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Primed Mesenchymal Stem Cells Alter and Improve Rat Medial Collateral Ligament Healing

Erin E. Saether[1] · Connie S. Chamberlain[2] · Erdem Aktas[3] · Ellen M. Leiferman[2] · Stacey L. Brickson[2] · Ray Vanderby[1,2]

Abstract Cell therapy with mesenchymal stem cells (MSCs) can improve tissue healing. It is possible, however, that priming MSCs prior to implantation can further enhance their therapeutic benefit. This study was then performed to test whether priming MSCs to be more anti-inflammatory would enhance healing in a rat ligament model, *i.e.* a medial collateral ligament (MCL). MSCs were primed for 48 h using polyinosinic acid and polycytidylic acid (Poly (I:C)) at a concentration of 1 μg/ml. Rat MCLs were surgically transected and administered 1×10^6 cells in a carrier solution at the time of injury. A series of healing metrics were analyzed at days 4 and 14 post-injury in the ligaments that received primed MSCs, unprimed MSCs, or no cells (controls). Applying primed MSCs beneficially altered healing by affecting endothelialization, type 2 macrophage presence, apoptosis, procollagen 1α, and IL-1Ra levels. When analyzing MSC localization, both primed and unprimed MSCs co-localized with endothelial cells and pericytes suggesting a supportive role in angiogenesis. Priming MSCs prior to implantation altered key ligament healing events, resulted in a more anti-inflammatory environment, and improved healing.

Keywords Mesenchymal stem cells · Ligament · Macrophage · Inflammation · Angiogenesis · Polycytidylic acid

Introduction

Musculoskeletal injuries account for 33 million of the injuries in the US annually with 50 % involving soft tissue such as ligament and tendon [1]. The high prevalence of ligament injuries combined with poor healing makes this an important area of study. Ligament repair after 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 [2]. The resultant tissue is often less organized, made up of smaller collagen fibrils [3–5] and exhibits decreased mechanical strength [4, 6]. 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 as well as 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. [7] compiled data and divided the therapeutic benefits into 6 categories: immunomodulation, anti-apoptosis, angiogenesis, support of stem/progenitor cells, anti-scarring, and

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✉ Ray Vanderby
Vanderby@ortho.wisc.edu

[1] Department of Biomedical Engineering, University of Wisconsin, 1111 Highland Ave, WIMR Room 5059, Madison, WI 53705, USA

[2] Department of Orthopedics and Rehabilitation, University of Wisconsin, Madison, WI 53705, USA

[3] Department of Orthopedics, Ankara Oncology Research and Training Hospital, Ankara, Turkey

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chemoattraction. There is overlap and complex interplay among these categories and thus illustrates the difficulty in trying to isolate the fundamental mechanisms for improved healing.

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 [8]. Interestingly the best results were found in ligaments that were administered a lower dose (1×10^6 cells) versus a higher dose (4×10^6 cells) of cells. Due to improved healing 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 prior to administration into an injured environment to stimulate a more robust paracrine response and optimize healing.

There is a body of literature demonstrating the requirement of some form of stimulation or 'licensing' [9] 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 IL-1α/β, IFNγ, and TNFα, and reported that this exposure was necessary to stimulate MSCs immunosuppressive abilities [10, 11]. 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 [12, 13], others report the opposite [14]. Disagreement in the literature may be due to different cell types (mouse vs. human), *in vivo* versus *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 naïve, unprimed MSCs and primed MSCs. Polyinosinic and polycytidylic acid (poly(I:C)) was used as a primer due to its specificity to toll-like receptor 3 (TLR3) and anti-inflammatory behavior [12, 13]. Since our previous study showed improved healing using 1×10^6 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 primed MSCs would result in a less inflammatory environment leading to improved ligament healing demonstrated by increased ligament strength, a more normal composition, better organization of the extracellular matrix, and a faster rate of healing.

Materials and Methods

Experimental Design

The healing model used for this study examined extra-articular ligament healing. The rat medial collateral ligament (MCL) served as an appropriate tissue of study in this category and has been well characterized by our lab [15]. 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 receiving carrier solution only (Hanks Balanced Saline Solution, (HBSS; Hyclone Laboratories Inc, Logan UT) 2) unprimed MSCs (1×10^6 cells) and 3) primed MSCs (1×10^6 cells). The cell number used in this study was chosen due to dose optimization performed in a previous study [8]. Forty-two adult male Wistar rats (275–299 g) 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 treatment administration. For the unprimed and primed MSC groups, 1×10^6 cells were suspended in 25 ul of HBSS and administered using a sterile pipette at the location of the ligament transection. The control group received 25 ul of HBSS without cells. Identical 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. Cells were analyzed for specific cell marker expression using flow cytometry and were positive for CD44 and CD90 (>70 %), and negative for CD34, CD11b, CD45 (<5 %). Along with expression analysis of specific markers, tri-lineage differentiation (osteogenic, chondrogenic, and adipogenic) was performed by the vendor. Upon arrival, cells 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 containing 1 μg/ml Poly (I:C) (Sigma Aldrich). Cells were maintained in the primed media for 48 h and collected for surgery. 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 (IHC)/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 (5 μm), 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 h 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 min 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 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 1α (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), interleukin-1 receptor antagonist (IL-1Ra, 1:200, 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 Picrosirius 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 a custom image analysis program using Matlab (MathWorks, Inc., Natick MA) [16]. Using FA, lower values approaching 1 suggest a more organized matrix. The opposite is true when using FFT. Higher values approaching 2 indicate increased matrix organization.

