Immune Modulation With Primed Mesenchymal Stem Cells Delivered Via Biodegradable Scaffold to Repair an Achilles Tendon Segmental Defect

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ABSTRACT: Tendon healing is a complex coordinated series of events resulting in protracted recovery, limited regeneration, and scar formation. Mesenchymal stem cell (MSC) therapy has shown promise as a new technology to enhance soft tissue and bone healing. A challenge with MSC therapy involves the ability to consistently control the inflammatory response and subsequent healing. Previous studies suggest that preconditioning MSCs with inflammatory cytokines, such as IFN-γ, TNF-α, and IL-1β may accelerate cutaneous wound closure. The objective of this study was to therefore elucidate these effects in tendon. That is, the in vivo healing effects of TNF-α primed MSCs were studied using a rat Achilles segmental defect model. Rat Achilles tendons were subjected to a unilateral 3 mm segmental defect and repaired with either a PLG scaffold alone, MSC-seeded PLG scaffold, or TNF-α-primed MSC-seeded PLG scaffold. Achilles tendons were analyzed at 2 and 4 weeks post-injury. In vivo, MSCs, regardless of priming, increased IL-10 production and reduced the inflammatory factor, IL-1α. Primed MSCs reduced IL-12 production and the number of M1 macrophages, as well as increased the percent of M2 macrophages, and synthesis of the anti-inflammatory factor IL-4. Primed MSC treatment also increased the concentration of type I procollagen in the healing tissue and increased failure stress of the tendon 4 weeks post-injury. Taken together delivery of TNF-α primed MSCs via 3D PLG scaffold modulated macrophage polarization and cytokine production to further accentuate the more regenerative MSC-induced healing response. © 2016 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: MSC; priming; tendon healing; TNF-α; Achilles

Achilles tendon ruptures are one of the most frequent tendon injuries, with the incidence rising due to demographic changes and an increase in sports activities.[1–3] Although treatment modalities include both nonsurgical and surgical options, surgical repair remains the gold standard due to a lower risk of re-tear.[4] Despite advanced surgical techniques, the repaired tendon usually heals sub-optimally as scar tissue. The success rate of regeneration and functional recovery decline further if the defect size is too large to allow the tendon ends to re-appose. Current treatment modalities for these segmental defects include allograft or autograft reconstruction. Allograft reconstruction can result in allograft rejection or disease transmission whereas autografts are a limited resource with donor site morbidity. The quality and speed of tendon healing thereby remains problematic.

Mesenchymal stem/stromal cell (MSC) therapy for musculoskeletal regeneration has emerged as a new technology to enhance soft tissue and bone healing.[5] MSCs are thought to exert regenerative effects through immunomodulation and paracrine regulation. Specifically, MSCs may modulate the macrophages and T-lymphocytes of the innate and adaptive immune response to stimulate healing. Previous in vivo research demonstrated that MSC-secreted prostaglandin E_2 (PGE_2), stimulated production of interleukin-10 (IL-10) from the M2 macrophages and prevented further tissue damage by inhibiting free oxygen radicals and migration of neutrophils towards the damaged tissue.[6] MSCs have also been reported to directly affect T-cell function and shift the T-helper lymphocyte (Th) balance by reducing Th1 and Th17 lymphocytes while favoring the development of Th2 and regulatory T-cells (Treg).[7,8] Although MSC therapy has potential to improve tendon healing,[9] significant barriers remain including the ability to consistently control the inflammatory response. Thus, a challenge for therapeutic healing involves the appropriate expression of MSC-induced immunoregulatory signaling factors to suppress inflammation in a temporal and localized manner.

MSC-mediated immunosuppression may be more robust after exposure to a pro-inflammatory environment. Acute tissue damage is characterized by inflammation and infiltration of macrophages and neutrophils, which in turn release inflammatory factors such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and free radicals. These inflammatory cells and associated cytokines present at the time of injury may be an important prerequisite for MSCs to stimulate a reparative response, suggesting that MSC-mediated immunosuppression has to be induced by

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specific pro-inflammatory cytokines. Indeed, previous research demonstrated that injected MSCs preferentially migrated to inflammatory sites likely due to the immune cell-produced cytokines and chemokines.[10] Further research indicated the immunosuppression potential of MSCs required stimulation by inflammatory factors, such as interferon-γ (IFN-γ) and TNF-α.[11–13] Based on these results, preconditioning, or "priming," of MSCs via pro-inflammatory cytokines prior to implantation may stimulate an enhanced immunosuppressive response by the MSCs, in vivo.

Therapeutic benefits of MSCs are frequently observed without evidence of engraftment. Instead, the cells enhance repair by paracrine secretion or cell-cell contacts that modulate the inflammatory reactions or enhance propagation and differentiation of endogenous stem cells. However, the implanted cells rapidly disappear from tissues with a half-life of approximately 24 h.[14] Culturing MSCs in three dimensional (3D) scaffolds may facilitate greater cell-cell contacts and interactions of cells with the developing extracellular matrix (ECM), thus allowing cells to adapt to their native morphology. Indeed, previous studies have reported that pre-seeding MSCs into 3D scaffolds improves neotissue formation, stimulates immune cell chemoattraction, and increases anti-inflammatory cytokines while retaining MSC properties.[15–19] In this study, we present an experiment where TNF-α primed MSCs are incorporated within a biodegradable 3D porous poly(lactide-co-glycolide) (PLG) scaffold and used for segmental repair of a ruptured tendon. We hypothesized that TNF-α primed MSCs delivered via a scaffold would effectively provide localized control of inflammation and reparative healing in an injured Achilles tendon. Herein, the in vivo healing effects of TNF-α primed MSCs are elucidated using a rat Achilles segmental defect model.

METHODS

Mesenchymal Stem Cell Culture and In Vitro Priming Assay

Adult Fisher 344 rat bone marrow-derived mesenchymal stem cells were purchased from a commercial vendor (Cyagen Biosciences Inc, Santa Clara, CA) at passage 5. MSCs tested positive for CD44 and CD90 (>70%), negative for CD34, CD11b, CD45 (<5%), and were able to differentiate along osteogenic, chondrogenic, and adipogenic lineages. Upon arrival from the vendor, MSCs were seeded in T175 flasks and administered Mesenchymal Stem Cell Growth Medium (Cyagen Biosciences Inc, Santa Clara, CA) consisting of MSC basal medium, MSC-qualified fetal bovine serum (FBS), penicillin-streptomycin, and glutamine. Cells were maintained at 37°C and 5% CO_2 atmosphere. Media was changed every 3–4 days and passaged at 70% confluency. Cell morphology was monitored throughout passaging to ensure a spindle-like appearance.

In Vitro Priming Experiment

MSCs were used for in vitro and in vivo experiments. For the in vitro priming experiment, 3.8×10^4 MSCs were seeded into 24-well plates using media containing 10% FBS. Cells were primed by the addition of 10 ng/ml TNF-α ("pMSC" group). The concentration of TNF-α was determined based on previous publications.[20,21] Another group of cells received only media containing 10% FBS and served as the control ("MSC" group). MSC supernatants were then collected at 1, 6, 12, and 24 h post-treatment and used for cytokine multiplex analysis.

