# IL-10 Cytokine Released from M2 Macrophages Is Crucial for Analgesic and Anti-inflammatory Effects of Acupuncture in a Model of Inflammatory Muscle Pain

**Morgana D. da Silva · Franciane Bobinski · Karina L. Sato · Sandra J. Kolker · Kathleen A. Sluka · Adair R. S. Santos**

**Abstract** Muscle pain is a common medical problem that is difficult to treat. One nonpharmacological treatment used is acupuncture, a procedure in which fine needles are inserted into body points with the intent of relieving pain and other symptoms. Here we investigated the effects of manual acupuncture (MA) on modulating macrophage phenotype and interleukin-10 (IL-10) concentrations in animals with muscle inflammation. Carrageenan, injected in the gastrocnemius muscle of mice, induces an inflammatory response characterized by mechanical hyperalgesia and edema. The inflammation is initially a neutrophilic infiltration that converts to a macrophage-dominated inflammation by 48 h. MA of the Sanyinjiao or Spleen 6 (SP6) acupoint reduces nociceptive behaviors, heat, and mechanical hyperalgesia and enhanced escape/avoidance and the accompanying edema. SP6 MA increased muscle IL-10 levels and was ineffective in reducing pain behaviors and edema in IL-10 knockout (IL-10$^{-/-}$) mice. Repeated daily treatments with SP6 MA induced a phenotypic switch of muscle macrophages with reduced M1 macrophages (pro-inflammatory cells) and an increase of M2 macrophages (anti-inflammatory cells and important IL-10 source). These findings provide new evidence that MA produces a phenotypic switch in macrophages and increases IL-10 concentrations in muscle to reduce pain and inflammation.

**Keywords** Acupuncture · SP6 · Nociception · Inflammation · Pain · Hyperalgesia · Edema · Muscle · IL-10 cytokine · Macrophage

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## Introduction

Muscle pain is one of the most frequent symptoms encountered by primary care providers [1]. The underlying mechanisms of muscle pain are complex and involve changes in the peripheral as well as the central nervous system [2]. Numerous pain conditions, such as myositis, strains, or exercise-induced muscle pain, are associated with muscle inflammation, and inflammatory cytokines, growth factors, and prostaglandins that are released during inflammation sensitize nociceptive pathways [3-5].

In muscle injury, macrophages are required for the resolution of inflammation and restoration of tissue integrity by removing debris and promoting the proliferation and differentiation of parenchymal cells [6]. Classically activated macrophages (M1 macrophages) release inflammatory cytokines; mediate defense of the host from a variety of bacteria, protozoa, and viruses; and have roles in antitumor immunity. Alternatively activated macrophages (M2 macrophages) have an anti-inflammatory function and can secrete interleukin-10 (IL-10) in response to Fc receptor-$\gamma$ binding [7-9]. Macrophages

play dual roles in orchestrating the onset of inflammation primarily through the M1 phenotype and then can switch to an M2 phenotype to promote healing and repair [6, 10].

Manual acupuncture (MA) is used to manage a variety of painful conditions including muscle pain. It involves insertion of fine needles into an individual at discrete points that are then manipulated, with the intent of relieving pain [11]. Acupuncture has been shown to have both analgesic and anti-inflammatory effects through actions in the nervous system and the local tissues [12-14]. Prior studies reveal involvement of sensory fibers, central spinal pathways, and molecules released locally [13-15]. Our previous work shows that unilateral Sanyinjiao or Spleen 6 (SP6) MA elicited significant anti-inflammatory effects in a mouse model of peritonitis, and this effect depended on the adrenal glands and increased circulating levels of IL-10 [16]. For this reason, the current study sought to identify if SP6 MA could reduce muscle pain and inflammation and if this was mediated through IL-10. For this purpose, we used a well-characterized model of muscle inflammation induced by carrageenan to demonstrate the analgesic and anti-inflammatory effects of SP6 MA. In addition, the participation of IL-10 and anti-inflammatory macrophages in the effect of SP6 MA was analyzed through the use of IL-10 knockout (IL-10$^{-/-}$) mice and by immunohistochemistry, respectively.