Immunohistochemistry Quantification

In order to measure the spatial distribution of cells and protein, 5 ligament regions were imaged at 400× using a camera-assisted microscope (Nikon Eclipse microscope, model E6000 with Olympus camera, model DP79). The 5 areas represented included 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 (average of all regions) 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 maintained at −80 °C until dissected. 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/s 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). Results were considered significant if the overall p-value for the F-test in ANOVA was<.05. Post-hoc comparisons were performed using Fisher's least significant difference (LSD). Results were considered a trend with ANOVA overall p-values between 0.05 and 0.10. Experimental data are 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. Day 4 poly (I:C) ("primed") and HBSS-treated ligaments exhibited increased procollagen 1α in the healing region compared to unprimed MSCs (PMSC vs. MSC p=.005, HBSS vs. MSC p=.008, Fig. 1a–d). By day 14, primed MSCs tended to (p=.057) increase procollagen 1α within the healing region edges compared to the MSC and control groups (PMSC vs. MSC p=.027, PMSC vs. HBSS p=.055, Fig. 1e–h).

Apoptosis, cellular proliferation, and overall cellularity were measured at days 4 and 14 to determine whether either treatment had an effect on these general cellular functions in a healing environment. No significant differences were detected in the number of apoptotic cells at day 4 (Fig. 2a–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=.008, PMSC vs. HBSS p=.003, Fig. 2e–h). A significant increase in cell proliferation was detected throughout the MCL at day 4 in the primed MSC group compared to the unprimed MSC (p=.048) and control group (p=.019) (Fig. 3a–d). No significant differences between groups were noted at day 14 (Fig. 3e–h). Additionally, 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=.003, MSC vs. HBSS p=.034, Fig. 4a–d). At day 14, the primed MSC group exhibited the fewest number of endothelial cells in the healing region and healing region edges (PMSC vs. MSC p=.016, PMSC vs. HBSS p=.059, Fig 4e–h).

Factors involved in the inflammatory response including type 1 macrophages (M1) and type 2 macrophages (M2) were analyzed. At D4, M2s were increased in the healing region and healing region edges in the primed MSC group compared to the control group with a similar trend when compared to the MSC group (PMSC vs. HBSS p=.017, PMSC vs. MSC p=.056, Fig. 5a–d). However, at day 14 a different trend (ANOVA p=.056) emerged with increased M2s throughout the MCL in the MSC group compared to both the primed MSC and control groups (MSC vs. PMSC p=.056, MSC vs. HBSS p=.037, Fig. 5e–h). The ratio of M2s to M1s were then measured to determine the balance of macrophage phenotypes since this may better represent inflammation in the healing environment (Fig. 5i). At day 4, the primed MSCs (ANOVA p=.069) tended to exhibit higher ratios of type 2 macrophages to type 1 macrophages throughout the MCL compared to the MSC and control groups (PMSC vs. MSC p=.035, PMSC vs. HBSS p=.054). Although the day 14 MSC group had significantly more M2s throughout the MCL compared to both treatment groups, the ratio of M2s to M1s was not significantly different between groups.

Along with examining various cellular responses in the healing MCL after administration of unprimed and primed MSCs, cytokines known to play an essential role in healing, such as IL-1Ra, transforming growth factor beta (TGFβ) and vascular endothelial growth factor (VEGF), were also measured. IL-1Ra is an anti-inflammatory cytokine that competes with binding sites for pro-inflammatory IL-1β and IL-1α. At day 4, primed MSCs increased IL-1Ra within the healing region and healing region edges compared to the control group (PMSC vs. HBSS p=.006, Fig. 6a–d). Primed MSCs exhibited greater IL-1Ra compared to the MSC group, but not to a level of significance (PMSC vs. MSC p=.112). No treatment differences were noted in TGFβ (p=.625) and VEGF (p=.388).

Mechanical Properties and Matrix Organization

Functional mechanical properties were measured at day 14 of healing. Ligaments treated with primed MSCs tended (p=.068) to increase failure strength (13.699 N±0.850) compared to ligaments that received unprimed MSCs (9.988 N±

