications and priming MSCs has the ability to change both these microenvironments. Previous studies have shown systemic changes upon systemic delivery[145], however, less research exists as to what happens throughout the body up local delivery. This is important to analyze in order to optimize dosage and minimize any potential side effects.

The patterns of cytokine expression outlined above can serve as a comparison for future studies looking at the potential of unprimed and primed MSC therapy and how it alters healing from an inflammatory and matrix building perspective. Capturing the correct time point for cytokine levels in ligaments is difficult and requires a lot of animals. Therefore monitoring serum levels for several days offers some insight and can minimize the number of research animals. It is difficult to predict synergies and antagonisms among cytokines due to the complexity in regulation. However, by continuing to analyze the various fluctuations paired with functional outcomes, knowledge regarding optimal temporal and spatial fluctuations will be obtained.

Table 4.1 Numerical Values for Cytokine Levels within MCLs (% total protein)

Cytokine	Group	Day 4 (% of total Protein)
IL-1α	HBSS	$1.8061^{-08} \pm 5.3475^{-10}$
	MSC	$7.2412^{-09} \pm 3.2914^{-10}$
	PMSC	$6.0294^{-09} \pm 1.7734^{-10}$
IL-1β	HBSS	$1.9675^{-07} \pm 3.2903^{-08}$
	MSC	$2.1098^{-07} \pm 1.5706^{-08}$
	PMSC	$2.8250^{-07} \pm 3.3012^{-08}$
IL-10	HBSS	$9.5710^{-09} \pm 5.5659^{-10}$
	MSC	$1.0039^{-08} \pm 1.6457^{-10}$
	PMSC	$1.0108^{-08} \pm 0$
IL-12	HBSS	$8.9535^{-09} \pm 1.5437^{-10}$
	MSC	$5.5955^{-09} \pm 4.3542^{-10}$
	PMSC	$1.0906^{-08} \pm 9.3418^{-10}$

Table 4.2 Numerical Values for Cytokine Levels within Serum (fold change from baseline)

Cytokine	Group	Day 1	Day 2	Day 3	Day 4
IL-1α	HBSS	.4486 ± .007	.6052 ± .011	.3679 ± .004	.3002 ± .011
	MSC	.5776 ± .049	1.754 ± .011	.5603 ± .038	.2866 ± .015
	PMSC	.7303 ± .017	.8764 ± .044	.5449 ± .027	.6180 ± .039
GM-CSF	HBSS	1.368 ± .053	1.421 ± 0	1.158 ± .053	1.211 ± .053
	MSC	1.211 ± .053	1.000 ± .053	1.211 ± .053	1.053 ± .053
	PMSC	.8000 ± .050	.8500 ± .050	.9000 ± .087	1.300 ± .132
IL-6	HBSS	1.154 ± 0	.9231 ± 0	1.077 ± .077	.8460 ± .077
	MSC	1.475 ± .109	1.100 ± .050	1.400 ± .050	.8000 ± .050
	PMSC	1.100 ± .100	.7000 ± .132	.5000 ± .050	.6500 ± .100
IL-4	HBSS	.9767 ± .070	1.105 ± .012	1.070 ± .023	1.116 ± .040
	MSC	1.094 ± .038	.9434 ± .019	1.113 ± .038	.9717 ± .041
	PMSC	1.114 ± .023	1.114 ± .045	.9773 ± .046	1.386 ± .060
IL-12	HBSS	.8944 ± .007	.8979 ± .019	.8330 ± .009	.8359 ± .050
	MSC	.7049 ± .035	.8053 ± .007	.9108 ± .011	.7939 ± .022
	PMSC	.8955 ± .013	.7957 ± .014	.7147 ± .013	.7804 ± .008
TNFα	HBSS	1.044 ± 0	.6957 ± 0	.5797 ± .058	.4058 ± .058
	MSC	1.625 ± .125	1.313 ± 0	1.188 ± .063	.9375 ± .165
	PMSC	1.545 ± .091	.5000 ± .045	.4545 ± .182	1.364 ± .182
IL-2	HBSS	.9854 ± .029	1.009 ± .018	.9532 ± .016	.9035 ± .018
	MSC	.8901 ± .032	.8717 ± .012	.9686 ± .014	.8482 ± .009
	PMSC	1.014 ± .024	.9190 ± .038	.8310 ± .035	.8873 ± .021
IFNγ	HBSS	.8675 ± 0	.8675 ± .021	.8313 ± .021	.7712 ± .012
	MSC	.9605 ± .035	1.000 ± .026	1.118 ± .013	.9342 ± .013
	PMSC	1.170 ± .038	1.132 ± .033	.9434 ± .038	1.311 ± .025
IL-10	HBSS	1.474 ± 0	1.535 ± .023	1.316 ± 0	1.281 ± .046
	MSC	1.431 ± .069	1.345 ± .030	1.466 ± .062	1.362 ± .017
	PMSC	1.217 ± .017	1.100 ± .029	1.083 ± .017	1.267 ± .017

Chapter 5: Discussion and Conclusions

Contributions to the field

Dosage and Cell Therapy

Using MSCs to improve ligament and tendon healing is not a new concept. There are several studies that have used MSCs in different injury models (ACL, rotator cuff) with varying results[41–43]. However, there are very few studies examining the effect of cell dosage. One of the key findings of our first study is that more MSCs do not lead to superior results. The lower dose proved to be more beneficial. This is an important point to consider with clinical application of MSCs. It seems there's a belief that all that is required is the application MSCs to an injured area and the cells and/or the body will take care of the rest. Although these cells have a variety of therapeutic capabilities, it would appear that a set of correct cues is still necessary to exploit their healing characteristics. Our study shows potential for improved ligament healing using MSCs, however, optimizing dosage for different injury/disease applications will be an important factor for successful outcomes.

