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# The influence of interleukin-4 on ligament healing

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## ABSTRACT

Despite a complex cascade of cellular events to reconstruct the damaged extracellular matrix, ligament healing results in a mechanically inferior scarred ligament. During normal healing, granulation tissue expands into any residual normal ligamentous tissue (creeping substitution), resulting in a larger region of healing, greater mechanical compromise and an inefficient repair process. To control creeping substitution and possibly enhance the repair process, the anti-inflammatory cytokine, interleukin-4 (IL-4), was administered to rats before and after rupture of their medial collateral ligaments. In vitro experiments showed a time-dependent effect on fibroblast proliferation after IL-4 treatment. In vivo treatments with IL-4 (100 ng/mL IV) for 5 days resulted in decreased wound size and type III collagen and increased type I procollagen, indicating a more regenerative early healing in response to the IL-4 treatment. However, continued treatment of IL-4 to day 11 antagonized this early benefit and slowed healing. Together, these results suggest that IL-4 not only influences the macrophages and T lymphocytes but also stimulates fibroblasts associated with the proliferative phase of healing in a dose-, cell-, and time-dependent manner. Although treatment significantly influenced healing in the first week after injury, IL-4 alone was unable to maintain this early regenerative response.

Ligament healing involves a complex cascade of events to reconstruct the damaged tissue, encompassing inflammation, proliferation, and remodeling processes. Early wound healing is normally characterized by an up-regulation of neutrophils, macrophages, and T lymphocytes infiltrating the injury and a concomitant synthesis and degradation of the extracellular matrix (ECM). As mediated by the immune cells, fibroblasts arrive in the wound-healing region via chemokine signaling, where they synthesize ECM constituents including type I and type III collagens. Simultaneously, macrophage-produced MMPs breakdown collagen, degrading the ECM as part of the remodeling process.[1,2] This coordinated ECM scar-forming response during remodeling lasts months or even years and the injured ligament never fully recovers its original functional properties.[3,4] Numerous reports have examined the influence of biological components on ligament healing, including matrix metalloproteinases,[5,6] cytokines and growth factors,[7–10] stem cells,[5,11] and platelet-rich plasma.[12,13] To date, no treatment resulted in the complete recovery of the injured ligament. Previous research from our lab characterized the expansion of granulation tissue as "creeping substitution" of the residual ECM.[14] Following ligament injury, granulation tissue forms beyond the original injury creeping into the surrounding tissue. The expansion of granulation tissue to replace residual ECM results in further ligament degeneration and an inefficient healing response. A desired healing scenario after injury would have minimal scar tissue, organized collagen fibers, restored concentrations of type I collagen, and limited creeping substitution. Such scenario would reduce type III collagen, and regenerate the ligament with nearly normal composition and mechanical properties.

| | |
|---|---|
| ANOVA | Analysis of variance |
| Daily Day 11 | Daily IL-4 injections day 11 |
| dHR | Distal healing region |
| DMEM | Dulbecco's modified Eagle's medium |
| ECM | Extracellular matrix |
| EL | Epiligament |
| FBS | Fetal bovine serum |
| FMT | Femur–MCL–tibia |
| H&E | Hematoxylin and eosin |
| HD Day 5 | High-dose IL-4 day 5 |
| HR | Healing region |
| IHC | Immunohistochemistry |
| IL-4 | Interleukin-4 |
| LD Day 5 | Low-dose IL-4 day 5 |
| MCL | Medial collateral ligament |
| MMP | Matrix metalloproteinase |
| MTS | [3-(4,5-Dimethylthizaol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] |
| N | Newtons |
| OCT | Optimal cutting temperature |
| PBS | Phosphate-buffered saline |
| pHR | Proximal healing region |
| Prox | Proximal |

A localized increase in M1 macrophages that function in tissue debridement, phagocytosis, and MMP synthesis, parallels the onset of creeping substitution and therefore may be a key mediator of the expanding granulation tissue. In contrast, the M2 macrophages promote regeneration by up-regulating antiinflammatory cytokines. A paucity of M2 macrophages infiltrates the healing ligament. The dominance of M1 over M2 cells may further exacerbate scar formation.

Interleukin-4 (IL-4) is a pleiotropic cytokine involved in cell growth, immune system regulation, antiinflammation, differentiation of T lymphocytes to Th2 lymphocytes, and promotion of macrophages to the M2 phenotype. Although normally found at low levels in uninjured tissue, IL-4 increases significantly 1 day after injury and peaks at 4 days before decreasing to normal levels by 21 days.[15] This up-regulation of macrophage-produced IL-4 results in exaggerated collagen and ECM production by fibroblasts.[16,17] In mouse dermal wounds, daily IL-4 treatments accelerated the formation of granulation tissue and wound closure.[15] In contrast, healing was delayed in wounds treated with IL-4-antisense oligonucleotides, although topical IL-4 administration overrode the delay.[15] These reported effects led to the present study, which was designed to investigate the potential of IL-4 to improve ligament healing. We hypothesized that IL-4 treatment accelerates healing to control inflammation and reduce scar formation. Our results indicate that IL-4 treatment to the injured ligament reduces wound size, decreases type III collagen, and increases type I procollagen. However, supplementation of IL-4 alone was unable to maintain these effects beyond 5 days or increase healing strength of the ligament.