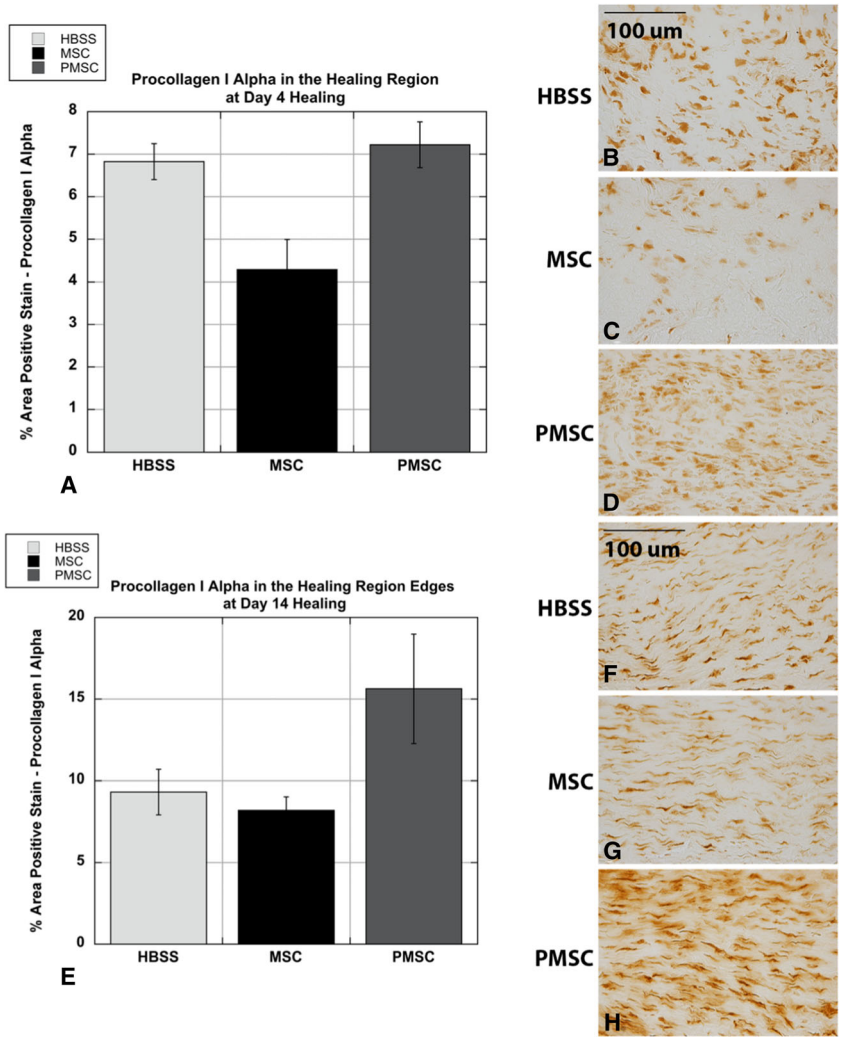

Fig. 1 **a**–**d** At day 4 healing, there was increased procollagen 1α in the healing region in the primed MSC group (p=.004) and the HBSS controls (p=.008) compared to the MSC group (PMSC 7.219±0.538 %, MSC 4.294±0.699 %, HBSS 6.826± 0.423 %). **a** Graph comparing average percentage area stain for 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, the same trend occurred (ANOVA p=.057) with increased procollagen 1α 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± 0.813 %, 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

1.221, p=.022, Fig. 7). This same pattern (ANOVA p=.091) was demonstrated with ligament stiffness. Primed MSC treatment increased stiffness (7.277 N/mm±.424) compared to the unprimed MSC group (5.771 N/mm±0.583, p=.033, Fig. 7). Although the primed MSC group had greater mean failure strength and stiffness compared to the control group, it did not reach significance. 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 (data not shown).

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=.806, Supplement 1, A-D). At day 14, although not significant, there was a trend with more DiI + MSCs in the unprimed group compared to the primed group (p=.065) within the healing region (Supplement 1, E-H). Due to the day 14 trend 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 did not 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).

Further examination of MSC localization indicated a congregation around blood vessel lumen. In order to study cell fate, MSCs were co-stained with an endothelial cell marker (CD31) or a pericyte marker (CD146). Neither the primed or unprimed MSCs co-expressed these markers, but the MSCs appeared in close proximity with both endothelial cells (Supplement 2, A-D) and pericytes (Supplement 2, E-H).