Another interesting finding with dosage of MSCs is that the larger dose had some pro-inflammatory characteristics. The literature consistently talks about MSCs and their anti-inflammatory properties, however, less is mentioned in regard to potential pro-inflammatory effects. The pro-inflammatory characteristics found using the higher dose was demonstrated by increased macrophages at day 14 along with increased pro-inflammatory cytokines within the healing ligaments at day 5 (IL-1α, IFNγ, IL-12). Although we did not specifically measure immune rejection of these cells, it is possible that the larger dose of cells were detected by the

animal's immune system. This is an interesting point to consider since allogeneic MSCs are considered immune privileged and one of the benefits of MSC therapy is the ability to use allogeneic cells without an immune response. Although MSCs are considered immune evasive due to low expression of MHC I and a lack of expression of MHC II molecules[146], they may not be as privileged as the literature would suggest. Others have shown immune detection and responses using allogeneic MSCs as well as MSCs increasing MHC I and II expression when placed in a pro-inflammatory environment[62–67]. The other possibility is that the higher dosage simply shifted the balance of cytokines in a pro-inflammatory direction due to paracrine interactions with other cells or released several of these cytokines themselves. Taken together, our dose study highlights a couple key factors that are often overlooked with MSC therapy.

Priming Altering the Function of Cells In Vivo

Based on our findings suggesting improved healing using a lower dose of MSCs combined with the possibility that the high dose of MSCs may have been detected by the immune system, we began researching ways to amplify the therapeutic capacity of a lower dose of cells. This lead us to the idea of cell priming prior to in vivo implantation. Priming and/or pretreating MSCs is not a novel concept. However, priming cells and applying them to a ligament model has not been done to the best of our knowledge and the outcome has not been well characterized. We found that priming the cells prior to administration altered the healing response and yielded different results compared to unprimed cells.

Priming the cells for 48 hours using poly (I:C) altered the healing cascade in a few specific ways that we believe to be beneficial. This includes early M2 infiltration, early endothelializtion

followed by decreased endothelialization later in healing (day 14), and early matrix deposition. The improved mechanical properties affiliated with these findings suggest that these are characteristics of healing to exploit and try to amplify with potential ligament therapeutic treatments. Normal ligament healing is well characterized[6], but less is known about the order of events desired to tip ligament healing more towards regeneration vs. scar-like tissue deposition. Our findings outline some key events that can be further optimized to enhance ligament healing.

Local Injection, Systemic Cytokine Changes

It has previously been reported that systemic injection of MSCs can decrease circulating pro-inflammatory cytokines[147], however, not many studies have looked at changes in systemic levels of cytokines upon a local injection of MSCs. Our study elucidated significant changes in several pro-inflammatory and anti-inflammatory cytokines upon primed and unprimed MSC application to an injured ligament. This is an important finding to consider during clinical application of MSCs for a few reasons. Dampening or exacerbating certain cytokines have the potential to have unintended effects, especially in patients with complex medical scenarios i.e. chronic disease or other pathologies. Many diseases, such as cancer and rheumatoid arthritis, have dysfunction surrounding immune system responses and therefore this should be considered before modulating the circulating levels of cytokines via local injections. More research is necessary to determine whether local injections alter circulating levels enough to be clinically relevant, however, these preliminary fluctuations seen with primed and unprimed MSCs can serve as a starting point.

A more specific outcome elucidated with testing serum cytokine levels is that using primed MSCs appeared to have a global dampening effect on several of the pro-inflammatory cytokines. The altered cytokine expression was most noticeable during the first 3 days after injury/cell administration and leveled out by day 4. This provides a pattern of cytokine changes that is associated with improved ligament healing by using primed MSCs. More studies are necessary and hopefully with more data, optimal levels and/or combinations of cytokines will be recorded. This has the potential to allow for easy monitoring of healing progress (via blood collection) when testing other therapeutics and/or cell therapies.

PGE2 In Vivo

Our PGE2 findings go against many references that suggest that this is the mechanism for MSCs immunomodulation and an M1 to M2 phenotype switch[57,58,87]. We found decreased PGE2 levels in the cell culture supernatent with in vitro priming and we also found a decrease in vivo throughout the matrix at day 4 in the primed MSC group. Interestingly, the primed MSC group had increased M2s even though there was decreased PGE2 throughout the ligament. A limitation to this finding is that we didn't collect any earlier time points examining PGE2 levels at days 1-3 post-injury. There is a lack of research examining PGE2 production by MSCs in vivo, and our findings point to the need to increase in vivo data regarding this molecule to enhance mechanistic understanding.

Co-localization with Endothelial Cells

The fate of MSCs is always questioned during cell therapy studies with very few conclusive results. In our study, we found that many MSCs co-localize with endothelial cells suggesting

pericyte-like activities after implantation. However, upon staining for a pericyte marker (CD146), we found that MSCs were not expressing this marker. This adds some evidence to the argument that not all MSCs are pericytes. Even though MSCs were not expressing a pericyte marker, it is interesting that many remained in close proximity to endothelial cells and CD146+ cells indicating a strong interaction with the angiogenic process. More research is necessary to fully elucidate this interaction and has the potential to guide future cell therapies that want to exploit this property or minimize it depending on the application.

Future Work

Priming Optimization

Priming MSCs with poly(I:C) at a concentration of 1 μg/ml for 48 hours had a positive healing effect in our injury model. We highlighted some key events that we believe lead to the improvements such as an early M2 presence and early endothelialization. However, this could be enhanced further. One way to optimize would be to prime MSCs using different concentrations and exposure times to poly (I:C). A series of in vitro experiments could look at what concentration/exposure time combination yields the greatest number of M2s when primed MSCs are co-cultured with macrophages. This combination of concentration/exposure time could then be applied to an in vivo healing environment with the goal being a greater infiltration of M2s early during healing. Other metrics could be used to determine an optimized cell type, such as VEGF for its angiogenic and anti apoptotic effects. Choosing this specific molecule has the potential to empower the resident cell (fibroblast) by minimizing hypoxic stress and cell death allowing a maximized endogenous healing response. Picking a factor released by MSCs

for exogenous healing would be less efficacious since MSCs do not remain in the healing environment in large numbers.