## MATERIALS AND METHODS

### Cell culture

Fibroblasts from injured and uninjured ligaments were obtained from MCLs at day 5 postinjury and from intact rat ligaments as controls. Ligaments were collected from rats that underwent bilateral surgical ruptures of their MCLs described below. Five days postinjury, ligaments were dissected and minced in Hanks' balanced saline solution using sterile techniques. Intact MCLs were similarly collected and processed. Ligament tissue was digested overnight in filtered 0.5% type IV collagenase (Worthington, Lakewood, NJ) centrifuged, resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), expanded in 75 $mm^2$ treated polystyrene flasks (Corning, Corning, NY) and grown to confluence. After reaching confluence, cells were trypsinized, counted, and plated at $3\times10^3$ cells/well in DMEM containing 2% FBS in tissue-culture-treated Falcon 12-well polystyrene plates (Becton Dickinson, Franklin Lakes, NJ). After 24 hours, fibroblasts obtained from the healing or intact MCLs were separately treated with recombinant rat IL-4 (Sigma-Aldrich, St. Louis, MO) at 0, 0.01, 1, or 100 ng/mL containing DMEM and 2% FBS. Untreated cells (0 ng/mL IL-4) were exposed to medium containing 2% FBS. Treatments were changed every 24 hours. At 24, 48, and 72 hours posttreatment, cell proliferation was determined using MTS assay (Cell Titer 96 $Aq_{ueous}$ One Solution Cell Proliferation Assay, Promega, Madison, WI).

### Animals

This study was approved by the University of Wisconsin Institutional Animal Use and Care Committee. Thirty-four skeletally mature male Wistar rats (275–299 g) were used as an in vivo animal model for ligament healing. All rats were purchased with fitted external jugular catheters to enable IV treatment administration. Animals were divided into four experimental groups based on time of collection and dose of IL-4. In *experiment 1*, animals were divided into groups of three and subjected to lower doses of 1 ng/mL of IL-4 (low-dose [LD] Day 5) or phosphate-buffered saline (PBS) IV until collection at day 5. For *experiment 2*, animals were treated with either high doses of 100 ng/mL IL-4 (high-dose [HD] Day 5) or PBS IV until collection at day 5 ($n=3$/treatment). *Experiment 3* used the same treatments as experiment 2 but then survived the rats until day 11 before collection providing a treated (HD Day 11) and control (PBS) group of animals ($n=8$/treatment). During all experiments, IL-4 or PBS was administered 2 days before surgery (d2), the day of surgery (d0), and daily thereafter until 4 days postinjury. Finally, *experiment 4* animals were treated with IV injections of 100 ng/mL IL-4 (Daily Day 11) or PBS until the time of sacrifice at day 11 ($n=3$/treatment). In *experiment 4* animals were subjected to IL-4 or PBS injections at d2 and d0 and daily until 10 days postinjury. Ligaments from three animals per treatment in all four of the above experiments were collected and used for immunohistochemistry (IHC) and histology. Another five animals/group were included in experiment 3 for mechanical testing. Mechanical testing was not performed on day 5 tissue, because the ligament is too compromised for meaningful mechanical data.

### Surgical procedure

Two days before surgery, animals were administered IL-4 or PBS via IV injections into their previously implanted jugular catheters. Rats were anesthetized (day 0) via isofluorane. Surgical group rats were then subjected to bilateral transactions of their MCLs using sterile techniques. MCLs were transected, rather than torn, to create a uniform defect for healing. A small, 1 cm skin incision was made over the medial aspect at both the left and right stifles. The subcutaneous tissue was dissected to expose the sartorius muscle and underlying MCL. The axial mid-point of the MCL (determined using a scaled scalpel handle) was completely transected and the muscular, subcutaneous, and subdermal tissue layers were each closed with 4-0 dexon suture. All animals were allowed unrestricted cage movement immediately after surgery. At 5 and 11 days postinjury, animals were sacrificed and the MCLs collected. MCLs were used for IHC or mechanical testing.

### Tissue harvest

At the time of sacrifice, the MCLs used for IHC were carefully dissected, measured, weighed, and immediately placed in OCT for flash freezing. Longitudinal cryosections were

then cut at a 5 μm thickness, mounted on Superfrost plus microscope slides, and maintained at −70 °C. Animals used for mechanical testing were sacrificed and stored in toto at −70 °C until animals were defrosted, MCLs femurs and tibia were dissected, and MCLs were tested.

### Histology

Ligament cryosections were H&E stained to observe general morphology of the healing ligaments. After staining, images were captured and the granulation tissue regions were measured using Image J.

### IHC

Immunostaining was performed on frozen sections using mouse monoclonal or rabbit polyclonal antibodies. Cryosections were fixed for 10 minutes with acetone, exposed for 5 minutes to 3% hydrogen peroxide to eliminate endogenous peroxidase activity, blocked for 30 minutes with Background Buster (Innovex Biosciences, Richmond, CA) and incubated with rabbit or mouse primary antibody. Sections were then incubated with biotin, and streptavidin conjugated to horseradish peroxidase using the Stat Q staining kit (Innovex Biosciences). The bound antibody complex was then visualized using diaminobenzidine. Stained sections were dehydrated, cleared, cover-slipped, and viewed using light microscopy. Negative controls omitting the primary antibody were included with each experiment. Positive controls of gut or spleen were also included.

Mouse monoclonal antibodies to cell surface markers, CD68, CD163, and CD3, were used to identify the classically activated macrophages (M2), alternatively activated macrophages (M1), and T-lymphocytes, respectively (all from Abcam-Serotec, Raleigh, NC, at a dilution of 1:100). To identify collagen production, type I procollagen (straight; SP1.D8; Developmental Hybridoma, Iowa City, IA) and type III collagen (1 : 8,000, Sigma-Aldrich) mouse antibodies were used. Endothelial cells were identified using the polyclonal rabbit antibody thrombomodulin (1 : 2,500; American Diagnostica, Stamford, CT) and myofibroblasts were identified using α-smooth muscle actin (straight; Abcam-Serotec).

### Quantification

After IHC staining, micrographs were collected using a camera-assisted microscope (Nikon Eclipse microscope, model E6000, Nikon Instruments, Inc, Mellville, NY, with an Olympus camera, model DP79, Olympus Imaging America, Inc, Center Valley, PA). Six blocked random pictures were obtained from each stained cryosection. Images were captured at the healing region (HR), the edges of the healing regions, including the distal healing region (dHR) and proximal healing region (pHR), the distal (distal) and proximal (prox) ends of the MCL, and the epiligament (EL) (Figure 1). Two to three sections were counted per animal. Granulation tissue measurements, endothelial cells, myofibroblasts, type I procollagen, and type III collagen were then quantified with Image J (National Institutes of Health). Images captured for T lymphocytes, and M1 and M2 macrophages were quantified manually.