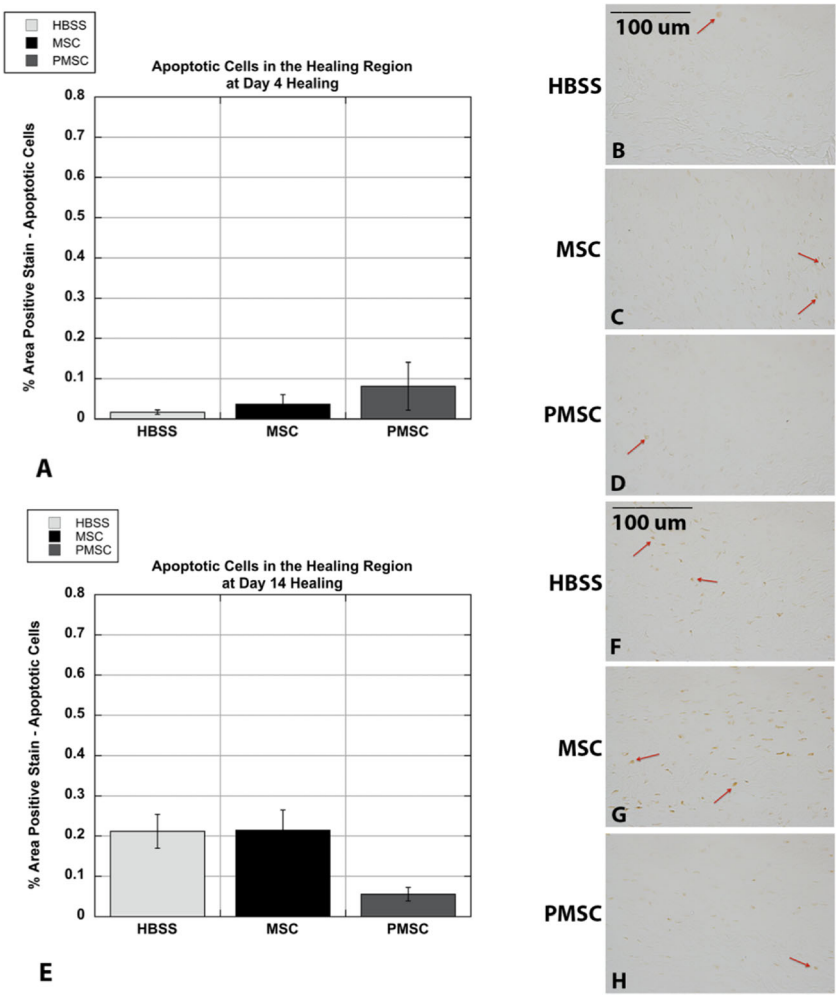

Fig. 2 **a**–**d** At day 4 healing, there were no significant differences between groups in apoptotic cells in the healing region. (PMSC 0.081±0.060 %, MSC 0.037±0.024 %, HBSS 0.017±0.006 %). **a** Graph comparing average percentage area stain for each condition. **b** Representative image of IHC in 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 apoptotic cells compared to the MSC group (p= 0.008) and the HBSS controls (.003) (PMSC 0.055±0.017 %, MSC 0.214±0.051 %, HBSS 0.212±0.042 %) **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

Discussion

Altered Healing with Primed MSCs

Allogeneic MSCs were used in this study to augment healing. Ligament strength was decreased from controls at 14 days when unprimed cells were used. Interestingly, the application of primed MSCs reversed this effect and significantly increased ligament strength compared to the unprimed group. Although the primed MSC group displayed the best mean mechanical properties, they were not significantly stronger than the control group at day 14. Modest changes were evident, however, in structure and composition (collagen 1 expression) of the healing ligament after 14 days of healing. Since the 14 days is still early in the healing cascade and before maturation of the neo matrix, further improvements (by the primed MSCs over control healing) in structure, composition, and biomechanical function are expected with later time points. Mechanical properties early in healing are only one set of metrics however. A closer examination of the healing cascade for unprimed and primed MSC groups revealed clear benefits in tissue composition that suggest a more regenerative process is occurring.

The primed MSC group exhibited more M2s within the healing region and increased the percentage of M2s to M1s during early healing (day 4). M2s are more reparative and anti-inflammatory whereas M1s are more pro-inflammatory [17–19]. M1s generally appear within the injured tissue and initiate 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 is important to note that both cell types appear essential for healing, and decreasing macrophage number has proven to be detrimental to ligament healing [20]. However, modulating the macrophage ratio 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 and macrophage plasticity.