Optimization does not have to stop here. Once a metric is determined and improved healing is shown, the cells can be further analyzed through RNA expression using qPCR. This technique would allow further characterization of factors that are up regulated with priming and better define a cellular phenotype. Since it's risky to suggest improved healing based off one molecule (i.e. VEGF), analyzing other factors being regulated can lead to a greater understanding of potential synergisms. To increase the potency of these factors in the healing environment, cells could be sorted prior to implantation to eliminate cells that didn't respond as robustly to the priming based on the desired expression of various molecules. The cells that do respond will be applied to the healing environment, which would increase the concentration of the desired factors. The overall goal of this level of optimization would be superb ligament healing and a protocol that yields more consistent outcomes from cell therapy due to high quality control prior to application.

Transfection of PGE2

Several studies have suggested the importance of PGE2 production by MSCs during healing and this molecule has been linked to enhancing the type 2 macrophage phenotype[57–60,87]. However, we measured a decrease in PGE2 levels both in vitro and in vivo when MSCs were primed with poly(I:C). Since the primed group demonstrated improved outcomes, this raises more questions about the function and importance of this molecule. Due to these findings, combined with a lack of in vivo data reported in the literature, one could design a study comparing MSCs with this

protein up regulated, down regulated, and unregulated (control). Mechanical properties and cellular response to the varying levels of PGE2 would offer novel insight and help elucidate the role of PGE2 during healing. This information has the potential to improve the efficacy of MSC therapyxq1.

Adjuvent to MSC Application

As discussed previously, MSCs require an inflammatory environment for activation and stimulation of their immunomodulatory actions[44–46]. Pro-inflammatory cytokines found within the injured region of a ligament or treating cells prior to application with a pro-inflammatory molecule can serve as the activator and was outlined in our studies. However, once the cells are administered, they are left to react to any changes that occur in their microenvironment, which can affect their in vivo function. A couple of researchers have found that altering the level of inflammation in vivo by co-administration of an immunosuppressant can inhibit MSCs modulatory activities[148,149]. Others have shown that MSCs are more effective during maximum inflammation and less effective when inflammatory symptoms are in remission during organ transplantation and autoimmune encephalitis, respectively[45,150–152]. This suggests that in our healing model, once the MSCs start guiding the microenvironment to be less inflammatory, their function may be decreasing. Therefore identifying a molecule that could be co-administered, released through a device, or injected may boost MSCs therapeutic potential. The key is finding a molecule that acts as an adjuvant to MSCs but does not have detrimental effects on the overall healing microenvironment. It's a delicate balance to strike, but once a molecule is identified, it has the potential to provide continuous cues to enhance MSC action.

Concluding Remarks

The motivation behind this research was to gain specific knowledge on the therapeutic actions of MSCs during ligament healing. Upon elucidating these details, we wanted to try to optimize them through priming.

The goal of aim 1 was to determine if there was a dosage effect using MSCs during ligament healing and whether an increased number of cells would yield better results. This was achieved by examining ligament healing using a low dose ($1x10^6$ cells) and high dose ($4x10^6$ cells) of cells and measuring functional ligaments properties (failure strength, stiffness), cellular response, and cytokine release. In summary, the lower dose of cells lead to greater functional gains demonstrated by increased ligament strength and stiffness. The lower dose also appeared to improve the rate of healing based on fewer proliferating cells and endothelial cells at day 14 combined with a smaller, more filled in healing region. The ligaments treated with the low dose had fewer pro-inflammatory measures such as macrophages and pro-inflammatory cytokines in the matrix. This result was somewhat surprising based on the thought that an increased number of cells would be more potent healers. These findings also demonstrated that the increased number of cells increased some inflammatory factors, which could be due to detection by the host's immune system in response to the application of allogeneic cells.

The goal of aim 2 was to measure the effects of priming MSCs with an inflammatory factor (poly (I:C)) in order to improve the efficacy while using a low dose of cells. This was completed by comparing primed MSCs and unprimed MSCs to control ligaments and measuring functional properties (failure strength, stiffness, matrix organization) along with cellular response, cytokine

production, and cellular fate. In summary, the unprimed low dose of MSCs did not yield improved results compared to control ligaments as was shown in our dose study. We used a different commercial vendor to obtain MSCs for this study, which highlights one of the struggles of getting consistent results with cell therapy. However, priming the cells lead to improved functional properties (strength and stiffness but not matrix organization), as well as an improved early cellular response demonstrated by increased M2s, procollagen 1α, and endothelial cells in the matrix at day 4. In this study, the priming rescued the therapeutic efficacy of these particular cells. This study also showed that many MSCs tend to localize around endothelial cells and pericytes, but do not express a common pericyte marker (CD146). A final observation is that very few primed MSCs remained in the matrix at day 14 which strengthens the argument that engraftment is not absolutely necessary for improved outcomes.

The goal of aim 3 was to examine the function of primed and unprimed cells in regard to cytokine production and document a pattern of comparative cytokine levels associated with improved healing seen in the primed MSC group. We accomplished this by measuring 13 different cytokines with pro- and/or anti- inflammatory properties locally in the matrix at day 4 and 10 cytokines systemically in the serum on days 1 – 4 post-injury. A complex scenario of cytokine regulation arose both locally and systemically. There was a decrease in pro-inflammatory IL-1α and an increase in anti-inflammatory IL-1Ra in ligaments that received primed MSCs. However, there was also an increase in pro-inflammatory IL-12 and a decrease in PGE2 (considered both pro and anti-inflammatory). In the blood serum, the primed MSC group diminished inflammation by dampening several pro-inflammatory cytokines. There were exceptions, most notably an increase in IFNγ which is considered both pro and anti-

inflammatory. This study demonstrated systemic effects upon local administration of MSCs and a less inflammatory pattern of cytokine expression using primed cells.

Important findings that were consistent between studies and were associated with stronger ligaments at day 14 were a decrease in IL1α levels locally within the matrix and an increased number of endothelial cells during the early stages of healing (day 4 and day 5). During later healing (day 14), there were decreased endothelial cells. This suggests that early endothelialization may be minimizing cell death (necrosis) leading to an increased rate of healing and less active tissue at day 14. Since this was found using 2 different sources of MSCs, early endothelialization may be a stable characteristic of MSCs that is less susceptible to different manufacturing and cell culture methods.