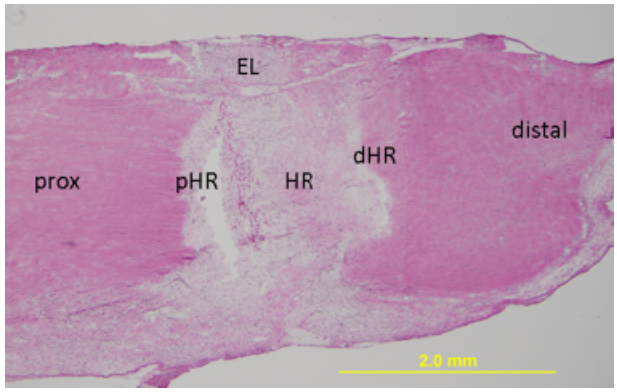


**Figure 1.** Representative cross section of an H&E-stained healing MCL, indicating the approximate locations subsequent images were captured for later cell enumeration. Two to three sections from each animal were examined accordingly. HR, healing region; pHR, proximal healing region; dHR, distal healing region; prox, proximal; EL, epiligament; H&E, hematoxylin and eosin. Original magnification ×40.

### Mechanical testing

The mechanical behavior of the MCLs was tested to determine the influence of macrophage inhibition on the functional integrity of the healing tissue. Pull-to-failure testing was performed as previously described by Provenzano et al.[18–20] Each MCL was removed with both femoral and tibial insertion sites intact. The surrounding tissue was carefully excised to avoid damaging the insertion sites. During preparation, the Femur–MCL–tibia (FMT) complex was kept hydrated using PBS. The width and thickness of the ligament were measured optically and the cross-sectional area for the ligament was estimated assuming an elliptical cross section. The FMT complex was mounted in a custom testing bath and mechanical testing machine. Optical markers were applied to the ligament on the insertion sites and the tests were recorded. A preload of 0.1 N was applied to the ligament and the MCL was preconditioned (cyclically loaded to approximately 1% strain for 10 cycles). Dimensional measurements for the ligament were recorded at the preload. The ligament was then pulled to failure at a rate of 10% strain per second.

Failure force, failure stress, and stiffness parameters were all measured to determine ligament functional behavior after treatment. Failure force was recorded as the highest load before failure of the ligament and failure stress was calculated by dividing the failure force by the initial cross-sectional area measurements. Failure strain was calculated by dividing the change in ligament length during testing by the initial length of the ligament. Ligament stiffness was defined as the slope of the linear region of the stress–strain curve. In the linear region, this number is nearly constant and thus can be calculated by identifying the linear region and average slope.

### Statistical analysis

For cell culture data, a three-way analysis of variance (ANOVA) analysis was implemented to determine the

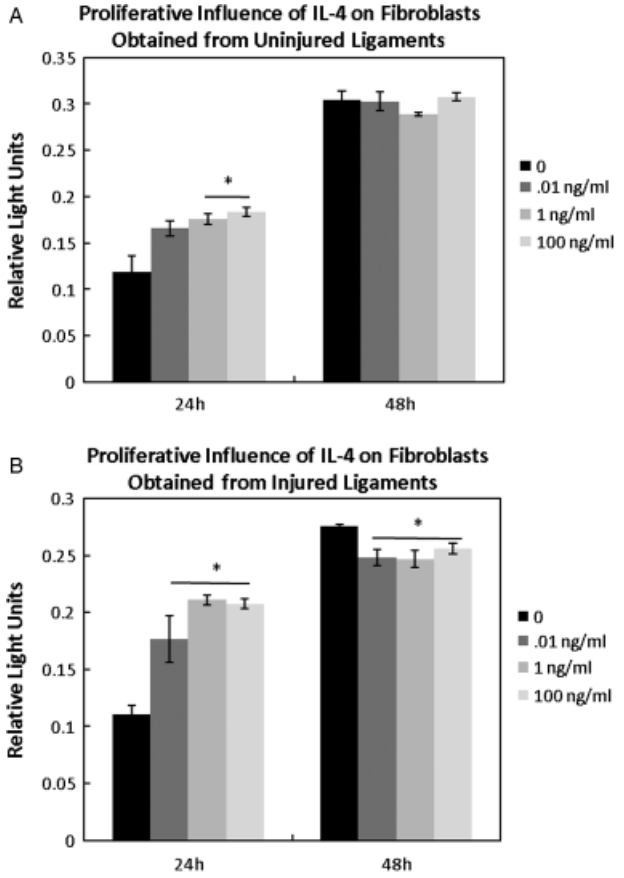


**Figure 2.** Dose response of IL-4 on fibroblast proliferation from cells obtained from uninjured (A) or day 5 postinjured ligaments (B). Cells were collected at 24, 48, and 72 (not shown) hours posttreatment and quantified. Error bars represent SEM.

significance of all main effects and interaction effects (up to three-factor interaction). Specifically, the main effect testing included cell effect (''intact'' vs. ''day 5''), time effect (''24 hours,'' ''48 hours,'' and ''72 hours''), and dose effect (''dose 0,'' ''dose 0.01,'' ''dose 1,'' and ''dose 100''). The interactions, cell×time, cell×dose, time×dose interaction, and cell×time×dose were also tested. The three-way ANOVA analysis of cell×time×dose interactions was insignificant. Therefore, a two-way ANOVA model in which all main effects and three two-factor interactions, i.e. (cell, time), (cell, dose), and (time, dose), was used. A pair-wise contrast *F*-test was used for testing the pair-wise differences between groups.