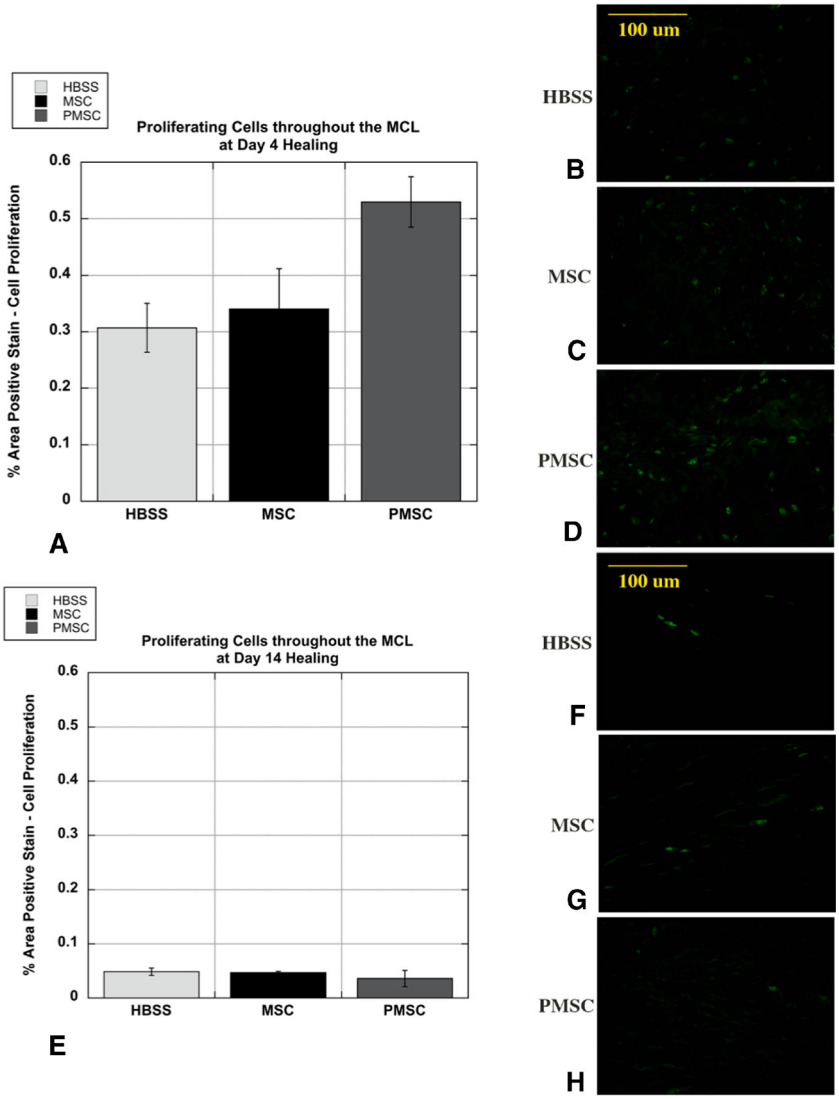

Fig. 3 **a**–**d** At day 4 of healing, the primed MSC group had increased cellular proliferation throughout the MCL compared to both the MSC group (p=.048) and control group (.019) (PMSC 0.530±0.045 %, MSC 0.341± 0.071 %, HBSS 0.307±0.044). **a** Graph comparing average percentage area stain for each 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 0.036±0.015, MSC 0.047±0.002, HBSS 0.049 ±0.007). **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

Along with increased M2s, primed MSCs increased procollagen 1α in the healing region at day 4 and in the healing region edges at day 14 compared to unprimed MSCs. However, unprimed MSC treatment demonstrated the lowest levels of procollagen 1α matrix deposition of all 3 groups at day 4 without a significant increase at day 14. As mentioned previously, procollagen 1α is the precursor to type 1 collagen, the most abundant matrix component in ligaments. Previous work from our lab demonstrated treatment with low MSC concentrations (1× 10^6) improved day 14 ligament strength but reduced day 5 procollagen levels compared to ligaments treated with high concentrations (8×10^6) of MSCs. Day 14 endothelial cells were also reduced by the treatment with a lower MSC concentration. Blood vessel formation is essential for healing, however, excessive formation appears detrimental due to mechanical deficits [21, 22]. Matrix metalloproteinase (MMP)-induced degradation that allows for vessel formation can disrupt matrix integrity and diminish mechanical strength. Less ECM degradation by blood vessel infiltration in the previous study was consistent with improved strength after MSC treatment. In the current study, unprimed MSCs reduced day 5 procollagen and did not reduce day 14 endothelial cells. Taken together, these assays are consistent with and help explain the day 14 reduction in strength.

VEGF and TGFβ were not significantly different between groups at day 4. However it is interesting to note that the primed MSC group had the greatest levels of VEGF, which correlated 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 [23]. However, it also plays a role in cell proliferation and migration [23]. Increased VEGF corresponds with ingrowth of vasculature in tendons which provides extrinsic cells, nutrients, and growth factors to the healing region [22]. Therefore initiating the cascade early after injury has the potential to improve healing, as shown in our study. Increased VEGF