## Materials and Methods

### Animals

A total of 112 mice were used in these studies (Swiss, $n$=80; C57BL/6, $n$=16; IL-10$^{-/-}$, $n$=16). Initial experiments were carried out in Swiss male mice (20 to 30 g) that were kept in a room with controlled temperature (22±1 °C) and humidity (50 to 80 %), under a 12-h:12-h light-dark cycle (lights on at 06:00 am) with food and water ad libitum. All of the procedures used in the present study were approved by the Institutional Ethics Committee of the Universidade Federal de Santa Catarina (CEUA/UFSC, protocol number PP00208) and were carried out in accordance with the "Principles of Laboratory Animal Care" from the National Institutes of Health publication No. 85-23. Other experiments were performed with C57BL/6 (20–30 g) (Jackson Laboratories) and congenic IL-10 knockout (IL-10$^{-/-}$) (20–30 g) (Jackson Laboratories) male mice at the University of Iowa (Iowa City, IA, USA-protocol number 0908193). Animals were housed in the Animal Care Facility with a 12-h light-dark cycle (lights on at 07:00 am). The animals were acclimatized to the laboratory settings for at least 1 h before testing and were used only once throughout the experiments. In addition, the experimental procedures were in agreement with the current guidelines for the care

and protection of animals used for scientific purposes (Directive 2010/63/EU revising Directive 86/609/EEC) and the National Institutes of Health Guidelines. Moreover, the experimental procedures were in agreement with the ethical guidelines for investigations of experimental pain in conscious animals as previously specified [17].

### Inflammatory Muscle Pain Model

Mice were briefly anesthetized with 4 % isoflurane, and 20 μL of 3 % carrageenan dissolved in sterile isotonic saline, which has a slightly acidic pH (6.0), was injected intramuscularly in the gastrocnemius muscle (right). In another group of animals, saline was injected intramuscularly (pH=6.0). Carrageenan causes an initial infiltration of neutrophils and this converts to a macrophage-dominated inflammation by 1 week [4, 18].

### SP6 MA Treatment

Treatments with MA were carried out into SP6 acupoint [19]. Mice were gently handled and lightly restrained in a plastic cylinder (7×2.5 cm) with the right hind limb out of the tube for needling. After cleaning the skin with alcohol, MA stimulation was performed by obliquely inserting a stainless steel needle (0.17×7 mm) to a depth of about 2–3 mm at SP6 acupoint, and the needle was then rotated slowly. The entire procedure was completed in less than 15 s. After inserting the needle, each mouse was placed in a transparent acrylic box (10×10×10 cm$^3$) for the entire 10-min treatment. The animals remained awake, and no signs of distress were observed during acupuncture stimulation. There was no sham acupuncture point in this study because previous studies in our group have demonstrated that sham acupuncture did not affect inflammatory parameters [16]. MA applications were given once per day for 1, 5, or 13 days according to each experimental protocol.

### Behavioral Testing

Animals were acclimated to the behavioral tests for 2 days prior to assessment. For cutaneous mechanical hyperalgesia, they were acclimated to the wire mesh platform for 5 min per session, twice per day. For muscle mechanical hyperalgesia, they were acclimated to a gardener's glove for 5 min per session, twice per day. Prior to testing, animals were acclimated to the behavioral testing room for 1 h.

### Cutaneous Mechanical Hyperalgesia

Mechanical hyperalgesia of the paw was assessed with von Frey filaments (Stoelting, Chicago, USA) using bending forces from 0.02 to 4 g. Fifty percent mechanical paw withdrawal threshold (the force of the von Frey hair in grams to

which an animal reacts in 50 % of the presentations) was determined according to the Dixon up-and-down method [20, 21]. On each testing day, the mice were habituated in individual Plexiglas boxes (9 cm×7 cm×11 cm) on an elevated wire mesh platform for 1 h. Testing was initiated with the 0.4-g filament. The filaments were applied and held for a period of approximately 3 s from underneath the grid floor perpendicular to the plantar surface. A positive response was recorded if the paw was withdrawn, in which case the next weaker filament was applied and the next measurement recorded. In the absence of a response, the next stronger filament was presented. This procedure continued until six responses in the immediate vicinity of the 50 % threshold were obtained. The resulting sequence of positive and negative response was used to interpolate the threshold. Mechanical nociceptive thresholds were evaluated before the carrageenan injection to characterize the baseline response as well as 24 h after (time course) and after the 1st, 2nd, 4th, 7th, 9th, 11th, and 13th days after MA.

## Muscle Mechanical Hyperalgesia

Muscle hyperalgesia was tested by squeezing the gastrocnemius muscle of the mice with a calibrated pair of tweezers until the mouse withdraw the limb as previously described [18]. The force at which the mouse withdrew was measured in millinewton (mN). Three trials, with 5 min in between, at each time point were taken and averaged. A decrease in threshold was interpreted as primary muscle hyperalgesia. Hyperalgesia in both the ipsilateral and contralateral muscles was measured. Muscle hyperalgesia was tested as follows: before and 24 h after carrageenan injection of the muscle and 3 and 5 days after MA.