Ligament regions (Figure 1) were separately analyzed and pooled into various subgroups (with italicized names below) to determine any spatial cellular or ECM factor differences. For IHC analysis, two to three MCL sections per rat were used (3 rats/treatment). Six blocked random regions per section were then counted. The *total* accounts for the average cell numbers throughout the entire ligament. The *MCL* includes the average means of all the subgroups excluding the EL. The *granulation tissue* contains the HR, pHR, and dHR regions (Figure 1). The *ends* include the proximal and distal ligament ends excluding the granulation tissue and epiligament. Finally, the EL only considers the EL measurements. All IHC assays were analyzed using one-way ANOVA to observe for treatment differences (IL-4 and PBS) within the specific subgroups. Specifically, an *F*-test was used for testing the overall difference among treatments. A pair-wise contrast *F*-test was used for testing the pair-wise differences between groups. Mechanical testing data were analyzed via *t*-tests (5 rats/treatment). $p < 0.05$ was used as the criterion for statistical significance of all experiments. All analyses were conducted using the statistical software package R-2.9.1.[21]

## RESULTS

### Cell proliferation

To determine the in vitro dose effects of IL-4 on fibroblasts obtained from healing and injured ligaments, IL-4 was tested on cultured cells. Twenty-four hours posttreatment, fibroblast proliferation from both the injured and the intact ligament significantly increased at all concentrations tested (0.01, 1, and 100 ng/mL IL-4; Figure 2). At 48 hours, IL-4 did not significantly influence fibroblast proliferation from the intact ligament cells. In contrast, all tested concentrations of IL-4 inhibited proliferation in injured ligament fibroblasts. No effects were observed at 72 hours, regardless of cell type (data not shown).

### LD (1 ng/mL) IL-4 results

In vivo administration of 1 ng/mL IL-4 (experiment 1; $n=3$ rats/treatment) resulted in no significant differences in granulation tissue area, M2 macrophages, T lymphocytes, procollagen, type III collagen, or endothelial cells between the PBS controls. Only the M1 macrophages within the EL were significantly different after IL-4 treatment. Based on these LD results, a higher dose of IL-4 (100 ng/mL) was tested in vivo for experiments 2–4.

### Morphological measurements/granulation tissue size

To determine the in vivo influence of IL-4 on ligament healing, area of granulation tissue was measured. Size of granulation tissue was normalized to the total ligament area. High doses of IL-4 significantly decreased granulation tissue size ($p=0.049$) at day 5 (Figures 3 and 6A and B). No other significant effects were observed.

### Macrophages and T lymphocytes

Because the probable targets of IL-4-mediated immune suppression are the T lymphocytes and macrophages, these cell types were evaluated after IL-4 treatment (Figure 4). The number of M1 macrophages was reduced within the EL ($p=0.001$) after LD IL-4 treatment ($103.1 \pm 9.5$ cells/mm$^2$ PBS vs. $47.1 \pm 8.3$ cells/mm$^2$ IL-4) (data not shown). Administering a higher dose of IL-4 tended to decrease the MCL M1 macrophages at day 5 ($p=0.078$), but no other experimental groups or time points were significantly altered (Figure 4A). On day 5, the number of M2 cells was not significantly altered after IL-4 treatment, but daily doses of IL-4 decreased

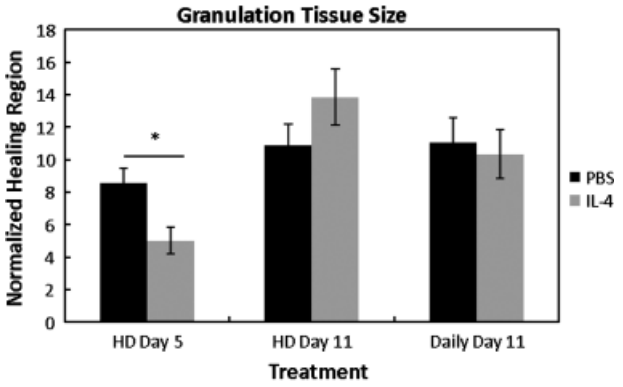


**Figure 3.** Graph of granulation tissue size after IL-4 treatment ($n$=3 rats/treatment; 21 total rats). High doses of IL-4 significantly reduced the normalized healing region at day 5 ($p < 0.05$). No other significant treatment effects were observed at day 11 or with low doses of IL-4. Values are the mean percentage of the granulation tissue area divided by the total ligament area ± SEM. IL, interleukin; SEM, standard error of mean.

($p$=0.004) the number of M2 cells within the granulation tissue at day 11 (Figure 4B). A paucity of T lymphocytes was identified in the ligament regardless of treatment (Figure 4C). IL-4 did not significantly influence T-lymphocytes numbers at day 5 ($p > 0.05$). However, T-lymphocytes numbers were diminished in the granulation tissue of the day 11 ligament from experiment 3 (Figure 4C; $p$=0.03).

### Type I and III pro/collagen

To determine whether IL-4 influences the primary collagens involved in ligament healing, collagen type I and III were analyzed. Type I procollagen increased significantly ($p$=0.03) in the day 5 ligament after treatment of HD IL-4 (Figures 5A and 6). Concomitantly, type III collagen MCL levels decreased ($p$=0.003) in response to IL-4 treatment (Figures 5B and 6). This modification of the normal healing process was transient, however. After cessation of IL-4 treatment, no other treatment effects were observed with type I or III collagen on day 11. Continuation of IL-4 treatment resulted in detrimental effects on collagen production. Daily doses decreased procollagen type I ($p$=0.057) while maintaining type III collagen ($p$=0.15), which is abundant in scar formation. These results agree with the in vitro data indicating time-dependent effects of IL-4 on fibroblast proliferation.

### Endothelial cells

Studies in other tissues report an inhibitory influence of IL-4 on angiogenesis.[22,23] Therefore, we investigated endothelial cell localization using IHC. In the day 5 specimens, a HD of IL-4 increased the average number of endothelial cells within the EL ($p$=0.01; data not shown). No other day 5 effects were observed. In contrast, IL-4 significantly reduced the number of MCL endothelial cells in both day 11 groups (HD day 11: $p$=0.03; Daily Day 11: $p$=0.01) when compared with PBS controls (Figure 7A).