In Vivo Priming Experiment

For the in vivo priming experiment, MSCs were collected the morning of Achilles tendon surgery. Briefly, MSCs were exposed to trypsin/EDTA (Cellgro, Manassas, VA) for 3 min. After washing and re-suspending, cells were fluorescently labeled with Celltracker CM-DiI (Life Technologies, Grand Island, NY) in order to visualize their spatial distribution within the scaffold and healing tendons. Once fluorescently tagged, 1×10^6 MSCs were suspended in 25 μl of DMEM and seeded into a 3D-PLG scaffold (Fig. 1a). Cells were allowed to incubate on 3D-PLG scaffolds at 37°C and 5% CO_2 for 2 h prior to surgery to allow cell adherence.

Fabrication of Poly(Lactide-co-Glycolide) (PLG) Scaffold

Three dimensional PLG scaffolds were fabricated in a polypropylene 96-well plate by a salt fusion/solvent casting/salt leaching technique as previously reported.[22] Sodium chloride particles (130 mg/well, 106–250 um), used as a porogen, were placed in the wells and incubated for 4 h at 37°C and 95% humidity for salt fusion. After the fused salt template was dried overnight at 50°C, the PLG solution in acetone (10w/v%, 30 μl) was added into each well and centrifuged at 2,000 rpm to wet the sodium chloride particles. After overnight acetone evaporation, the plate containing PLG scaffolds was immersed in deionized water for at least 48 h to leach out the sodium chloride and then lyophilized (Fig. 1b). The resultant scaffolds were retrieved from the wells and cut into the desired dimension ($3 \times 2 \times 1$ mm) for MSC priming and Achilles tendon surgery.

Surgical Approach

Experimental procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee. In order to identify the influence of MSC on Achilles tendon healing, seventy two skeletally mature male Wistar rats were randomized to one of the three treatment groups and subjected to a 3 mm Achilles tendon segmental defect. All surgeries were performed using aseptic techniques. Rats were placed in prone position and the right ankle was secured in neutral under general anesthesia. A 1.5 cm longitudinal midline skin incision was made over the Achilles tendon, exposing the Achilles and superficial digital flexor (SDF) tendons (Fig. 1c–f). A 3 mm segmental defect was created in the mid-substance of the Achilles, half way between the calcaneal insertion and the musculotendinous junction. Approximately 3 mm tendon remained on each side, distal and proximal to the gap defect. The defect was reconstructed with one of the three treatments: (i) 3-D PLG scaffold alone ($n = 24$); (ii) PLG scaffold engrafted with 1×10^6 allogenic MSCs (MSCs; $n = 24$); or (iii) PLG scaffolds engrafted with 1×10^6 TNF-α primed-MSCs (pMSCs; $n = 24$). Following Achilles tendon reconstruction, the epitenon and skin were closed with 5–0 Vicryl sutures using an interrupted stitch. The leg was immobilized using a long leg cast applied immediately after the surgical procedure and casting was continued for 10 days post-repair. Contralateral hindlimbs were unaltered and used as intact controls. At 14

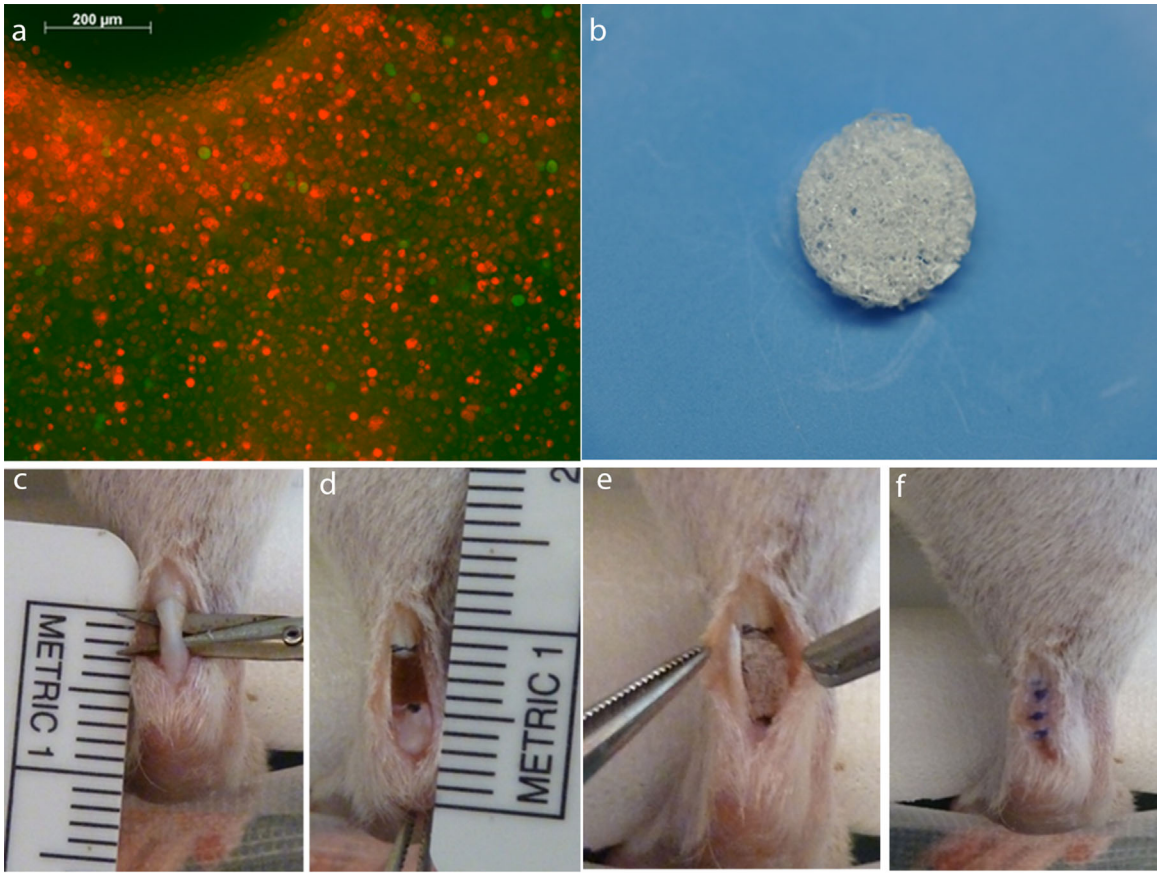


Figure 1. Representative images of the surgical procedure. (a) MSCs fluorescently tagged with CM-DiI and seeded onto a PLG scaffold prior to surgical implantation. (b) Example of a PLG scaffold used for the surgical procedure. During the surgery, (c) the Achilles tendon was exposed, (d) a 3 mm segmental defect was created, the (e) PLG with or without cells was inserted into the defect, and (f), the incision repaired with sutures.

($n = 36$) and 28 ($n = 36$) days post-injury, animals were euthanized and used for histology/immunohistochemistry ($n = 3$ rats/treatment/day), cytokine analysis ($n = 3$ rats/treatment/day), and mechanical testing ($n = 6$ rats/treatment/day). Tendons used for histology and IHC were dissected, embedded longitudinally in optimal cutting temperature (OCT) medium, and snap-frozen in liquid nitrogen. Tendons allocated for multiplex analysis were dissected and snap frozen in liquid nitrogen. Animals used for mechanical evaluation, were sacrificed and limbs were stored in toto at −80°C until testing.