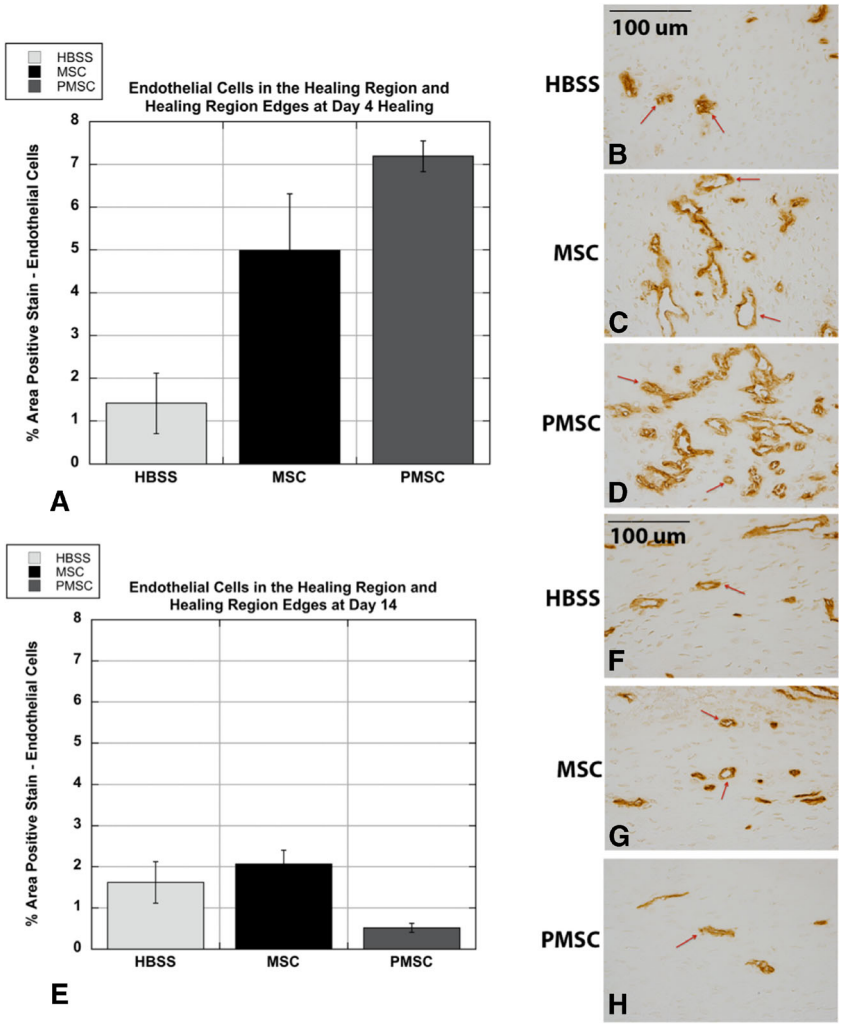

Fig. 4 **a–d** At day 4 healing, the primed MSC group (p=.003) and MSC group (p=.034) had increased endothelial cells in the healing region and healing region edges compared to the HBSS controls. (PMSC 7.193±0.908 %, MSC 4.986±1.324 %, HBSS 1.416±0.704). **a** Graph comparing average percentage area 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 0.512 ±0.111 %, MSC 2.067±0.337 %, HBSS 1.620±0.505 %). **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

production by MSCs is thought to be a key activity contributing to their anti-apoptotic and angiogenic properties [7, 24].

IL-1 cytokines (IL-1β, IL-1α, IL-1Ra) play a significant role in healing and have been a target for improving healing by our lab [25, 26]. IL-1 is a key initiator of the inflammatory process by signaling nearby cells, activating the clotting cascade, and releasing downstream cytokines and growth factors [27]. Therefore, modulation of IL-1 during early inflammation may impact subsequent healing. Altogether, increased IL-1Ra at day 4 by the primed MSCs may have contributed to improved healing.

Priming Effects on *In Vivo* MSC Function

Overall, regardless of priming, the number of MSCs localized to the injury was substantially reduced by 4 days post-injection. This reduction is likely attributable to large amount of MSC death soon after direct, scaffold less implantation and migration of cells away from the implantation site. Interestingly, fewer primed DiI+MSCS remained in the healing region compared to the unprimed cells, yet the primed MSC group demonstrated improved healing. One possible explanation may be that priming changed the cells migratory properties. Several studies examined this but no consensus was reached. An *in vitro* study showed decreased migration with TLR3 priming for 24 h in human MSCs, whereas priming for shorter times enhanced MSC migration [12]. Another study treated hMSCs for an even shorter amount of time (4 h) with poly(I:C) *in vitro* and reported increased cell migration [28]. A group of researchers used porcine MSCs and applied a higher concentration of poly(I:C) (4 μg/ml). They reported no change in migration *in vitro* upon exposure to poly(I:C) for 24 h [13]. Finally, others examined the expression of 2 key molecules involved in cell migration (CXCR4, CXCR7) upon exposing hMSCs to poly(I:C) (10 μg/ml) for 6 h and found that these molecules were significantly down-regulated [29]. 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