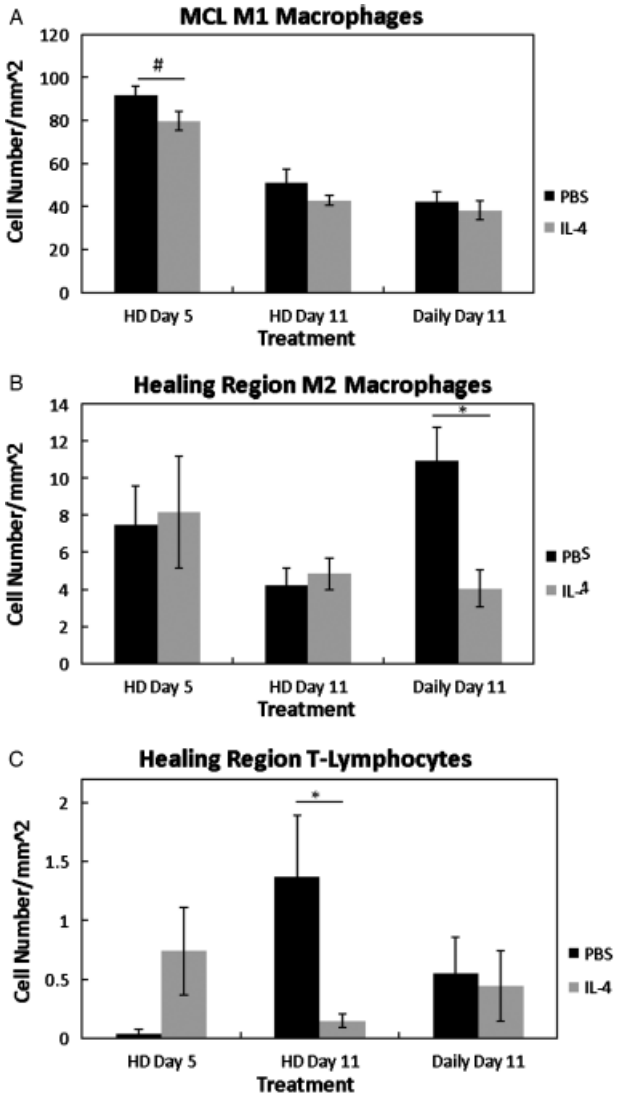


**Figure 4.** Graph of the M1 macrophages (A), M2 macrophages (B), and T lymphocytes (C), at 5 and 11 days postinjury after PBS or IL-4 treatment ($n$=3 rats/treatment; 21 total rats). On day 5, high doses of IL-4 (HD Day 5) tended to reduce the MCL M1 macrophages ($p$=0.078) (A). No differences within the MCL were observed at any other points. The day 11 healing region M2 macrophages were significantly reduced after continuous treatment of IL-4 (Daily Day 11) (B). High doses of IL-4 (HD Day 5) reduced the number of healing region T lymphocytes at day 11. #A trend ($p < 0.1$ between PBS and IL-4 at day 5). *Significance ($p < 0.05$) between PBS and IL-4 at day 11 (C). Values are expressed as mean cell numbers ± SEM. IL, interleukin; SEM, standard error of mean; MCL, medial collateral ligament; PBS, phosphate-buffered saline; HD, high dose.

### Myofibroblasts

Myofibroblasts are differentiated fibroblasts known to aid in wound contraction. The LD IL-4 treatment group exhibited no discernable effects, hence this group was omitted from further testing. No significant difference ($p > 0.05$) in

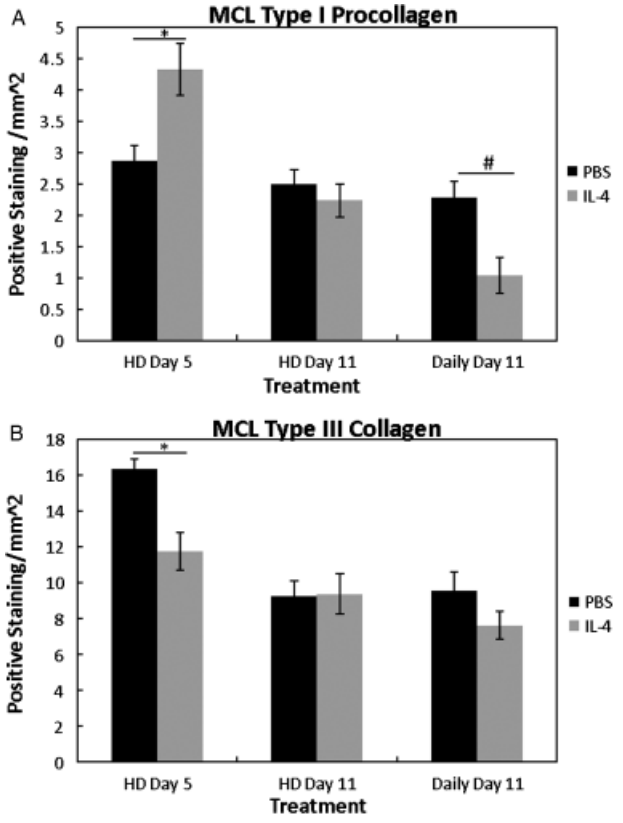


**Figure 5.** Graph of type I procollagen (A) and type III collagen (B) after PBS and IL-4 treatment ($n$=3 rats/treatment; 21 total rats). High doses of IL-4 (HD Day 5) significantly increased type I procollagen (A) and decreased type III collagen (B) at day 5 within the MCL. However, daily doses of IL-4 (Daily Day 11) reduced type I collagen ($p$=0.057) (A). No other effects were observed with type III collage at day 11 (B). *Significant difference ($p < 0.05$) between PBS and IL-4 at day 5 (A and B) or day 11 (A). #A trend ($p < 0.1$ between PBS and IL-4 at day 11 (Daily Day 11). Values are expressed as mean positive staining ± SEM. IL, interleukin; SEM, standard error of mean; MCL, medial collateral ligament; PBS, phosphate-buffered saline; HD, high dose.

granulation tissue-localized myofibroblasts was found in the day 5 or day 11 HD group when compared with PBS controls (Figure 7B). However, daily administration of IL-4 decreased the number myofibroblasts ($p$=0.03).