Enzyme-Linked Immunosorbent Assay (ELISA)

Two ELISA kits were used to quantify the amount of PGE_2 (R&D Systems, Minneapolis, MN) and IL-6 (R&D Systems, Minneapolis, MN) in MSC-conditioned media according to the manufacturer's instructions. Conditioned media from MSC and pMSC groups were collected at 1, 6, 12, and 24 h post-treatment then measured for PGE_2 and IL-6.

Imunohistochemistry (IHC)

IHC was performed in order to identify cellular and ECM changes within the 2 and 4 weeks healing Achilles tendon. Frozen tissue was longitudinally sectioned at 5 μm thickness, mounted on Colorfrost Plus (Fisher Scientific, Pittsburgh, PA) microscope slides and used for histology/IHC. Cryosections for IHC were fixed in acetone, exposed to 3% hydrogen peroxide, blocked with Background Buster (Innovex Biosciences, Richmond, CA) and incubated with mouse primary antibodies. Mouse monoclonal antibodies to CD68, CD163, CD3, and CD31 (all used at 1:100, Abcam-Serotec, Raleigh, NC) were used to identify the M1 macrophage, M2 macrophages, T-lymphocytes, and endothelial cells, respectively. Mouse antibodies were also used to identify type I procollagen (straight; SP1.D8; Developmental Hybridoma, Iowa City, Iowa) and type III collagen (1:8000, Sigma–Aldrich, St. Louis, MO). Samples were then incubated with biotin and streptavidin-conjugated to horseradish peroxidase using the Stat Q IHC staining kit (Innovex Biosciences, Richmond, CA). The bound antibody was visualized using diaminobenzidine (DAB). Stained sections were then dehydrated, cleared, cover-slipped, and visualized using light microscopy.

After staining, images of each marker were collected using a camera assisted microscope (Nikon Eclipse microscope, model E6000 with an Olympus camera, model DP79). Within each IHC stained tissue section, images were captured in selected areas including, the scaffold region, the tissue immediately proximal and distal to the scaffold, the intact tissue proximal and distal from the healing site (outside of the granulation tissue area), and the epitenon. A total of six images were captured per section. IHC data were analyzed to determine (i) spatial differences (scaffold region, edge of the scaffold, epitenon, and away from the scaffold region) within the tissue; and (ii) total differences between treatments (all regions of the tendon average together). Data

collected from the captured IHC images were quantified using Image J (National Institutes of Health, NIH, Bethesda, MD) and reported as density/mm^2.

Cytokine Multiplex Analysis

To identify the influence of MSC treatments on cytokine production, a rat 10-plex Luminex assay (Life Technologies, Grand Island, NY) was performed using the Achilles tendon samples. Achilles tendons and scaffolds were rinsed in Cell Wash Buffer (Bio-Rad, Hercules, CA) and placed in Navy Bead Lysis Kit tubes containing a 0.9–2.0 mm stainless steel bead blend, 3.2 mm stainless steel balls (Next Advance, Averill Park, NY) and Lysing Solution (Bio-Rad, Hercules, CA). Tendon samples were homogenized for 10 min using a Bullet Blender (Next Advance, Averill Park, NY). To determine total protein concentration, a Bicinchoninic acid (BCA) assay was also performed. Multiplex cytokine assays (Life Technologies, Grand Island, NY) were subsequently performed according to the manufacturer's instructions. Diluted magnetic bead solution was vortexed, sonicated, and added to a 96-well plate. Standards and supernatant samples were added to the wells and incubated in the dark, overnight at 4°C on a plate shaker. The next day, samples were washed, incubated with biotinylated detector antibody and streptavidin-RPE solution. After washing and re-suspension in working wash solution, duplicate samples were measured on a Luminex 200 instrument (Luminex, Austin TX). Ten cytokines were quantified and included interleukin-1α (IL-1α), IL-1β, IL-10, interleukin-2 (IL-2), interleukin-12 (IL-12), interleukin-4 (IL-4), interleukin-6 (IL-6), tumor necrosis factor- α (TNF-α), IFN-γ, and granulocyte macrophage-colony stimulating factor (GM-CSF). All cytokines listed, except for IL-4 and IL-10, are believed to create an inflammatory response within the healing tendon. IL-4 and IL-10 are anti-inflammatory cytokines involved in the M2 phenotypic response. Assessment of the multiplex assay was performed by verifying each standard curve point was within 80–120% recovery, and two standard deviations above background. Cytokine concentrations were normalized to total protein concentration and expressed as percent cytokine concentration.

Mechanical Testing

Mechanical testing was performed to measure the effect of primed MSCs on functional outcome of the healing tendons. Achilles tendons were excised from the rats leaving the calcaneal insertion and the muscle-tendon junction (MTJ) intact. Tendon length was measured using a 0–150 mm (0.01 mm resolution) digital caliper. Tendon width and thickness were measured optically using Image J (measurements taken at three locations along the tendon length and averaged) and the cross-sectional area (assumed to be an ellipse) was calculated. Throughout testing, tendons remained hydrated using phosphate buffered saline (PBS). The calcaneus was trimmed and press-fit into a custom bone grip. The soft tissue end was fixed to strips of Tyvek with a cyanoacrylate adhesive, which were held in a soft-tissue grip. Tendons were tested in a custom-designed load frame along their longitudinal axis. Pre-loading was not performed due to the delicate nature of the 2 weeks healing tendon. Loading was constrained to prevent twisting of the grips or out-of-plane movements. The distance between grips at the onset of loading (7.13 ± 0.19 mm) was the gauge length. Pull-to-failure testing was applied at a rate of 6.0 mm/sec. This rate (84% per second) was standardized across all treatment groups and was chosen to produce clinically relevant failure modes. Axial force was measured with a load transducer. The load and displacement data from a linear variable differential transformer were collected and analyzed. Failure load and failure stress were determined as the maximum load and stress reached during testing, respectively. Stress was calculated by dividing the load by the initial cross-sectional area. Stiffness was calculated by determining the slope of the most linear portion of the load-displacement curve.

Statistical Analysis

After acquisition of metrics, a one-way analysis of variance (ANOVA) was used to examine differences among treatment groups for all mechanical, multiplex, and immunohistochemistry results. If the overall p value for the F-test in ANOVA was significant ($p \leq 0.05$), Fisher's Least Significant Difference (LSD) was performed. All data were reported as mean ± standard error of the mean (S.E.M.).

RESULTS

In Vitro Priming Optimization

To first determine the optimal timing of TNF-α-priming of MSCs (pMSCs), PGE_2 and IL-6 concentrations were quantified using the MSC supernatants. MSCs cultured for 1–12 h, PGE_2 was not significantly different (Fig. 2a). By 24 h, pMSCs significantly produced more PGE_2 than unprimed MSCs. IL-6 production was also increased by pMSCs, 6, 12, and 24 h post-incubation compared to unprimed MSCs (Fig. 2b). Based on these results, MSCs were primed 24 h with TNF-α for the remaining in vivo studies.