The collective research outlined elucidates cellular and cytokine temporal regulation using MSCs that resulted in improved ligament healing and functional properties (mechanical strength). One of the key findings is the improvement that occurred by priming cells prior to administration leading to increased M2 infiltration and procollagen 1α deposition. Taken together, this body of work will serve as a guide for future MSC studies and assist in the development of effective MSC therapeutics for clinical treatment of ligament and tendon injuries.

References:

1. Frank, C. B. & others. Ligament structure, physiology and function. *J. Musculoskelet. Neuronal Interact.* **4,** 199 (2004).
2. James, R., Kumbar, S. G., Laurencin, C. T., Balian, G. & Chhabra, A. B. Tendon tissue engineering: adipose-derived stem cell and GDF-5 mediated regeneration using electrospun matrix systems. *Biomed. Mater.* **6,** 025011 (2011).
3. Frank, C., McDonald, D. & Shrive, N. Collagen fibril diameters in the rabbit medial collateral ligament scar: a longer term assessment. *Connect. Tissue Res.* **36,** 261–269 (1997).
4. Frank, C. *et al.* Collagen fibril diameters in the healing adult rabbit medial collateral ligament. *Connect. Tissue Res.* **27,** 251–263 (1992).
5. Nakamura, N. *et al.* Decorin antisense gene therapy improves functional healing of early rabbit ligament scar with enhanced collagen fibrillogenesis in vivo. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **18,** 517–523 (2000).
6. Chamberlain, C. S., Crowley, E. & Vanderby, R. The spatio-temporal dynamics of ligament healing. *Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc.* **17,** 206–215 (2009).
7. Frank, C. *et al.* Medial collateral ligament healing. A multidisciplinary assessment in rabbits. *Am. J. Sports Med.* **11,** 379–389 (1983).
8. Jensen, K. T., Rabago, D. P., Best, T. M., Patterson, J. J. & Vanderby, R. Response of knee ligaments to prolotherapy in a rat injury model. *Am. J. Sports Med.* **36,** 1347–1357 (2008).

9. Scavenius, M. *et al.* Isolated total ruptures of the anterior cruciate ligament--a clinical study with long-term follow-up of 7 years. *Scand. J. Med. Sci. Sports* **9,** 114–119 (1999).
10. Frank, C. B., Loitz, B. J. & Shrive, N. G. Injury location affects ligament healing. A morphologic and mechanical study of the healing rabbit medial collateral ligament. *Acta Orthop. Scand.* **66,** 455–462 (1995).
11. Provenzano, P. P., Hurschler, C. & Vanderby, R. Microstructural morphology in the transition region between scar and intact residual segments of a healing rat medial collateral ligament. *Connect. Tissue Res.* **42,** 123–133 (2001).
12. Lo, I. K. Y., Marchuk, L. L., Leatherbarrow, K. E., Frank, C. B. & Hart, D. A. Collagen fibrillogenesis and mRNA levels in the maturing rabbit medial collateral ligament and patellar tendon. *Connect. Tissue Res.* **45,** 11–22 (2004).
13. Chamberlain, C. S., Crowley, E. M., Kobayashi, H., Eliceiri, K. W. & Vanderby, R. Quantification of collagen organization and extracellular matrix factors within the healing ligament. *Microsc. Microanal. Off. J. Microsc. Soc. Am. Microbeam Anal. Soc. Microsc. Soc. Can.* **17,** 779–787 (2011).
14. Chamberlain, C. S. *et al.* Gene profiling of the rat medial collateral ligament during early healing using microarray analysis. *J. Appl. Physiol. Bethesda Md 1985* **111,** 552–565 (2011).
15. Nordin, M., Lorenz, T. & Campello, M. Biomechanics of tendons and ligaments. *Basic Biomech. Musculoskelet. Syst. 3rd Ed Phila. Lippincott Williams Wilkins* 102–125 (2001).
16. Tidball, J. G. Inflammatory processes in muscle injury and repair. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **288,** R345–353 (2005).

17. Schulze, P. C. & Lee, R. T. Macrophage-mediated cardiac fibrosis. *Circ. Res.* **95,** 552–553 (2004).

18. Wynn, T. A. Cellular and molecular mechanisms of fibrosis. *J. Pathol.* **214,** 199–210 (2008).

19. Mu, X., Bellayr, I., Walters, T. & Li, Y. Mediators leading to fibrosis - how to measure and control them in tissue engineering. *Oper. Tech. Orthop.* **20,** 110–118 (2010).

20. Barron, L. & Wynn, T. A. Fibrosis is regulated by Th2 and Th17 responses and by dynamic interactions between fibroblasts and macrophages. *Am. J. Physiol. Gastrointest. Liver Physiol.* **300,** G723–728 (2011).

21. Caplan, A. I. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J. Cell. Physiol.* **213,** 341–347 (2007).

22. Caplan, A. I. Mesenchymal stem cells. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **9,** 641–650 (1991).

23. Caplan, A. I. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. *Tissue Eng.* **11,** 1198–1211 (2005).

24. Groh, M. E., Maitra, B., Szekely, E. & Koç, O. N. Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells. *Exp. Hematol.* **33,** 928–934 (2005).

25. Singer, N. G. & Caplan, A. I. Mesenchymal stem cells: mechanisms of inflammation. *Annu. Rev. Pathol.* **6,** 457–478 (2011).

26. Prockop, D. J. & Oh, J. Y. Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. *Mol. Ther. J. Am. Soc. Gene Ther.* **20,** 14–20 (2012).