### Spatial localization of IHC factors

To target any cellular or ECM spatial differences after IL-4 treatment, the total ligament, MCL body, granulation tissue region, extraneous granulation tissue region, and the EL were individually analyzed. Treatment primarily affected IHC factors within the granulation tissue, including the T lymphocytes, type I procollagen, endothelial cells, M2 macrophages, and myofibroblasts. IL-4 was less influential within the EL and uninjured ligament regions, affecting only endothelial cells and M1 cells within the EL, and type I procollagen and type III collagen within the uninjured regions.

### Mechanical testing

To determine if IL-4 treatment affected ligament function, the day 11 samples (HD day 11) (Figure 8) were mechanically tested. Ligament failure force, failure stress, and stiffness were measured. No significance difference was found between IL-4-treated specimens and the PBS control for any parameter tested ($p > 0.05$). These results therefore suggest that IL-4 treatment did not reduce the mechanical properties of the healing ligament or inhibit functional recovery.

## DISCUSSION

We hypothesized that IL-4 treatment would accelerate healing by controlling inflammation and reducing scar formation. Results indicate beneficial, albeit transient effects of IL-4 on ligament healing. The in vitro study reveals a time-dependent effect of IL-4 on fibroblasts obtained from normal and injured tissue. It also shows different behaviors in fibroblasts from normal and injured tissues. In vivo administration of IL-4 stimulates early biological healing in a dose-dependent manner without degrading mechanical recovery compared with normal healing. Finally, continued IL-4 treatment negates the early benefits in the observed healing response.

IL-4 is known to control the inflammatory response by modulating the inflammatory M1/Th1 cells toward a M2/Th2 pathway, respectively. In the current study at day 5, high doses of IL-4 accelerated in vivo ligament repair by reducing wound size, stimulating type I procollagen, and inhibiting type III collagen. High doses of IL-4 also tended to reduce M1 macrophages and increase T lymphocytes without affecting the number of myofibroblasts or endothelial cells. However, the in vivo and in vitro results consistently showed a time-limited response to IL-4. After 24 hours, IL-4 stimulated the in vitro proliferation of fibroblasts from tissue obtained from injured and uninjured tissue. In contrast, fibroblasts collected from the injured ligament were inhibited by IL-4 after 48 hours, and cells from the uninjured ligament were not. Similarly, in vivo IL-4 administration up to 4 days beyond injury showed early regenerative effects (observed at day 5), but these effects were lost by day 11.

In an attempt to sustain the regenerative effects of IL-4, an experiment was performed in which IL-4 was administered daily until the time of collection at day 11 with the expectation that sustained IL-4 treatment would maintain the regenerative response. Studies administering IL-4 to chronic inflammatory models support this hypothesis. For example, Horsfall et al.[24] administered daily IL-4 treatments to a collagen-induced arthritis (CIA) model. CIA results in a chronic macrophage response, coinciding with enhanced tissue damage. Daily IL-4 treatments suppressed CIA for 28 days. Once IL-4 treatments were halted, arthritis resumed, inflammation elevated, and treated joints were comparable with the controls.[4] These results suggest that