In Vivo Localization of the MSCs

To determine the time MSCs remained localized within the scaffold after in vivo implantation into the Achilles tendon injury, cells were pre-labeled with DiI. At 2 weeks post implantation, more primed MSCs remained within the scaffold compared to the unprimed MSCs (Fig. 3). By 4 weeks, fewer cells remained within the scaffold compared to the 2 weeks group and no obvious difference in cell engraftment was noted between treatments.

Immunohistochemistry (IHC)

Immunohistochemistry results indicated type I macrophages were significantly reduced after treatment with pMSCs compared to the scaffold and unprimed MSCs at 2 weeks (Figs. 4a and 5a–c). No significant treatment differences were noted at 2 weeks. In contrast, the anti-inflammatory M2 macrophages were significantly increased by both MSC groups at 2 weeks regardless of priming (Figs. 4b and 5d–f). By 4 weeks, no differences were noted. Further analysis of total macrophages at 2 weeks indicated the primed MSCs significantly increased the percent of M2 macrophages compared to the unprimed MSCs and scaffold group (Fig. 4c). Unprimed MSCs were also significantly increased in comparison to scaffold. T-lymphocytes were increased by the unprimed MSCs at 2 weeks within the scaffold

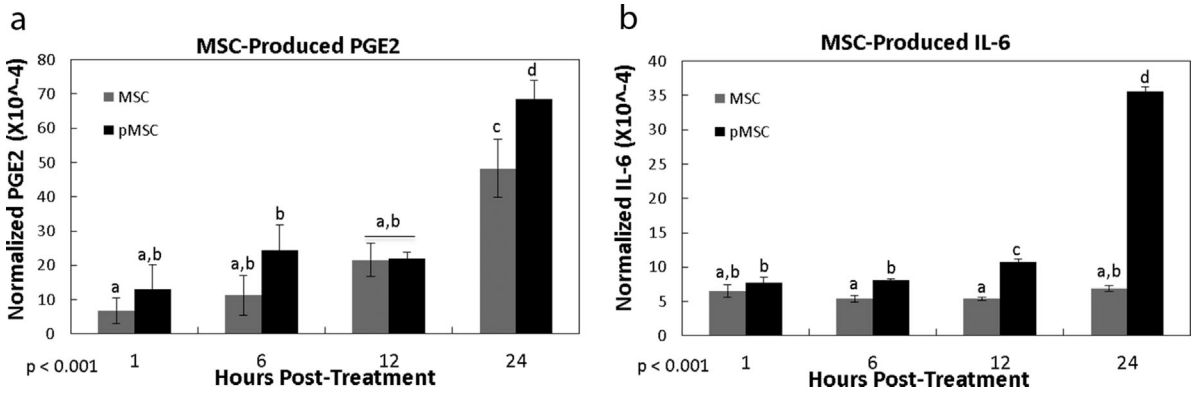


Figure 2. PGE_2 and IL-6 ELISA results of MSC conditioned media after pretreatment with TNF-α at 1, 6, 24, 48 h post-treatment. (a) PGE_2 levels between MSC and pMSC were not significantly different at 1–12 h post-treatment. In constrast, PGE_2 was highest at 24 h, with TNF-α-primed MSCs (pMSC) having the greatest effect. (b) IL-6 results of MSC-conditioned media indicated that TNF-α-primed MSCs stimulated production of IL-6 at 6 h post-treatment. Levels remained high at 12 h and significantly peaked at 24 h post-treatment. Beneath graph, *p* value indicates ANOVA results. [abcd]Within each graph indicate the results from Fisher's LSD post-hoc pairwise analysis ($p < .05$). Within each graph, treatment significance is denoted by different letters. Treatments (within each graph), containing similar letters indicate no significance. Values are expressed at normalized cytokine concentrations (percent cytokine/total protein) ± S.E.M. pMSC: primed MSCs.

(Figs. 4d and 5g–i). Priming did not provide an additive effect. The MSCs continued to increase the T-lymphocytes at 4 weeks compared to repair with scaffold (Figs. 4d,c and 5j–l). Within the 2 weeks epitenon, T-lymphocytes were significantly upregulated by the unprimed MSCs whereas priming attenuated the MSC-induced increase (Figs. 4e). Endothelial cells were not significantly different among treatment groups at 2 or 4 weeks (Fig. 4f). Treatments also did not significantly influence type I procollagen at 2 weeks. However, at 4 weeks, pMSCs increased production of type I procollagen compared to controls (Figs. 4g and 5p–r). Lastly, type III collagen was not significantly influenced by MSC treatments (Fig. 4h). Taken together, primed MSCs increased the anti-inflammatory macrophages decreased the pro-inflammatory macrophages, and epitenon-localized T-lymphocytes, and increased type I collagen production.

Multiplex Analysis

Multiplex results indicated that IL-12 and IL-1α were most prevalent after 2 weeks of healing. Unprimed MSCs significantly increased IL-12 over scaffolds without cells, whereas pMSCs attenuated this MSC-stimulated increase (Fig. 6a). By 4 weeks, IL-12 levels were significantly reduced by the MSCs compared to the scaffold alone, with the pMSCs having the greatest significant effect. IL-1α production was significantly reduced by MSC treatment, regardless of priming at week 2, compared to the scaffold alone (Fig. 6b). In contrast, MSCs increased week 4 IL-1α synthesis with the pMSCs having the greatest influence. The anti-inflammatory cytokine, IL-4, was significantly

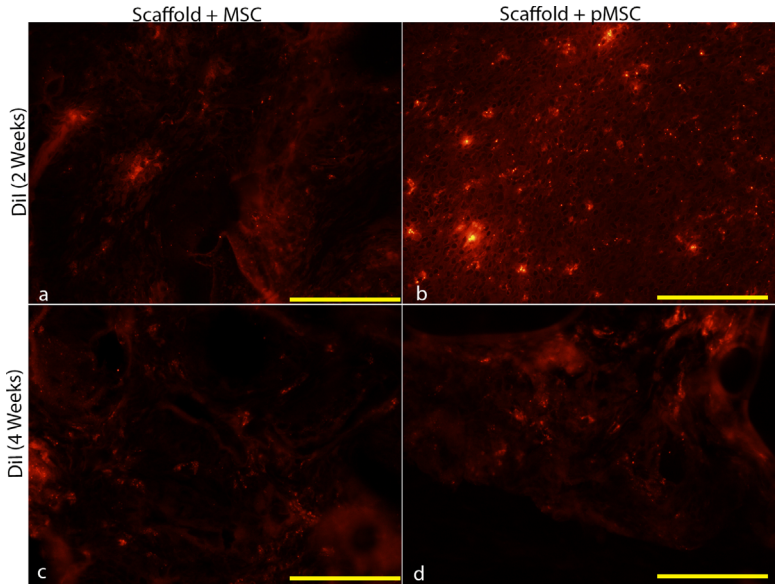


Figure 3. Representative micrographs DiI-labeled MSCs within the Achille tendon 2 and 4 weeks post-injury. Images demonstrate DiI-labeled unprimed (a, c) and primed (b, d) MSCs at 2 (top row) and 4 weeks (bottom row). Scale bar = 100 um.