27. Németh, K. *et al.* Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat. Med.* **15,** 42–49 (2009).
28. Kim, J. & Hematti, P. Mesenchymal stem cell-educated macrophages: a novel type of alternatively activated macrophages. *Exp. Hematol.* **37,** 1445–1453 (2009).
29. Maggini, J. *et al.* Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. *PloS One* **5,** e9252 (2010).
30. Zhang, Q.-Z. *et al.* Human gingiva-derived mesenchymal stem cells elicit polarization of m2 macrophages and enhance cutaneous wound healing. *Stem Cells Dayt. Ohio* **28,** 1856–1868 (2010).
31. Mosser, D. M. The many faces of macrophage activation. *J. Leukoc. Biol.* **73,** 209–212 (2003).
32. Martinez, F. O., Sica, A., Mantovani, A. & Locati, M. Macrophage activation and polarization. *Front. Biosci. J. Virtual Libr.* **13,** 453–461 (2008).
33. Gordon, S. & Martinez, F. O. Alternative activation of macrophages: mechanism and functions. *Immunity* **32,** 593–604 (2010).
34. Augello, A. *et al.* Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. *Eur. J. Immunol.* **35,** 1482–1490 (2005).
35. Lanz, T. V. *et al.* Mouse mesenchymal stem cells suppress antigen-specific TH cell immunity independent of indoleamine 2,3-dioxygenase 1 (IDO1). *Stem Cells Dev.* **19,** 657–668 (2010).

36. Karlsson, H. *et al.* Mesenchymal stem cells exert differential effects on alloantigen and virus-specific T-cell responses. *Blood* **112,** 532–541 (2008).

37. González, M. A., Gonzalez-Rey, E., Rico, L., Büscher, D. & Delgado, M. Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells. *Arthritis Rheum.* **60,** 1006–1019 (2009).

38. Schena, F. *et al.* Interferon-γ-dependent inhibition of B cell activation by bone marrow-derived mesenchymal stem cells in a murine model of systemic lupus erythematosus. *Arthritis Rheum.* **62,** 2776–2786 (2010).

39. Prigione, I. *et al.* Reciprocal interactions between human mesenchymal stem cells and gammadelta T cells or invariant natural killer T cells. *Stem Cells Dayt. Ohio* **27,** 693–702 (2009).

40. Raffaghello, L. *et al.* Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. *Stem Cells Dayt. Ohio* **26,** 151–162 (2008).

41. Chong, A. K. S. *et al.* Bone marrow-derived mesenchymal stem cells influence early tendon-healing in a rabbit achilles tendon model. *J. Bone Joint Surg. Am.* **89,** 74–81 (2007).

42. Kanaya, A. *et al.* Intra-articular injection of mesenchymal stromal cells in partially torn anterior cruciate ligaments in a rat model. *Arthrosc. J. Arthrosc. Relat. Surg. Off. Publ. Arthrosc. Assoc. N. Am. Int. Arthrosc. Assoc.* **23,** 610–617 (2007).

43. Gulotta, L. V. *et al.* Application of bone marrow-derived mesenchymal stem cells in a rotator cuff repair model. *Am. J. Sports Med.* **37,** 2126–2133 (2009).

44. Krampera, M. Mesenchymal stromal cell 'licensing': a multistep process. *Leukemia* **25,** 1408–1414 (2011).

45. Ren, G. *et al.* Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* **2,** 141–150 (2008).

46. Shi, Y. *et al.* Mesenchymal stem cells: a new strategy for immunosuppression and tissue repair. *Cell Res.* **20,** 510–518 (2010).

47. Waterman, R. S., Tomchuck, S. L., Henkle, S. L. & Betancourt, A. M. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. *PloS One* **5,** e10088 (2010).

48. Mastri, M. *et al.* Activation of Toll-like receptor 3 amplifies mesenchymal stem cell trophic factors and enhances therapeutic potency. *Am. J. Physiol. Cell Physiol.* **303,** C1021–1033 (2012).

49. Liotta, F. *et al.* Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell modulatory activity by impairing Notch signaling. *Stem Cells Dayt. Ohio* **26,** 279–289 (2008).

50. Kalinski, P. Regulation of immune responses by prostaglandin E2. *J. Immunol. Baltim. Md 1950* **188,** 21–28 (2012).

51. Goto, T., Herberman, R. B., Maluish, A. & Strong, D. M. Cyclic AMP as a mediator of prostaglandin E-induced suppression of human natural killer cell activity. *J. Immunol. Baltim. Md 1950* **130,** 1350–1355 (1983).

52. Bankhurst, A. D. The modulation of human natural killer cell activity by prostaglandins. *J. Clin. Lab. Immunol.* **7,** 85–91 (1982).

53. Walker, C., Kristensen, F., Bettens, F. & deWeck, A. L. Lymphokine regulation of activated (G1) lymphocytes. I. Prostaglandin E2-induced inhibition of interleukin 2 production. *J. Immunol. Baltim. Md 1950* **130,** 1770–1773 (1983).

54. Betz, M. & Fox, B. S. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J. Immunol. Baltim. Md 1950* **146,** 108–113 (1991).

55. Van der Pouw Kraan, T. C., Boeije, L. C., Smeenk, R. J., Wijdenes, J. & Aarden, L. A. Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. *J. Exp. Med.* **181,** 775–779 (1995).

56. Baratelli, F. *et al.* Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells. *J. Immunol. Baltim. Md 1950* **175,** 1483–1490 (2005).

57. Huang, M. *et al.* Non-small cell lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: up-regulation of interleukin 10 and down-regulation of interleukin 12 production. *Cancer Res.* **58,** 1208–1216 (1998).

58. Stolina, M. *et al.* Specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis. *J. Immunol. Baltim. Md 1950* **164,** 361–370 (2000).

59. Ylöstalo, J. H., Bartosh, T. J., Coble, K. & Prockop, D. J. Human mesenchymal stem/stromal cells cultured as spheroids are self-activated to produce prostaglandin E2 that directs stimulated macrophages into an anti-inflammatory phenotype. *Stem Cells Dayt. Ohio* **30,** 2283–2296 (2012).

60. Ryan, J. M., Barry, F., Murphy, J. M. & Mahon, B. P. Interferon-γ does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. *Clin. Exp. Immunol.* **149,** 353–363 (2007).

61. Awad, H. A. *et al.* Autologous mesenchymal stem cell-mediated repair of tendon. *Tissue Eng.* **5,** 267–277 (1999).