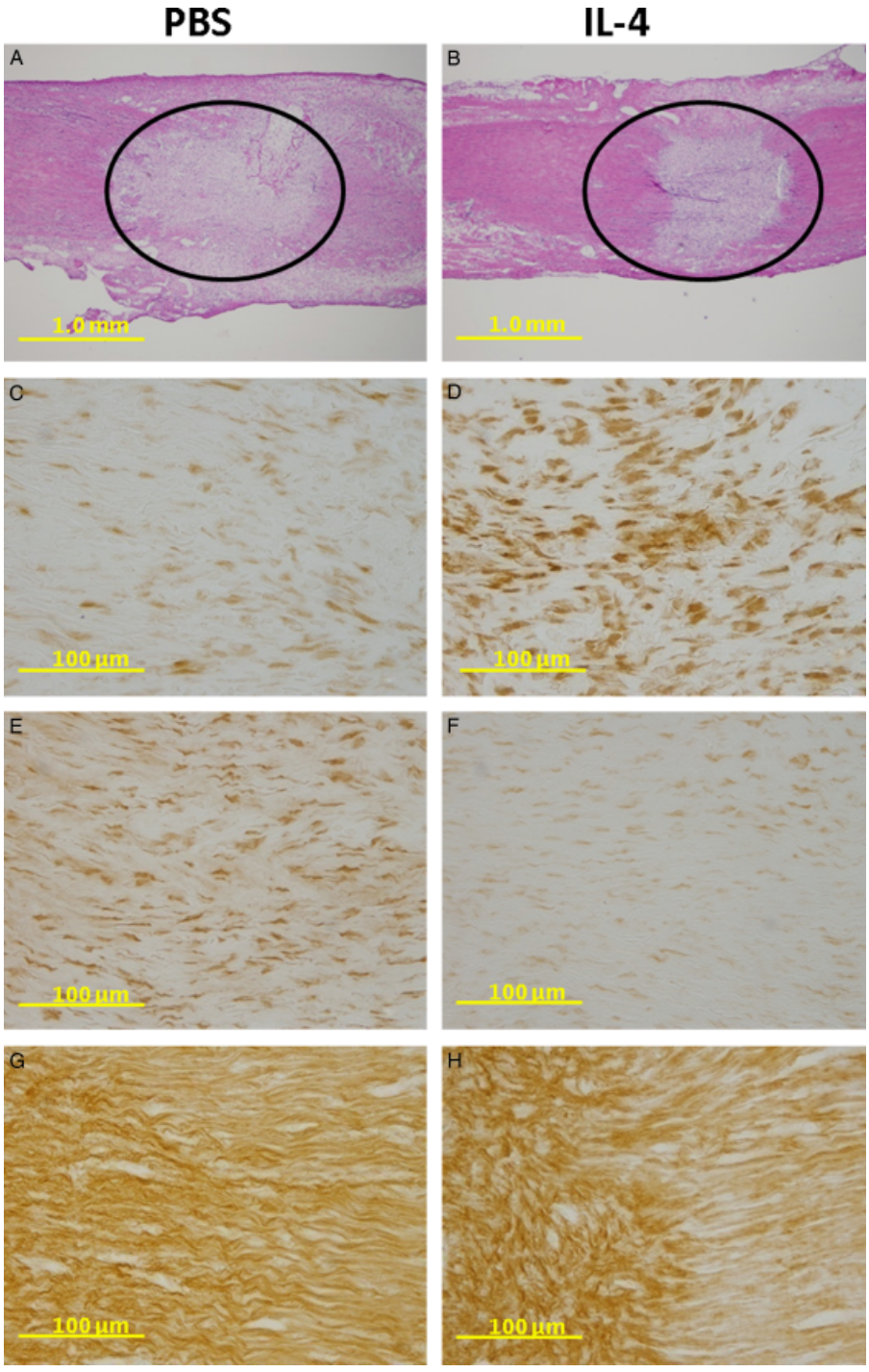


**Figure 6.** Representative micrograph of H&E (A and B), Type I procollagen (C–F) and Type III collagen (G and H) after PBS (left column) or IL-4 (right side) treatment. Size of the day 5 granulation tissue after PBS (A) or IL-4 (B) treatment. Black circles are the same size to compare the granulation size differences between the two groups (A and B). Type I procollagen IHC of the day 5 ligament after treatment with PBS (C) or high doses of IL-4 (D). Type I procollagen IHC of the day 11 ligament after PBS treatment (E) or daily high doses of IL-4 (F). Type III collagen IHC of the day 5 ligament after PBS (G) or high doses of IL-4 (H) treatment. IL, interleukin; PBS, phosphate-buffered saline; IHC, immunohistochemistry.

IL-4 acts on the persistently produced macrophages that are common to chronic inflammatory conditions.[25] Discontinuing IL-4 treatments no longer provided the necessary signals to control the immune cell response, thus enabling chronic inflammation to resume.[25] Our results indicate that continuous IL-4 administration beyond inflammation did not maintain the regenerative response and in fact inhibited healing and cell proliferation as confirmed by the in vivo experiment 4 and the in vitro 48-hour experiments, respectively. These results suggest that IL-4 accelerates healing during the inflammatory phase, but is unable to sustain the repair response beyond the inflammatory phase of healing at the concentrations of IL-4 tested in this study.

IL-4 is known to influence macrophages and T lymphocytes during inflammation but our in vitro and in vivo

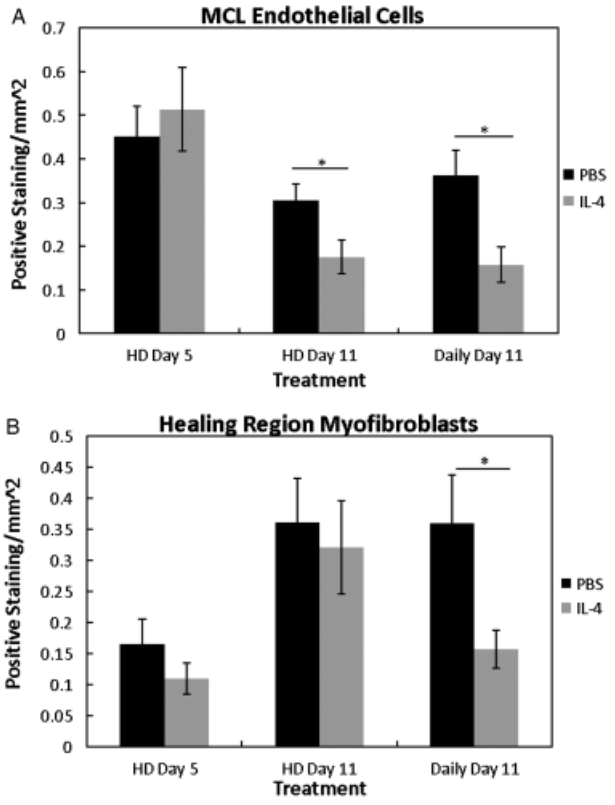


**Figure 7.** Response of endothelial cells (A) and myofibroblasts (B) to PBS or IL-4 ($n$=3 rats/treatment; 21 total rats). No treatment effects were observed at day 5 (A and B). In contrast, IL-4 significantly reduced the number of MCL endothelial cells at day 11 (A). Healing region myofibroblasts were also reduced with daily doses of IL-4 at day 11, whereas injections up to day 4 were not effective (B). *Significant difference ($p < 0.05$) between PBS and IL-4 at day 11 (A and B). Values are expressed as mean positive staining ± SEM. IL, interleukin; SEM, standard error of mean; MCL, medial collateral ligament; PBS, phosphate-buffered saline.