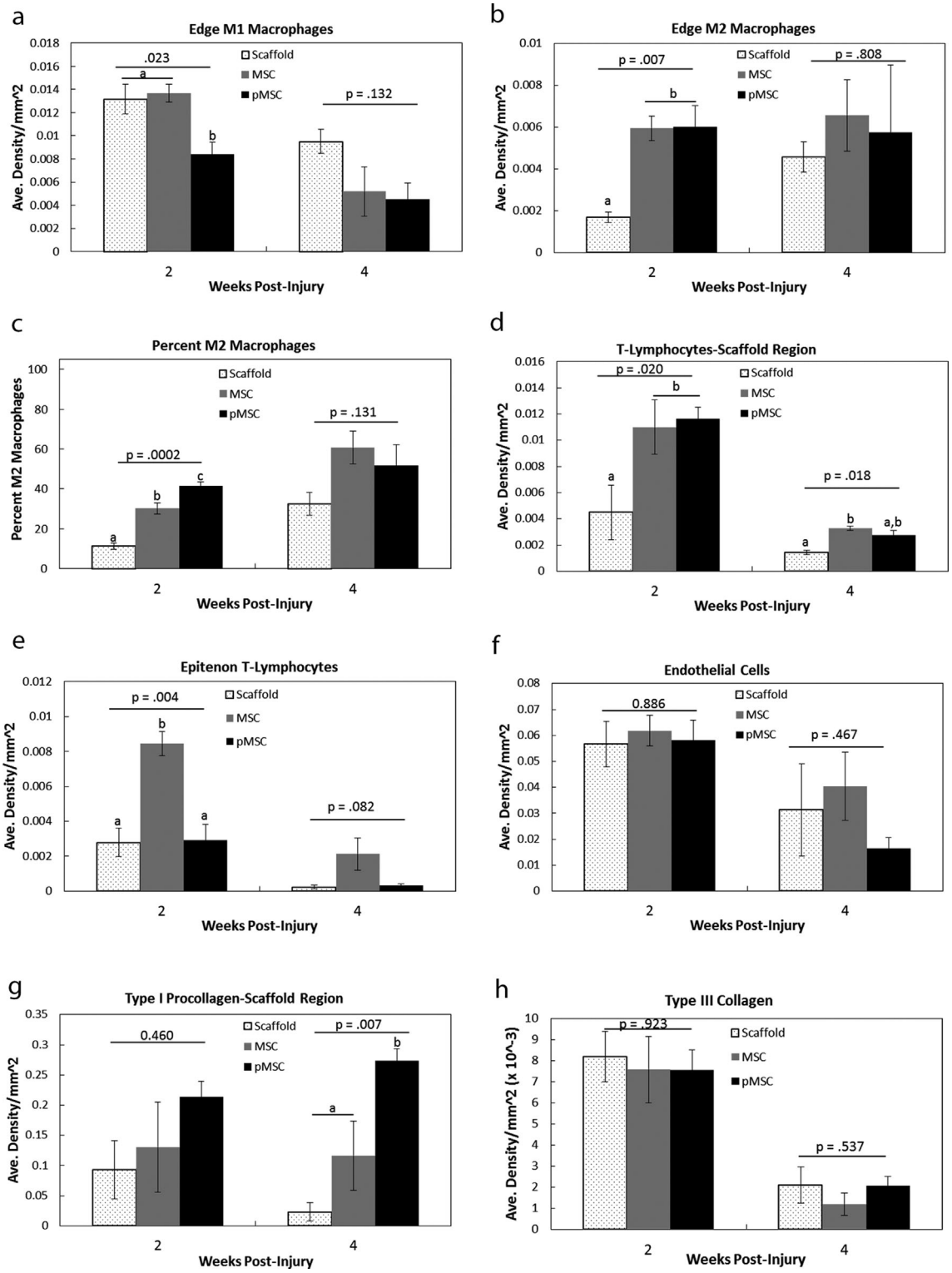


Figure 4. Immunohistochemistry results of the Achilles tendon collected at 2 and 4 weeks post-injury. (a) At 2 weeks post-injury the scaffold alone increased the M1 macrophages. Treatment with pMSC reduced the scaffold-mediated increase. Although MSCs with or without priming reduced the number of M1 macrophages at 4 weeks, no significance was noted. (b) The M2 macrophages were significantly increased by MSCs, regardless of priming at 2 weeks, but no changes were noted at 4 weeks. (c) Further analysis of total macrophages indicated the percent of M2 macrophages were significantly increased by unprimed MSCs. Priming further increased the percent. No changes were noted at 4 weeks. (d) T-lymphocytes were increased at 2 and 4 weeks by the unprimed MSCs within the scaffold region. (e) Within the epitenon, T-lymphocytes were significantly increased by the unprimed MSCs. However, pMSC treatment reduced the MSC-stimulated increase. No significant differences were noted at 4 weeks. (f) No significant differences were noted by the endothelial cells at 2 or 4 weeks. (g) Type I procollagen levels were not significantly different at 2 weeks, but were increased by pMSCs at 4 weeks. (h) No significant changes were noted in type III collagen. [abc]Within each graph indicate the results from Fisher's LSD post-hoc pairwise analysis ($p < .05$). Within each graph, treatment significance is denoted by different letters. Treatments (within each graph), containing similar letters indicate no significance. Values are expressed as mean density/mm2 ± S.E.M.