## Thermal Sensitivity to Heat

Thermal sensitivity was tested with the Hargreaves test [22]. The animals were placed in acrylic cubicles on a glass surface. Infrared light (40 °C) was irradiated onto the hind paw and the latency (in s) to withdrawal was recorded automatically. Three trials, with 5 to 10 min in between, were taken at each time point and averaged. If an animal failed to withdraw its hind paw within 12 s, the heat stimulus was stopped (cutoff time 12 s) and this time was recorded. Thermal sensitivity to heat was evaluated before and 24 h after carrageenan muscle injection and on the 1st, 3rd, 8th, and 10th days after MA.

## Hind Limb Grip Strength

Hind limb grip strength was used as a measure of muscle sensitivity as previously described [23]. Hind paw grip force (Force Gauge, Instrutherm, Brazil) was tested on each individual hind limb. Each animal was gently restrained so that it could grasp with only one hind limb to a wire mesh screen (10×12 cm) and the animal was moved in a rostrocaudal direction, and the maximal force the animal gripped was recorded (g). Each animal was tested twice at intervals of 2–3 min and averaged. Grip force was expressed with the difference between injured limb (right) and the noninjured limb (left) to correct for individual differences. Grip strength was evaluated before and 24 h after induction of muscle inflammation and on the 1st, 3rd, 8th, and 10th days after the MA.

## Escape/Avoidance

To assess supraspinal processing of nociception, we used the escape/avoidance test as previously published in mice [24]. The testing box was made of Plexiglas (16 cm×7 cm×13 cm) with two chambers and placed on top of a wire mesh screen. One half of the box was white with vertical black lines, while the other half was solid white, so that there was no preference. Mice were allowed to move unrestricted to either side of the box for a 30-min period. Mechanical stimulation was applied with a 0.07-g von Frey filament to the plantar surface of either the right or left hind paw. The ipsilateral hind limb was stimulated when the animal was on the white with vertical black lines side and the contralateral hind limb was stimulated when the animal was on the other side of the box (solid white side). Stimuli were given to the hind paw once per second. Measurements were assessed before and 24 h after induction of carrageenan and 5 days after MA. The time the animals spent on each side of the chamber was recorded and percent of time spent on the side ipsilateral to muscle inflammation was calculated.

## Edema

As a measure of inflammation, the degree of swelling was measured using a plethysmometer (Ugo Basile, VA, Italy). Measurements were assessed three times and averaged at each time point for both the ipsilateral and the contralateral hind limb in C57BL/6 mice. Data were expressed as the difference between the inflamed muscle and the contralateral muscle. Edema was evaluated before and 24 h after induction of muscle inflammation and after 5 days of MA.

## Measurement of IL-10 in Muscle

One or 5 days after carrageenan injection, mice were anesthetized with isoflurane (2–3 %, with 100 % $O_2$) and killed by decapitation. The gastrocnemius muscle (about 200 mg) was removed from the injected leg. In the treated animals, the muscle was removed 1 h after SP6 MA. All tissue samples were weighed, frozen in liquid nitrogen, and stored at −70 °C. For cytokine assays, samples were homogenized in phosphate-buffered saline (PBS, pH 7.4) containing Tween

20 (0.05 %), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 2 ng/mL aprotinin, and 0.1 mM benzemethonium chloride. Tissues were homogenized and centrifuged at 3,000×$g$ for 10 min at 4 °C, and the supernatant obtained was stored at −70 °C until further analysis. The total protein content of the supernatant was measured using the Bradford method. The levels of IL-10 were measured using sample aliquots of 100 μL and mouse cytokine ELISA kits from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's instructions (IL-10-DY417 kit, protein range of 31.25–2,000 pg). The level of cytokine was estimated by interpolation from a standard curve by colorimetric measurements at 450 nm (correction wavelength 540 nm) on an ELISA plate reader (Berthold Technologies—Apollo 8—LB 912, KG, Germany). All results were expressed as picograms per milligram of protein.

## Immunohistochemistry of Macrophages in Muscle

Immunohistochemistry of the inflamed muscle was done in the following groups: (1) saline, (2) control, (3) IL-10$^{-/-}$, (4) SP6, and (5) IL-10$^{-/-}$SP6. Mice were deeply anesthetized (100 mg/kg, sodium pentobarbital) and transcardially perfused with heparinized saline followed by freshly prepared 4 % paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline. The gastrocnemius muscles were removed and postfixed in 4 % PFA overnight (4 °C) (day 1). Next, the solution was changed to 15 % sucrose in PBS (4 °C) for 24 h (day 2); then to 30 % sucrose in PBS (4 °C) for 24 h (day 3); then to 1:1, 30 % sucrose in PBS plus OCT (4 °C) (day 4); and finally samples were frozen with Gentle Jane in small cryomolds (day 5). Serial cross sections of 20 μm were cut from each muscle on a cryostat and placed on slides.