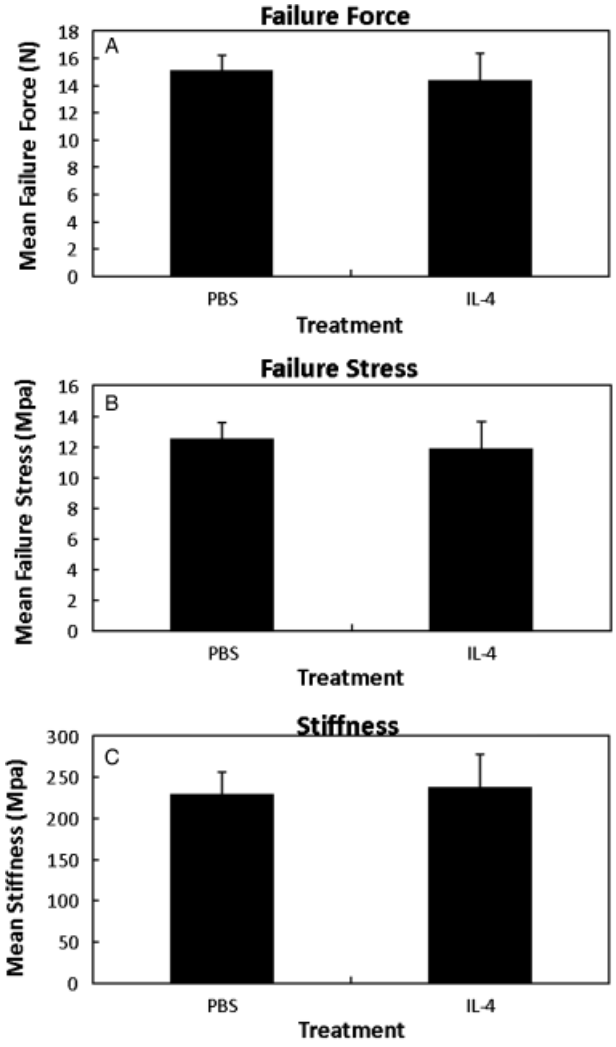


**Figure 8.** Failure force (A), failure stress (B), and stiffness (C) in response to PBS or IL-4 treatment 11 days postinjury ($n$=5 rats/treatment; 10 total rats). No significance in failure force (A) failure stress (B) or stiffness (C) was observed. $p > 0.05$. Results are expressed as mean ± SEM. IL, interleukin; SEM, standard error of mean; PBS, phosphate-buffered saline.

results indicate that IL-4 also influences MCL fibroblasts. Fibroblasts contain high-affinity IL-4 receptors and express collagen after IL-4 stimulation.[26–28] Huaux et al.[29] reported a dual role for IL-4, suggesting that IL-4 acts on the immune cells during early inflammation and on fibroblasts during fibrosis. Although our study only found a tendency for IL-4 to reduce macrophage numbers, treatment may have altered the macrophage-induced cytokine release. Numerous studies confirm the ability of IL-4 to block mononuclear phagocytic cell production of proinflammatory cytokines.[30–32] In this capacity, IL-4 may play a beneficial role by limiting early inflammatory signals, but may also play a detrimental role by promoting scarring after macrophages subside. Additionally, IL-4 may also influence the T lymphocytes in our wound-healing model. At day 11, daily doses of IL-4 significantly reduced the number of T lymphocytes, possibly signifying a regenerative role for the few T lymphocytes found in the ligament. The signals that govern regeneration vs. scar during early and late healing are not fully understood but are believed to be a coordinated response between macrophages, T lymphocytes, and fibroblasts.

The discrepancy in the IL-4-induced proliferative response between fibroblasts obtained from injured ligament and fibroblasts obtained from uninjured ligament is a new finding. During the first 24 hours posttreatment, fibroblasts from intact and injured ligaments responded to all doses of IL-4 tested. By 48 hours posttreatment, IL-4 treatment inhibited fibroblast proliferation from injured tissue but was not effective on fibroblasts from uninjured tissue. The difference in cell response to IL-4 may be

attributed to a change in IL-4 receptor number, a change in affinity of IL-4 to its receptor, the change in cytokine environment induced by the wound-healing response, or the difference in receptor internalization or processing mechanisms to induce proliferation. Further studies are required to test these concepts.

A desired healing scenario after injury would regenerate the ligament with nearly normal composition, microstructure, and mechanical properties. Although IL-4 promoted early ligament healing, treatment with this factor failed to improve mechanical or functional properties. The current results suggest that treatment of multiple IL-4 bolus injections to the injured ligament is insufficient to suppress scar tissue formation and improve mechanical properties during healing. A number of investigators reported enhanced biological responses after exogenous growth factor/cytokine treatment but only a few showed increased healing strength.[7–10,33] Continual release of a cytokine treatment rather than a bolus injection with a limited half-life may increase efficacy and improve the healing outcome. Thomopoulos et al.[34] demonstrated enhanced biological tendon healing after bFGF treatment, but no improvement in healing strength. In contrast, gene transfer of bFGF, resulting in controlled delivery, improved tendon healing strength.[33] In our lab, Provenzano et al.[35] showed increases in healing tissue strength with insulin-like growth factor-1 and growth hormone. These results suggest that not only the type and concentration of the cytokine/growth factor delivered but also the time, duration, and method of delivery influence healing outcome and mechanical behavior.

The current study also compared the spatial differences of the tested factors between the MCL, EL, healing regions, and uninjured regions after IL-4 treatment. The effects of IL-4 were primarily observed within the granulation tissue of the healing ligament, whereas few cellular changes occurred within the EL or the uninjured regions. Ligament vascularity, cellular differentiation, phagocytosis, and collagen synthesis are primarily localized to the EL, during normal healing.[36–39] However, the current results imply that IL-4 influences the fibroblasts and/or immune cells within the ligament body, suggesting a direct influence of IL-4 on the MCL.

Numerous reports have shown macrophage-induced cytokine modulation, but this study did not. The current study was not focused on which cells IL-4 influenced, but rather if IL-4 affected ligament healing via structural and compositional criteria. Furthermore, fibroblasts were only studied for the dose-dependent effects of IL-4. Certainly, the influence of macrophages, T lymphocytes and the coculture of these cells with fibroblasts would provide additional information about the specific cellular dose–response of IL-4.

In summary, IL-4 enhances ligament regeneration during the early phase of MCL healing. With administration of IL-4, the normally high production of type III collagen in the healing tissue was reduced while type I procollagen was stimulated. The response appears more regenerative of native tissue. However, IL-4 was insufficient to maintain these regenerative effects throughout the remodeling phase of tissue healing, suggesting that other downstream factors must be considered and modulated in order to achieve regenerative healing.

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