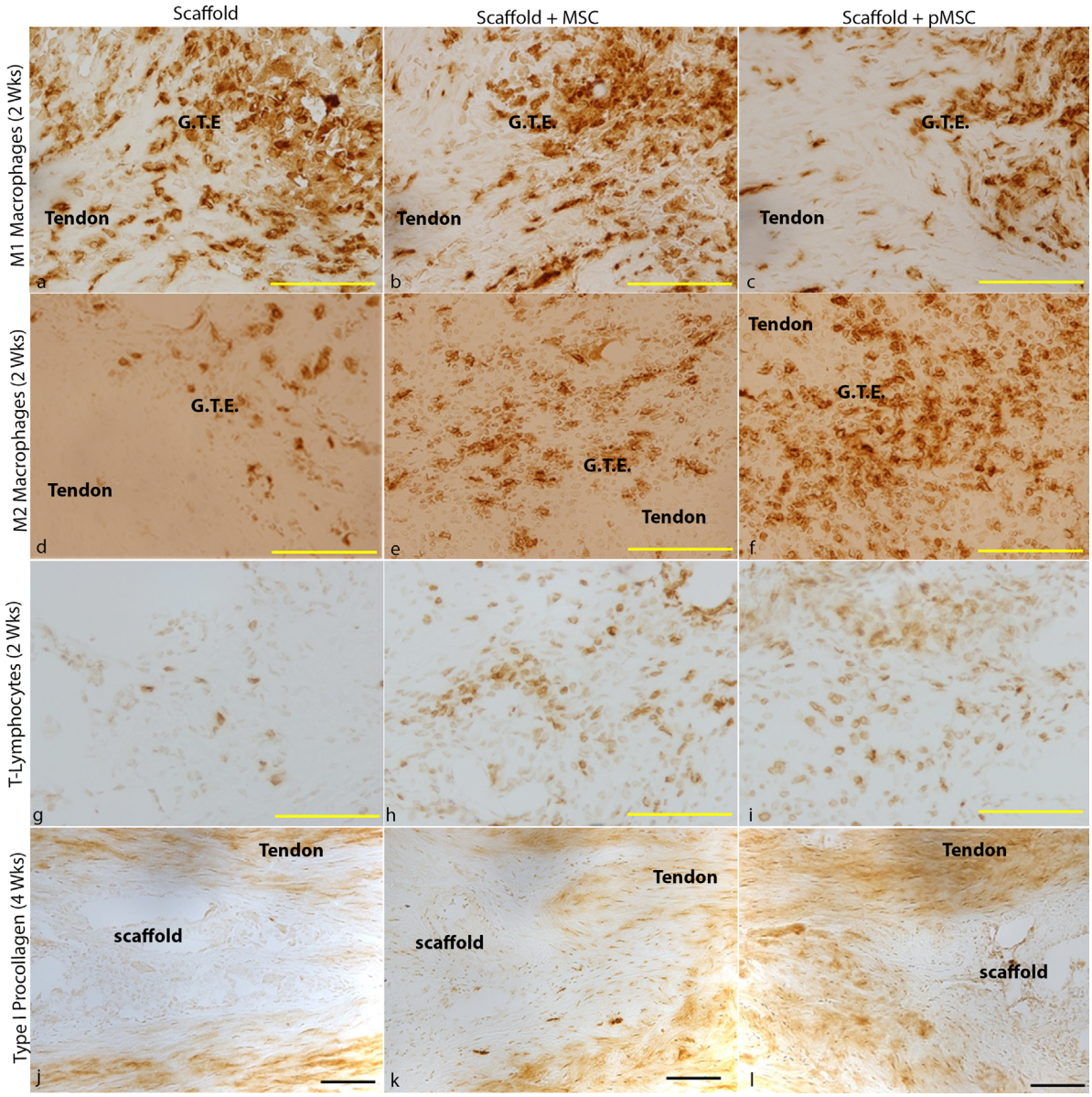
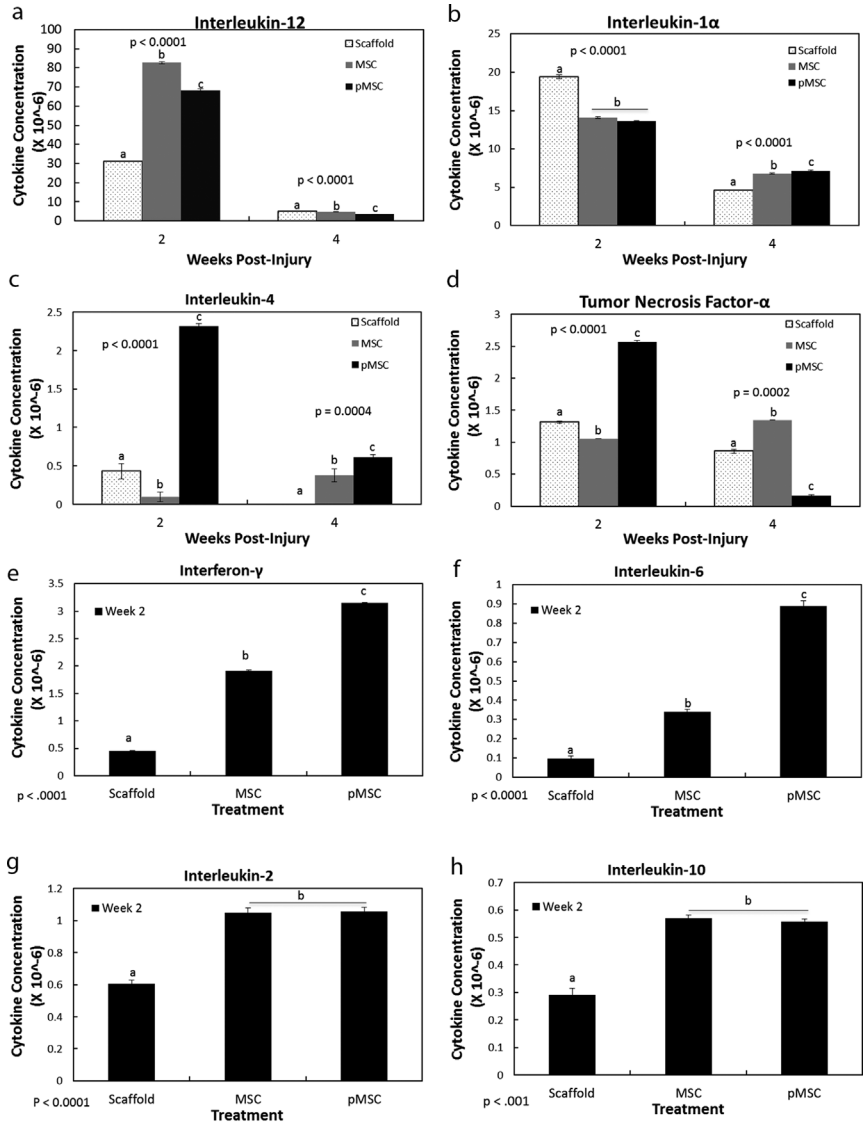


Figure 5. Representative IHC micrographs of the healing Achilles tendon. Images demonstrate staining of the M1 (a–c) and M2 macrophages (d–f) at 2 weeks, T-lymphocytes at 2 (g–i) and type I procollagen at 4 weeks (j–l), after repair with scaffold (first column; a, d, g, j), unprimed MSCs (second column; b, e, h, k,), and pMSC (third column; c, f, i, l). Scale bar = 100 um. G.T.E, granulation tissue edge; Tendon, area of tendon away from scaffold and granulation tissue edge; Scaffold, region of scaffold localization.

increased by pMSCs but was decreased with unprimed MSCs compared to the scaffold group at 2 weeks (Fig. 5c). By 4 weeks, unprimed MSCs increased IL-4, but pMSCs stimulated production further. TNF-α production was significantly increased by pMSCs whereas unprimed MSCs reduced TNF-α levels at 2 weeks, compared to the scaffold (Fig. 6d). In contrast, unprimed MSCs and pMSCs increased and decreased TNF-α production, respectively. IFN-γ and IL-6 were both upregulated by unprimed MSC at 2 weeks (Fig. 6e and f); pMSCs further accentuated the response. In contrast, regardless of priming, IL-2 and IL-10 were increased by MSC treatment (Fig. 6g and h). Week 4 levels of IL-10, IL-6, IL-2, and IFN-γ, as well as weeks 2 and 4 levels of GM-CSF and IL-1β were below the detectable sensitivity range. Overall, treatment with priming further accentuated the anti-inflammatory influence of the MSCs.