Sections from all animals were stained simultaneously for each macrophage marker to eliminate variability between staining. Standard immunohistochemical procedures were used. Briefly, sections were blocked with Fc receptor block followed by 10 % normal goat serum (NGS). For M1 staining, sections were incubated overnight with primary antibody hamster anti-mouse CD11c in 1 % NGS with 0.1 % Triton X-100 (Abdserotec, 1:100). For M2 staining, we used a biotinylated rat anti-mouse αCD206 (Abdserotec, 1:200). On the 2nd day, for M1 staining, sections were incubated with unconjugated (JIR) goat anti-rat Fab fragment (1:100, 30 min), fixed with 1 % PFA (10 min), incubated for 3 h in the primary antibody rat anti-mouse F4/80 (Abdserotec, 1:250, 3 h) followed by the secondary antibodies goat anti-hamster IgG-Rhod-Red-X (1:500, Jackson Immuno Research), and Fab fragment goat anti-rat Dylight488 (1:500, Jackson Immuno Research). On the 2nd day for M2 staining, sections were incubated with unconjugated (JIR) goat anti-rat Fab fragment (1:100; 30 min), fixed with 1 % PFA (10 min), incubated in the primary antibody rat anti-mouse F4/80 (Abdserotec,

1:250, 3 h) with Streptavidin-568 (Vector Labs, 1:5,000, 3 h), followed by Streptavidin-568 (1:1,000, 1 h) with Fab fragment goat anti-rat Dylight488 (1:500, Jackson Immuno Research, 1 h). All the antibodies were diluted (100+1 % NGS+0.1 % Triton X-100+1:100 sodium azide). Slides were coverslipped with Vectashield.

BioRad Laser Sharp 2000 was used to capture the images. The location captured was chosen visually using×20 and×60 objective lens. All images for a given stain were taken under the same conditions and pictures were stored for off-line analysis. Three pictures in each capture image (layers), and five captures per animal were obtained. We used ImageJ (NIH) and manually counted the total number of macrophages (F4/80) and those that were double-labeled for the M1 (CD11c) or the M2 (αCD206) marker. The percent of M1 and the percent of M2 out of the total number of macrophages were calculated for each animal.

## Experimental Protocol

### Experiment 1: Effect of SP6 MA on Inflammatory Muscle Pain Model

The first experiment examined the effects of SP6 MA in male mice injected with 3 % carrageenan or saline into the gastrocnemius muscle. The following groups were compared: (1) saline ($n=8$), (2) control (carrageenan i.m.) ($n=8$), and (3) SP6 (carrageenan i.m.+MA in SP6 acupoint) ($n=8$). Behavioral tests were performed before and 24 h after induction of hyperalgesia and after MA. The first experiment examined (a) cutaneous mechanical hyperalgesia of the paw; (b) thermal sensitivity of the paw; and (c) mechanical hyperalgesia of the muscle with grip strength analysis as outlined above. In these experiments, researchers were double-blinded.

### Experiment 2: Levels of IL-10 in Muscle After Repeated MA

In another set of experiments, the levels of IL-10 in the gastrocnemius muscle were quantified with ELISA in saline, control (inflamed), and SP6 groups, 1 or 5 days after saline or carrageenan injection and after acupuncture. In these experiments, researchers were double-blinded.

### Experiment 3: Effects of SP6 MA in IL-10$^{-/-}$ Mice

Since IL-10 levels were increased by MA in animals with inflammation, we examined if removal of IL-10 would prevent the analgesic effects of MA. Since available IL-10$^{-/-}$Mice are congenic on a C57BL/6 strain, preliminary data confirmed the analgesic effects of SP6 MA in C57BL/6 mice. The final experiment compared the following: group 1—saline (C57BL/6 mice with saline i.m.) ($n=8$); group 2—control (C57BL/6 mice with carrageenan i.m.) ($n=8$); group 3—IL-

$10^{-/-}$ (IL-10$^{-/-}$Mice with carrageenan i.m.) ($n=8$); group 4—SP6 (C57BL/6 mice with carrageenan i.m.+MA in SP6 acupoint) ($n=8$); and group 5—IL-10$^{-/-}$SP6 (IL-10$^{-/-}$Mice with carrageenan i.m.+MA in SP6 acupoint) ($n=8$). After 24 h, we performed the following behavioral tests: (a) mechanical hyperalgesia of the hind paw to stimulation with von Frey filaments; (b) mechanical hyperalgesia of the calf of the hind limb to stimulation with tweezers; (c) escape/avoidance behaviors; and (d) lower limb edema with plethysmometer.