62. Griffin, M. D. *et al.* Anti-donor immune responses elicited by allogeneic mesenchymal stem cells: what have we learned so far? *Immunol. Cell Biol.* **91,** 40–51 (2013).

63. Zangi, L. *et al.* Direct imaging of immune rejection and memory induction by allogeneic mesenchymal stromal cells. *Stem Cells Dayt. Ohio* **27,** 2865–2874 (2009).

64. Schu, S. *et al.* Immunogenicity of allogeneic mesenchymal stem cells. *J. Cell. Mol. Med.* **16,** 2094–2103 (2012).

65. Camp, D. M., Loeffler, D. A., Farrah, D. M., Borneman, J. N. & LeWitt, P. A. Cellular immune response to intrastriatally implanted allogeneic bone marrow stromal cells in a rat model of Parkinson's disease. *J. Neuroinflammation* **6,** 17 (2009).

66. Eliopoulos, N., Stagg, J., Lejeune, L., Pommey, S. & Galipeau, J. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. *Blood* **106,** 4057–4065 (2005).

67. Gebler, A., Zabel, O. & Seliger, B. The immunomodulatory capacity of mesenchymal stem cells. *Trends Mol. Med.* **18,** 128–134 (2012).

68. Xie, J. *et al.* Interleukin-1 beta influences on lysyl oxidases and matrix metalloproteinases profile of injured anterior cruciate ligament and medial collateral ligament fibroblasts. *Int. Orthop.* **37,** 495–505 (2013).

69. Wang, Y. *et al.* Combined effects of TNF-α, IL-1β, and HIF-1α on MMP-2 production in ACL fibroblasts under mechanical stretch: an in vitro study. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **29,** 1008–1014 (2011).

70. Riley, G. P. *et al.* Tendon degeneration and chronic shoulder pain: changes in the collagen composition of the human rotator cuff tendons in rotator cuff tendinitis. *Ann. Rheum. Dis.* **53,** 359–366 (1994).

71. Sharma, P. & Maffulli, N. Tendon injury and tendinopathy: healing and repair. *J. Bone Joint Surg. Am.* **87,** 187–202 (2005).

72. Sahin, H., Tholema, N., Petersen, W., Raschke, M. J. & Stange, R. Impaired biomechanical properties correlate with neoangiogenesis as well as VEGF and MMP-3 expression during rat patellar tendon healing. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **30,** 1952–1957 (2012).

73. Petersen, W., Pufe, T., Pfrommer, S. & Tillmann, B. [Overload damage to the Achilles tendon: the importance of vascularization and angiogenesis]. *Orthop.* **34,** 533–542 (2005).

74. Frank, C., Schachar, N. & Dittrich, D. Natural history of healing in the repaired medial collateral ligament. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **1,** 179–188 (1983).

75. Meirelles, L. da S., Fontes, A. M., Covas, D. T. & Caplan, A. I. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine Growth Factor Rev.* **20,** 419–427 (2009).

76. Saether, E. E. *et al.* Enhanced medial collateral ligament healing using mesenchymal stem cells: dosage effects on cellular response and cytokine profile. *Stem Cell Rev.* **10,** 86–96 (2014).

77. Frisch, K. E. *et al.* Quantification of collagen organization using fractal dimensions and Fourier transforms. *Acta Histochem.* **114,** 140–144 (2012).

78. Chamberlain, C. S. *et al.* The influence of macrophage depletion on ligament healing. *Connect. Tissue Res.* **52,** 203–211 (2011).

79. Di Nicola, M. *et al.* Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* **99,** 3838–3843 (2002).

80. Sotiropoulou, P. A., Perez, S. A., Gritzapis, A. D., Baxevanis, C. N. & Papamichail, M. Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells Dayt. Ohio* **24,** 74–85 (2006).

81. Chang, J., Thunder, R., Most, D., Longaker, M. T. & Lineaweaver, W. C. Studies in flexor tendon wound healing: neutralizing antibody to TGF-beta1 increases postoperative range of motion. *Plast. Reconstr. Surg.* **105,** 148–155 (2000).

82. Marui, T. *et al.* Effect of growth factors on matrix synthesis by ligament fibroblasts. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **15,** 18–23 (1997).

83. Candela, M., Barker, S. C. & Ballou, L. R. Sphingosine synergistically stimulates tumor necrosis factor alpha-induced prostaglandin E2 production in human fibroblasts. *J. Exp. Med.* **174,** 1363–1369 (1991).

84. Diaz, A., Chepenik, K. P., Korn, J. H., Reginato, A. M. & Jimenez, S. A. Differential regulation of cyclooxygenases 1 and 2 by interleukin-1 beta, tumor necrosis factor-alpha, and transforming growth factor-beta 1 in human lung fibroblasts. *Exp. Cell Res.* **241,** 222–229 (1998).

85. Nathan, C. Points of control in inflammation. *Nature* **420,** 846–852 (2002).

86. Cassatella, M. A. *et al.* Toll-like receptor-3-activated human mesenchymal stromal cells significantly prolong the survival and function of neutrophils. *Stem Cells Dayt. Ohio* **29,** 1001–1011 (2011).

87. Murphy, M. B., Moncivais, K. & Caplan, A. I. Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. *Exp. Mol. Med.* **45,** e54 (2013).

88. Saltzman, L. E., Moss, J., Berg, R. A., Hom, B. & Crystal, R. G. Modulation of collagen production by fibroblasts. Effects of chronic exposure to agonists that increase intracellular cyclic AMP. *Biochem. J.* **204,** 25–30 (1982).

89. Guller, S., Wozniak, R., Leibman, M. I. & Lockwood, C. J. Negative regulation of placental fibronectin expression by glucocorticoids and cyclic adenosine 3',5'-monophosphate. *Ann. N. Y. Acad. Sci.* **734,** 132–142 (1994).

90. Riquet, F. B. *et al.* Suppression of type I collagen gene expression by prostaglandins in fibroblasts is mediated at the transcriptional level. *Mol. Med. Camb. Mass* **6,** 705–719 (2000).