Mechanical Evaluation

During mechanical testing, all of the intact tendons failed at the insertion sites. At 2 weeks post injury, all of the unprimed MSC and five of the six scaffold repaired tendons failed in the mid-substance. Interestingly, three of the pMSC treated tendons failed in the mid-substance and three failed at the insertion site. By 4 weeks all but one sample (in the pMSC group) regardless of treatment, failed at their insertions. Mechanical results indicated stiffness was highest within the pMSC group and was similar to the intact



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	Scaffold		MSC		pMSC	
Anti-inflam.	**2 wk**	**4 wk**	**2 wk**	**4 wk**	**2 wk**	**4 wk**
IL-10	---	0.29 ± .02	---	0.57 ± .01	---	0.56 ± .01
IL-4	0.43 ± .10	0	0.10 ± .06	0.38 ± .08	2.31 ± .04	0.62 ± .03
Inflam.						
IL-12	31.22 ± .27	5.23 ± .08	82.65 ± .64	4.80 ± .09	68.31 ± .60	3.55 ± .06
IL-1α	19.39 ± .25	4.60 ± .03	14.06 ± .10	6.80 ± .05	13.65 ± .04	7.17 ± .04
TNF-α	1.31 ± .02	.86 ± .03	1.05 ± 0	1.34 ± 0	2.57 ± .02	0.16 ± .02
IFN-γ	0.46 ± 0	---	1.91 ± .01	---	3.15 ± .01	---
IL-6	0.10 ± .01	---	0.33 ± .01	---	0.89 ± .03	---
IL-2	0.60 ± .02	---	1.05 ± 03	---	1.06 ± .03	---

Figure 6. Multiplex results of the 2 and 4 week-post healing Achilles tendon samples after pMSC treatment. (a) At 2 weeks post-injury, MSC treatment increased IL-12 whereas pMSC reduced the MSC-stimulated IL-12 increase. Although overall IL-12 levels were significantly reduced at 4 weeks, pMSCs had the greatest inhibitory influence, followed by MSC treatment. (b) MSC treatment, regardless of priming, reduced the scaffold-stimulated increase in IL-1α at 2 weeks. By 4 weeks, pMSC and MSC increased IL-1α significantly more than the scaffold treated group. (c) pMSC significantly increased IL-4, at 2 weeks whereas unprimed MSCs reduced it. The pMSC treated group remained significantly higher compared to the treatment groups at 4 weeks. MSCs stimulated an increase in IL-4 at 4 weeks, compared to the scaffold group. (d) At 2 weeks, pMSCs increased TNF-α whereas MSCs reduced it. At 4 weeks, TNF-α was reduced by pMSC and increased by MSCs. (e) IFN-γ and (f) IL-6 levels were both increased by MSC and pMSC at 2 weeks with pMSC having the greatest effect. An MSC-stimulated increase in (g) IL-2 and (h) IL-10, regardless of priming, was also detected at week 2. Levels of INF-γ, IL-6, IL-2, and IL-10 were below the detectable sensitivity range at 4 weeks. (i) Summary table of inflammatory and anti-inflammatory cytokines influenced by MSC treatments. Beneath graph, p value indicates ANOVA results. ^{abc}within each graph indicate the results from Fisher's LSD post-hoc pairwise analysis ($p < .05$). Within each graph, treatment significance is denoted by different letters. Treatments (within each graph), containing similar letters indicate no significance. Values are expressed at normalized cytokine concentrations (percent cytokine/total protein × 10^-6) ± S.E.M. --- in table indicates values were below detectable levels.

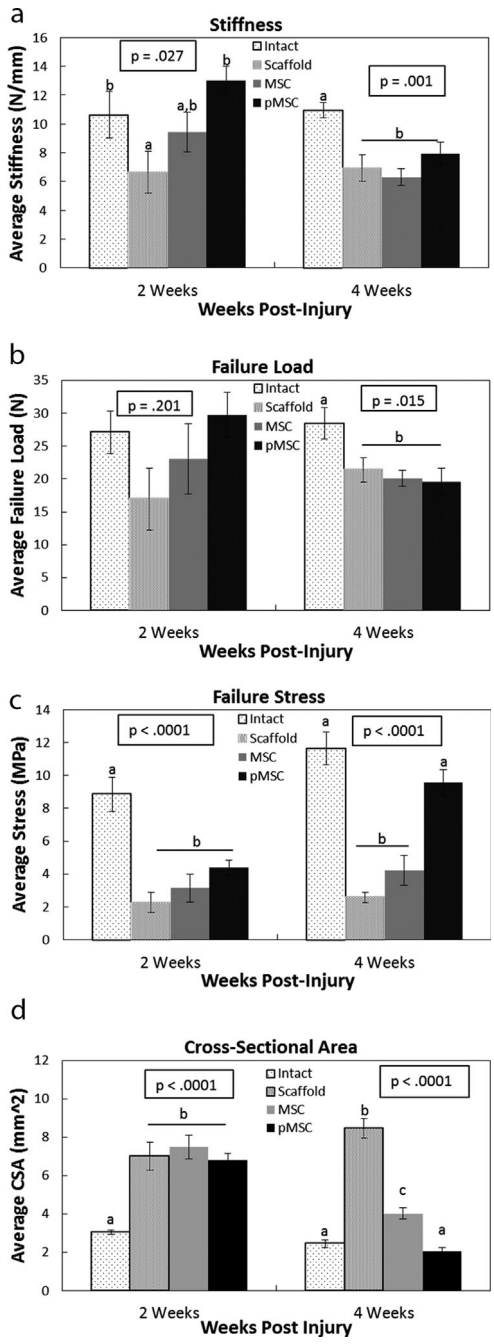

Figure 7. Mechanical results of the 2 and 4 week-post healing Achilles tendon samples after pMSC treatment. (a) Stiffness of the Achilles tendon was significantly higher when treated with the pMSC, 2 weeks post-injury. Treatments were not different at 4 weeks. The intact tendon was included and served as the overall control. (b) Failure load at 2 weeks indicated no significant difference between any groups. By 4 weeks, treatments were similar except to the higher intact control. (c) Except compared to the intact tendon, no significance was noted in stress between treatment groups at 2 weeks. By 4 weeks, pMSC significantly increased stress compared to the scaffold and MSC groups. (d) Cross-sectional area (CSA) of the 2 weeks injured tendons, regardless of treatment, was significantly larger than the intact tendon. By 4 weeks, tendons repaired with scaffold alone resulted in significantly larger CSA whereas MSC and pMSC treatments reduced the area. The CSA was similar in size to the intact tendons after pMSC treatment. [ab]within each graph indicates the results from Fisher's LSD post-hoc pairwise analysis ($p < .05$). Within each graph, treatment significance is denoted by different letters. Treatments (within each graph), containing similar letters indicate no significance. Values are expressed as mean ± S.E.M.

tendon at 2 weeks (Fig. 7a). No significant effects were noted at 4 weeks. Failure load was not significantly different between healing groups at 2 or 4 weeks (Fig. 7b). Failure stress (Fig. 7c) and cross-sectional area (Fig. 7d) were likewise not different at 2 weeks post-injury among treatment groups. However, pMSC treatment increased stress and cross sectional area to intact tendon levels by 4 weeks, compared to the scaffold and unprimed MSCs (Fig. 7c).

DISCUSSION

The overall goal of this study was to improve regenerative healing of segmental defects in ruptured Achilles tendons via a novel in vitro priming method to enhance the efficacy of stem cell therapy. A significant clinical need exists to modulate inflammation, accelerate healing and ameliorate scar during healing. To address this, we examined the use of TNF-α primed MSCs on a PLG scaffold in a segmental Achilles tendon injury. To our knowledge, this is the first study to report that delivery of TNF-α primed MSCs via a 3D PLG scaffold stimulates a more anti-inflammatory response by the macrophages, increases type I collagen production, and improves tendon strength. Thus, results support our hypothesis.