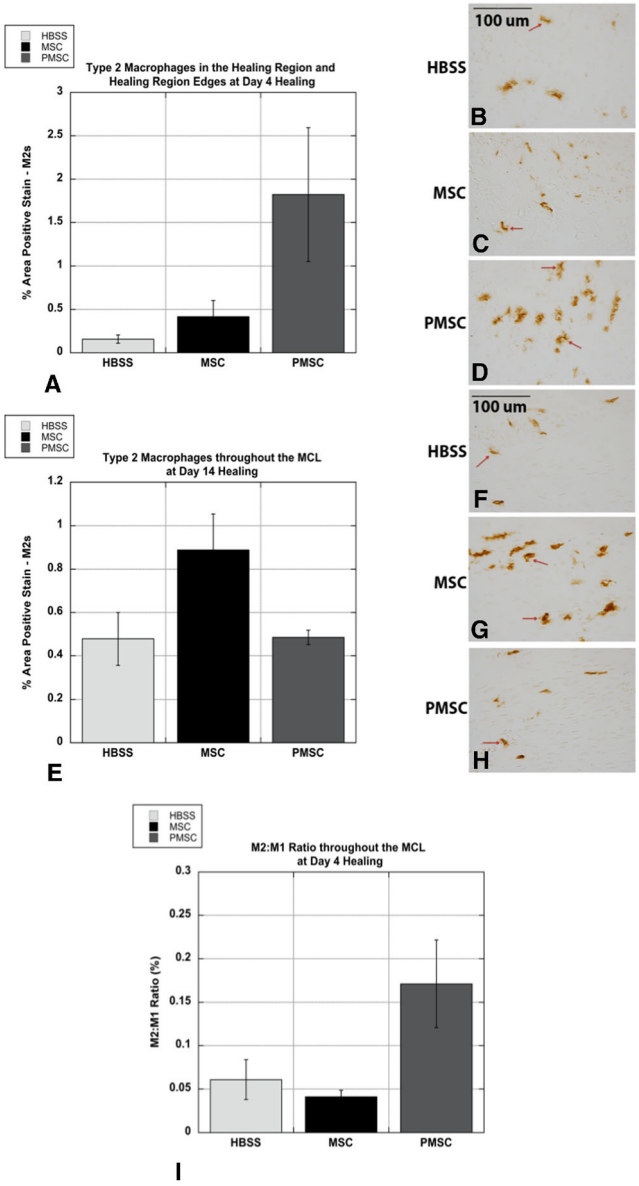

Fig. 5 **a**–**d** At day 4 healing, the primed MSC group exhibited more M2s in the healing region and healing region edges compared to the MSC group (p=.046) and the HBSS group (p=.017; PMSC 1.823±0.772 %, MSC 0.418±0.183 %, HBSS 0.157±0.048). **a** Graph comparing percent M2 macrophages at day 4. **b** Representative images of M2 macrophages in the day 4 control (**b**), MSC-treated (**c**) and PMSC-treated ligaments (**d**). **e**–**h** At day 14 healing, a different trend (ANOVA p=.056) emerged with the primed MSC group (p=.056) and HBSS control group (p=.037) having fewer M2s throughout the MCL compared to the MSC group. (PMSC 0.485±0.033 %, MSC 0.888±0.165 %, HBSS 0.478±0.122 %). **e** Graph comparing percent M2 macrophages at day 14. **f** Representative images of M2 macrophages in the day 14 control (**f**), MSC-treated (**g**), and PMSC-treated (**h**) ligaments. The primed MSC-treated ligaments tended (ANOVA p=.069) to exhibit an increase in M2:M1 ratio throughout the MCL compared to the MSC- (p=.035) and HBSS-treated ligaments (**i**; p=.054; PMSC 0.171±0.050, MSC 0.041±0.008, HBSS 0.061±0.036). Values are expressed as mean area stain± S.E.M

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 h used in our current study may have altered these migratory properties during rat ligament healing.

Another explanation for decreased MSCs in the primed group at day 14 involves 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 with studies showing either decreased or no change in proliferation *in vitro* with TLR3 activation of MSCs [13, 30].

Lastly, the differences found in MSC numbers could be explained by increased apoptosis leading to fewer cells in the matrix at day 14. However, our apoptotic data at day 4 and 14 suggest that this is unlikely. No significant differences in apoptosis were noted among groups at day 4, and 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 whether TLR3 ligands have the same effect [31, 32]. If there was increased cell death immediately after administration in the primed MSC group, the cells may have been cleared from the environment via the circulatory system before we could accurately measure through IHC.

MSC 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 of MSCs is via interaction with endothelial cells and pericytes. Both primed MSCs and unprimed MSCs localize to blood vessels at day 14 of healing. It was also shown that MSCs localize near pericytes during healing. This suggests that 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. [33] 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