91. Nathan, C. & Ding, A. Nonresolving inflammation. *Cell* **140,** 871–882 (2010).

92. Molloy, T., Wang, Y. & Murrell, G. The roles of growth factors in tendon and ligament healing. *Sports Med. Auckl. NZ* **33,** 381–394 (2003).

93. Rehman, J. *et al.* Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* **109,** 1292–1298 (2004).

94. Prockop, D. J., Kota, D. J., Bazhanov, N. & Reger, R. L. Evolving paradigms for repair of tissues by adult stem/progenitor cells (MSCs). *J. Cell. Mol. Med.* **14,** 2190–2199 (2010).

95. Von Bahr, L. *et al.* Analysis of tissues following mesenchymal stromal cell therapy in humans indicates limited long-term engraftment and no ectopic tissue formation. *Stem Cells Dayt. Ohio* **30,** 1575–1578 (2012).

96. Tomchuck, S. L. *et al.* Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. *Stem Cells Dayt. Ohio* **26,** 99–107 (2008).

97. Tomchuck, S. L., Henkle, S. L., Coffelt, S. B. & Betancourt, A. M. Toll-like receptor 3 and suppressor of cytokine signaling proteins regulate CXCR4 and CXCR7 expression in bone marrow-derived human multipotent stromal cells. *PloS One* **7,** e39592 (2012).

98. Lombardo, E. *et al.* Toll-like receptor-mediated signaling in human adipose-derived stem cells: implications for immunogenicity and immunosuppressive potential. *Tissue Eng. Part A* **15,** 1579–1589 (2009).

99. Wang, Z. *et al.* Lipopolysaccharides can protect mesenchymal stem cells (MSCs) from oxidative stress-induced apoptosis and enhance proliferation of MSCs via Toll-like receptor(TLR)-4 and PI3K/Akt. *Cell Biol. Int.* **33,** 665–674 (2009).

100. Yao, Y. *et al.* Lipopolysaccharide preconditioning enhances the efficacy of mesenchymal stem cells transplantation in a rat model of acute myocardial infarction. *J. Biomed. Sci.* **16,** 74 (2009).

101. Crisan, M. *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* **3,** 301–313 (2008).

102. Armulik, A., Genové, G. & Betsholtz, C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev. Cell* **21,** 193–215 (2011).

103. Feng, J., Mantesso, A., De Bari, C., Nishiyama, A. & Sharpe, P. T. Dual origin of mesenchymal stem cells contributing to organ growth and repair. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 6503–6508 (2011).

104. Chamberlain, C. S. *et al.* Interleukin-1 receptor antagonist modulates inflammation and scarring after ligament injury. *Connect. Tissue Res.* **55,** 177–186 (2014).

105. Chamberlain, C. S. *et al.* Interleukin expression after injury and the effects of interleukin-1 receptor antagonist. *PloS One* **8,** e71631 (2013).

106. Chamberlain, C. S. *et al.* The influence of interleukin-4 on ligament healing. *Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc.* **19,** 426–435 (2011).

107. Barrientos, S., Stojadinovic, O., Golinko, M. S., Brem, H. & Tomic-Canic, M. Growth factors and cytokines in wound healing. *Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc.* **16,** 585–601 (2008).

108. Murphy, J. E., Robert, C. & Kupper, T. S. Interleukin-1 and cutaneous inflammation: a crucial link between innate and acquired immunity. *J. Invest. Dermatol.* **114,** 602–608 (2000).

109. Chen, G. Y. & Nuñez, G. Sterile inflammation: sensing and reacting to damage. *Nat. Rev. Immunol.* **10,** 826–837 (2010).

110. Wang, X., Feuerstein, G. Z., Gu, J. L., Lysko, P. G. & Yue, T. L. Interleukin-1 beta induces expression of adhesion molecules in human vascular smooth muscle cells and enhances adhesion of leukocytes to smooth muscle cells. *Atherosclerosis* **115,** 89–98 (1995).

111. Gabay, C., Lamacchia, C. & Palmer, G. IL-1 pathways in inflammation and human diseases. *Nat. Rev. Rheumatol.* **6,** 232–241 (2010).

112. Kono, H., Karmarkar, D., Iwakura, Y. & Rock, K. L. Identification of the cellular sensor that stimulates the inflammatory response to sterile cell death. *J. Immunol. Baltim. Md 1950* **184,** 4470–4478 (2010).

113. Chen, C.-J. *et al.* Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat. Med.* **13,** 851–856 (2007).

114. Cohen, I. *et al.* Differential release of chromatin-bound IL-1alpha discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 2574–2579 (2010).

115. Liao, W., Lin, J.-X. & Leonard, W. J. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. *Immunity* **38,** 13–25 (2013).

116. Ouyang, W., Rutz, S., Crellin, N. K., Valdez, P. A. & Hymowitz, S. G. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu. Rev. Immunol.* **29,** 71–109 (2011).

117. Chan, B. P., Chan, K. M., Maffulli, N., Webb, S. & Lee, K. K. Effect of basic fibroblast growth factor. An in vitro study of tendon healing. *Clin. Orthop.* 239–247 (1997).

118. Leask, A. & Abraham, D. J. TGF-β signaling and the fibrotic response. *FASEB J.* **18,** 816–827 (2004).

119. Serini, G. *et al.* The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1. *J. Cell Biol.* **142,** 873–881 (1998).

120. Tsunawaki, S., Sporn, M., Ding, A. & Nathan, C. Deactivation of macrophages by transforming growth factor-beta. *Nature* **334,** 260–262 (1988).

121. Yönem, A., Cakir, B., Güler, S., Azal O, O. & Corakçi, A. Effects of granulocyte-colony stimulating factor in the treatment of diabetic foot infection. *Diabetes Obes. Metab.* **3,** 332–337 (2001).

122. Mann, A., Breuhahn, K., Schirmacher, P. & Blessing, M. Keratinocyte-derived granulocyte-macrophage colony stimulating factor accelerates wound healing: Stimulation of keratinocyte proliferation, granulation tissue formation, and vascularization. *J. Invest. Dermatol.* **117,** 1382–1390 (2001).