TNF-α-preconditioned MSCs stimulated PGE_2 and IL-6 production, in vitro, and suppressed inflammatory macrophages, in vivo, demonstrating that TNF-α can synergistically enhance MSC immunomodulation. TNF-α has been implicated as an important component of inflammation and is secreted by immune cells during the early phase of tissue repair. Previous research[20] demonstrated that TNF-α acts on the tumor necrosis factor receptor 1 (TNFR-1) of the MSCs, which results in the translocation of NF-κB into the nucleus.[21] Activation of nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB) stimulates cyclooxygenase two COX2, which in turn increases production of PGE_2. PGE_2 binds to its receptors on the macrophages, which subsequently increases IL-10 secretion, regulates macrophage polarization, and reduces inflammation.[6,23,24] Similar to those studies, our results also indicated that TNF-α induced production of PGE_2 by the MSCs, which then led to a decrease in M1 macrophages and the M1-produced cytokine, IL-12, and also resulted in an increase in IL-6 and the anti-inflammatory factor, IL-4. The anti-inflammatory cytokine, IL-10 also increased in the healing tendon, but the effects were not dependent on TNF-α-induced stimulation. Although this experiment did not discern whether the modulated cytokines originated from the added MSCs or from host cells, results suggest a primary interactive role between the MSCs and macrophages. Indeed, past studies have demonstrated immunomodulation of MSCs on macrophage function.[23] One study demonstrated that MSC treatment to mice after sepsis induction reduced mortality and improved organ function.[23] The positive effect of MSCs

was eliminated by macrophage depletion. Another study co-cultured human monocytes with MSCs, resulting in an anti-inflammatory M2-like macrophage phenotype with increased IL-10, IL-6, and decreased IL-12.[25] Taken together, preconditioning the MSCs with TNF-α prior to in vivo application, created a synergistic effect by enhancing the anti-inflammatory environment of the healing Achilles tendon. Although MSCs are considered to be non-immunogenic, treatment with unprimed MSCs, promoted more T-lymphocyte accumulation within the healing tendon in the current study. The upregulated $CD3^+$ T-lymphocyte response may indicate an immunogenic response to the allogeneic MSCs. MSCs express low levels of major histocompatibility complex (MHC) and co-stimulatory molecules,[26] but recent research indicates that MSCs may be immunogenic after administration, in vivo.[27,28] Treatment of allogeneic MSCs were found to promote rejection of donor bone marrow transplants in sub-lethally irradiated mice.[27] Similar findings reported allogeneic MSCs used in a rat skin transplant model accelerated donor skin graft rejection.[29] Based on these results, MSCs may be able to sensitize for MHC directed immune responses, in vivo. During healing, when inflammation is elevated, MHC expression on MSCs is upregulated, which could subsequently increase recognition of antigens and thereby enhance sensitization against the MSCs. In contrast, MHC recognition is a prerequisite for desensitization, suggesting that treatment with donor MSCs in combination with immunosuppressive factors could lead to desensitization against donor cells.[29] Indeed, in the current study, the MSC-induced T-lymphocyte increase within the epitenon was reduced after priming the cells with TNF-α. A study by Crop et al., further suggested that not only does TNF-α enhance MSC potency to inhibit lymphocyte proliferation, but it also boosts the speed of lymphocyte inhibition.[30] Taken together, if use of autologous MSCs is not a realistic option, preconditioning of allogeneic MSCs may beneficially inhibit potential adverse immune responses.

Our lab previously examined the effects of unprimed MSCs on ligament healing.[28] We reported that that injection of 1×10^6 unprimed MSCs into a surgically transected ligament model, reduced the number of M1 macrophages and IL-1α, and subsequently, increased type I procollagen and ligament strength. In the current study, treatment with MSCs on a PLG scaffold into a segmental defect, regardless of preconditioning, increased IL-2, and IL-10, while decreasing IL-1α, demonstrating the immunomodulatory effects of unprimed MSCs within the healing tissue. However, preconditioning MSCs with TNF-α further enhanced the MSC-stimulated anti-inflammatory response (increased percent of M2 macrophages and IL-4). Although unprimed MSCs were adequate to improve ligament strength in our previously reported study,[28] they were not adequate for the current Achilles segmental defect, PLG scaffold model. Treatment with TNF-α primed MSCs subsequently led to improved strength. Repair with the PLG scaffold induced an M1 macrophage response; a typical reaction observed with the implantation of "foreign" material, including PLG.[31,32] Preconditioning activated the MSCs, allowing for a more immediate response to the injured region at the time of treatment. Taken together, pre-conditioning MSCs may further improve the potency and efficacy of MSC-based therapies by priming the cells to secrete immunomodulatory as well as anti-scarring and chemotactic paracrine factors immediately on transplantation. Additional development of pre-conditioning strategies could better utilize MSC's immunomodulation potential and increase the effectiveness of MSC-based therapies for specific inflammatory and immune diseases.[33]

With advancement in tissue bioengineering, the primary delivery method of MSCs has shifted towards 3-D scaffolds composed of various organic/synthetic materials. PLG was chosen for scaffold fabrication because it is one of the most commonly used synthetic polymers with various applications that include medical devices, tissue engineering, and drug delivery. The PLG salt fusion/solvent casting/salt leaching technique results in a structure with numerous uniformly distributed interconnected macro-pores observed throughout the scaffold.[22,34] The highly interconnected scaffold bridge tissue gap, promoted uniformity of in vitro cell seeding, facilitated migration of seeded cells and cells migrating from a contiguous in vivo site, and encouraged ingrowth of new tissue. A previous study reported the implantation of MSC-seeded macroporous PLG scaffold into a critical-sized bone defect.[35] The cell-seeded scaffolds resulted in bridging of bone constructs 4 weeks post-injury.[35] Taken together, use of a PLG interconnected porous scaffold may benefit a broad range of defects to provide mechanical support and control cell migration for repair and regeneration.

In conclusion, tendon injury is a prevalent problem that results in scar formation, a slow healing process, and compromised function. To address this issue, we tested a novel treatment using a rat Achilles tendon segmental defect. TNF-α primed MSCs were seeded on a PLG scaffold and implanted to bridge the defect. Results showed improved strength, less inflammation, and a more favorable composition with collagen I, supporting our hypothesis that primed MSCs delivered via a scaffold would provide localized control of inflammation and improve reparative healing in an injured Achilles tendon. In a broader sense, this study shows the potency of MSC's immunomodulatory paracrine secretion and its promise for future cell-based therapies. MSCs have the potential to regulate multiple cell types arising in multiple pathologic scenarios involving inflammation and immunity. Additional

studies exploring this area will enhance understanding of MSC-based therapies and allow for more optimal clinical utilization.

AUTHORS' CONTRIBUTIONS

EA: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing. CSC: design, collection and/or assembly of data, data analysis and interpretation, manuscript writing. EES: collection and/or assembly of data, data analysis. SED-K, JK-M, MS, JSL, AEC: collection and/or assembly of data. WLM, RV (corresponding author): financial support, final approval of manuscript. All authors have read and approved the final submitted manuscript.

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