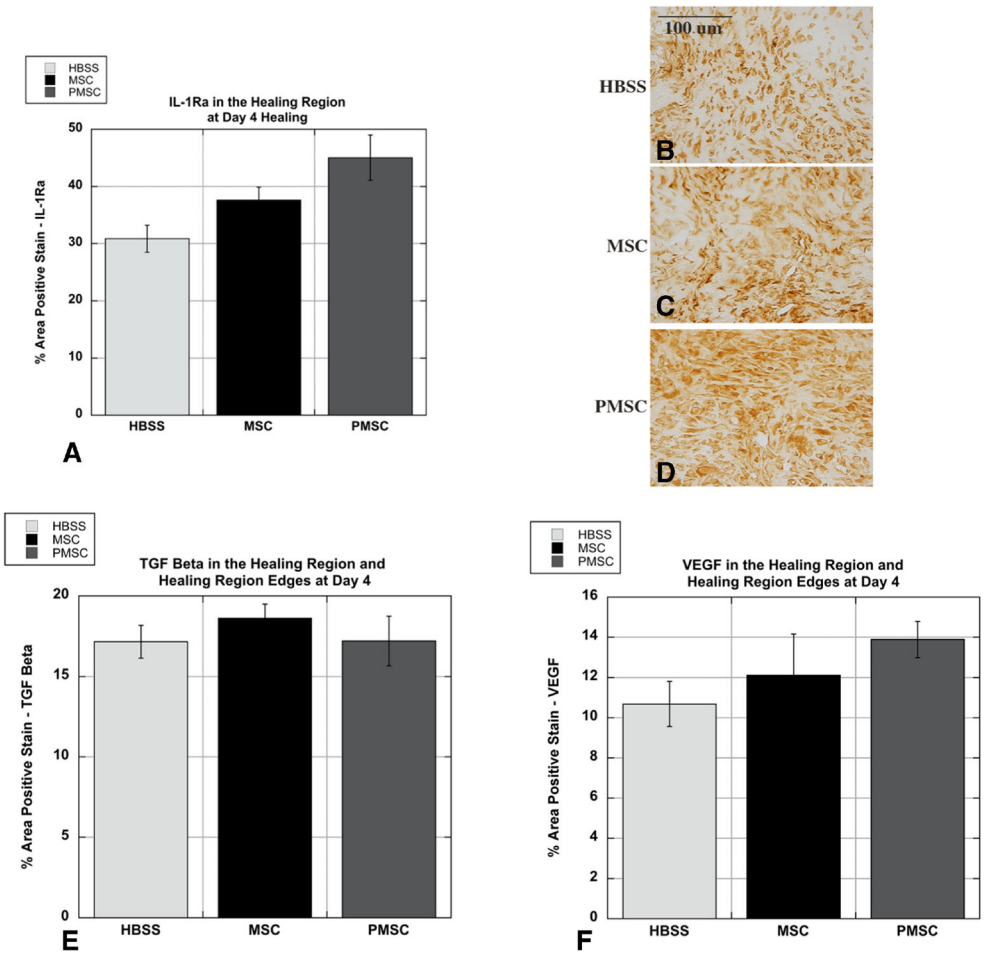

Fig. 6 **a**–**d** At day 4 healing, the primed MSC group had more IL-1Ra in the healing region and healing region edges compared to the HBSS group (p=.006) and the MSC group, although not significant (p=.112) (PMSC 45.021±3.962 %, MSC 37.575±2.276, HBSS 30.832±2.384 %). **a** Graph comparing average percentage area stain for each condition. **b** Representative images of IL1-Ra in D4 control (**b**), MSC- (**c**) and PMSC-treated (**d**) ligaments. No treatment differences were noted in TGF-β (**e**) and VEGF (**f**). Values are expressed as mean area stain± S.E.M

to whether MSCs are identical to a pericyte and if pericytes demonstrate greater plasticity [34]. Feng et al. [35] reported a dual origin of MSCs during tissue repair with some MSCs of pericyte origin whereas others were not of pericyte origin.

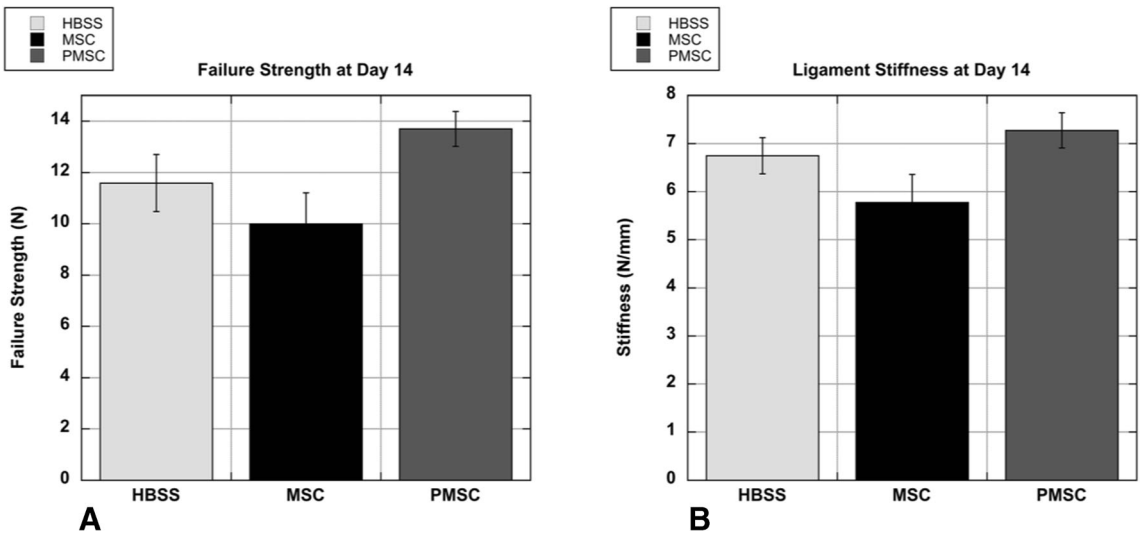

Fig. 7 **a** At day 14, a trend (ANOVA p=.068) emerged with the primed MSC group having increased failure strength compared to the MSC group (p=.022) (PMSC 13.699±0.850 N, MSC 9.988±1.222 N, HBSS 11.586±1.107 N). **b** At day 14, a similar trend (ANOVA p=.091) occurred with the primed MSC group having increased stiffness compared to the MSC group (p=.033) (PMSC 7.277±0.424 N/mm, MSC 5.771±0.584 N/mm, HBSS 6.747±0.376 N/mm). Values are expressed as mean±S.E.M

Although our focus has been more on the paracrine effects of MSCs, it's also interesting to observe 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 suggest 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.

Conclusion

We hypothesized that the primed MSCs would result in a less inflammatory environment and yield a faster rate and quality of healing. This was supported in our study by increased anti-inflammatory M2s and IL-1Ra, increased early endothelialization and procollagen 1α matrix deposition.

MSC therapy for ligaments and tendons have varying results with some studies demonstrating enhanced healing, while others show 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 activates cells and has the potential to increase their efficacy. Continued research is necessary to optimize priming protocols in order to maximize beneficial healing outcomes. Improving the efficacy of MSC therapy through priming has the potential to reduce the time needed to rehabilitate ligament injuries and decrease the risk of re-injury.

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Compliance with Ethical Standards

Competing of Interest No competing financial interests exist.

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