123. Singer, A. J. & Clark, R. A. Cutaneous wound healing. *N. Engl. J. Med.* **341,** 738–746 (1999).

124. Scheller, J., Chalaris, A., Schmidt-Arras, D. & Rose-John, S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim. Biophys. Acta* **1813,** 878–888 (2011).

125. Chomarat, P., Banchereau, J., Davoust, J. & Palucka, A. K. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat. Immunol.* **1,** 510–514 (2000).

126. Kaplanski, G., Marin, V., Montero-Julian, F., Mantovani, A. & Farnarier, C. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. *Trends Immunol.* **24,** 25–29 (2003).

127. Unemori, E. N., Hibbs, M. S. & Amento, E. P. Constitutive expression of a 92-kD gelatinase (type V collagenase) by rheumatoid synovial fibroblasts and its induction in normal human fibroblasts by inflammatory cytokines. *J. Clin. Invest.* **88,** 1656–1662 (1991).

128. So, T., Ito, A., Sato, T., Mori, Y. & Hirakawa, S. Tumor necrosis factor-alpha stimulates the biosynthesis of matrix metalloproteinases and plasminogen activator in cultured human chorionic cells. *Biol. Reprod.* **46,** 772–778 (1992).

129. Rawdanowicz, T. J., Hampton, A. L., Nagase, H., Woolley, D. E. & Salamonsen, L. A. Matrix metalloproteinase production by cultured human endometrial stromal cells: identification of interstitial collagenase, gelatinase-A, gelatinase-B, and stromelysin-1 and their differential regulation by interleukin-1 alpha and tumor necrosis factor-alpha. *J. Clin. Endocrinol. Metab.* **79,** 530–536 (1994).

130. Agren, M. S., Taplin, C. J., Woessner, J. F., Eaglstein, W. H. & Mertz, P. M. Collagenase in wound healing: effect of wound age and type. *J. Invest. Dermatol.* **99,** 709–714 (1992).

131. Mast, B. A. & Schultz, G. S. Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. *Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc.* **4,** 411–420 (1996).

132. DiPietro, L. A. Wound healing: the role of the macrophage and other immune cells. *Shock Augusta Ga* **4,** 233–240 (1995).

133. Gastl, G. & Huber, C. The biology of interferon actions. *Blut* **56,** 193–199 (1988).

134. Pober, J. S. *et al.* Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. *J. Immunol. Baltim. Md 1950* **137,** 1893–1896 (1986).

135. Hodge-Dufour, J. *et al.* Inhibition of interferon gamma induced interleukin 12 production: a potential mechanism for the anti-inflammatory activities of tumor necrosis factor. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13806–13811 (1998).

136. Gillery, P. *et al.* Gamma-interferon inhibits extracellular matrix synthesis and remodeling in collagen lattice cultures of normal and scleroderma skin fibroblasts. *Eur. J. Cell Biol.* **57,** 244–253 (1992).

137. Ghosh, A. K., Yuan, W., Mori, Y., Chen Sj, null & Varga, J. Antagonistic regulation of type I collagen gene expression by interferon-gamma and transforming growth factor-beta. Integration at the level of p300/CBP transcriptional coactivators. *J. Biol. Chem.* **276,** 11041–11048 (2001).

138. Scharffetter, K. *et al.* Synergistic effect of tumor necrosis factor-alpha and interferon-gamma on collagen synthesis of human skin fibroblasts in vitro. *Exp. Cell Res.* **181,** 409–419 (1989).

139. Granstein, R. D., Murphy, G. F., Margolis, R. J., Byrne, M. H. & Amento, E. P. Gamma-interferon inhibits collagen synthesis in vivo in the mouse. *J. Clin. Invest.* **79,** 1254–1258 (1987).

140. Siegel, J. P., Sharon, M., Smith, P. L. & Leonard, W. J. The IL-2 receptor beta chain (p70): role in mediating signals for LAK, NK, and proliferative activities. *Science* **238,** 75–78 (1987).

141. Mingari, M. C. *et al.* Human interleukin-2 promotes proliferation of activated B cells via surface receptors similar to those of activated T cells. *Nature* **312,** 641–643 (1984).

142. Davidson, T. S., DiPaolo, R. J., Andersson, J. & Shevach, E. M. Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. *J. Immunol. Baltim. Md 1950* **178,** 4022–4026 (2007).

143. Zheng, S. G., Wang, J., Wang, P., Gray, J. D. & Horwitz, D. A. IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. *J. Immunol. Baltim. Md 1950* **178,** 2018–2027 (2007).

144. Liao, W. *et al.* Priming for T helper type 2 differentiation by interleukin 2-mediated induction of interleukin 4 receptor alpha-chain expression. *Nat. Immunol.* **9,** 1288–1296 (2008).

145. Waterman, R. S. *et al.* Anti-inflammatory mesenchymal stem cells (MSC2) attenuate symptoms of painful diabetic peripheral neuropathy. *Stem Cells Transl. Med.* **1,** 557–565 (2012).

146. Le Blanc, K., Tammik, C., Rosendahl, K., Zetterberg, E. & Ringdén, O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp. Hematol.* **31,** 890–896 (2003).

147. Xu, J. *et al.* Prevention of endotoxin-induced systemic response by bone marrow-derived mesenchymal stem cells in mice. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* **293,** L131–L141 (2007).

148. Inoue, S. *et al.* Immunomodulatory effects of mesenchymal stem cells in a rat organ transplant model. *Transplantation* **81,** 1589–1595 (2006).

149. Chen, X. *et al.* The interaction between mesenchymal stem cells and steroids during inflammation. *Cell Death Dis.* **5,** e1009 (2014).

150. Sudres, M. *et al.* Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. *J. Immunol.* **176,** 7761–7767 (2006).

151. Rafei, M. *et al.* Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th17 T cells in a CC chemokine ligand 2-dependent manner. *J. Immunol.* **182,** 5994–6002 (2009).

152. Constantin, G. *et al.* Adipose-Derived Mesenchymal Stem Cells Ameliorate Chronic Experimental Autoimmune Encephalomyelitis. *Stem Cells* **27,** 2624–2635 (2009).