*Experiment 4: Effects of SP6 MA in Muscle Macrophages of Mice*

To examine the phenotype of macrophages in the muscle after MA, we removed and immunohistochemically stained the muscle for M1 and M2 macrophages in animals at the end of experiment 3. The percentage of M1 and M2 macrophages out of the total macrophages was examined as outlined above. In these experiments, researchers were double-blinded.

Statistical Analysis

Data are presented as mean±standard error of the mean (SEM). Behavioral experiments were statistically evaluated by repeated measures ANOVA followed by the post hoc Student-Newman-Keuls test. For ELISA and immunohistochemistry experiments, data were examined with a one-way ANOVA with multiple comparisons followed by the post hoc Tukey HSD test. Statistical analysis was performed with GraphPad Software (San Diego, CA, USA) or SPSS 13.0. The significance level in all cases was set at $p<0.05$.

**Results**

SP6 MA Reduces Nociceptive Behaviors Induced by Muscle Inflammation

To test if SP6 MA reduces pain behaviors, we tested a series of pain behaviors in animals with muscle inflammation. Initial tests using standard reflexive withdrawal threshold tests of the paw and muscle were performed in Swiss mice and later confirmed in C57BL/6 mice.

Intramuscular carrageenan induced marked decreases in mechanical withdrawal thresholds of the paw and muscle and decreased latency to heat in comparison with saline-injected control Swiss mice (Fig. 1a); these effects were observed as early as basal evaluation (24 h after carrageenan), 30 min after basal evaluation, and remained through 8–11 days (Figs. 1a, b and 2a).

A single treatment with SP6 MA significantly ($p<0.05$) increased the mechanical withdrawal threshold of the paw in Swiss mice with muscle inflammation for 1 h (Fig. 1a). To assess if there were continued effects, SP6 MA was done daily for 11 days. MA remained effective (increasing withdrawal threshold of the paw) with repeated treatments in Swiss mice with muscle inflammation ($p<0.05$ in 1, 2, 4, 7, and 11 days immediately after each daily treatment) (Fig. 1b). Similar effects were observed in C57BL/6 mice with reductions in withdrawal thresholds immediately after treatment with MA (Fig. 1c). Similarly, SP6 MA increased withdrawal threshold of the muscle in C57BL/6 mice with muscle inflammation (Fig. 1d). Repeated MA remained effective in increasing the muscle withdrawal threshold on days 3 and 5 ($p<0.001$) (Fig. 1d). A single SP6 MA did not have an immediate effect on thermal withdrawal latency. However, daily SP6 MA significantly increased the thermal withdrawal latency to baseline levels on the 3rd day of treatment ($p<0.01$) in Swiss mice (Fig. 2a). No effects of MA were observed for changes in withdrawal thresholds of the paw or muscle, or withdrawal latency to heat in animals without inflammation that received intramuscular injection of vehicle instead of carrageenan (Figs. 1 and 2).

To test for movement-evoked pain, we examined grip force of hind limbs in Swiss mice. Intramuscular carrageenan significantly decreased grip force through 8 days (Fig. 2b). A single SP6 MA had no immediate effect on the reduced grip force produced by carrageenan inflammation. However, daily SP6 MA acupuncture significantly increased grip force returning to that of uninflamed controls on the 3rd day ($p<0.001$) (Fig. 2b).

To test for cortical involvement in processing of nociception, we used the escape/avoidance test. In this test, C57Bl/6 mice with muscle inflammation spend less time on the side of the box when stimulation is applied to paw ipsilateral to the inflamed muscle; this effect lasts for up to 5 days after muscle inflammation (Fig. 2c). After a single SP6 MA, there was no preference (similar to baseline). MA was still effective after the 5th day of treatment (1st day: $p<0.05$ and 5th day: $p<0.01$) (Fig. 2c).

SP6 MA Reduced Edema in Muscle

We then tested if SP6 MA had any effect on edema formation by measuring the volume of the lower limb with a plethysmometer. Muscle inflammation significantly increases lower limb volume for at least 5 days when compared to the saline group (Fig. 3). SP6 MA significantly reduced limb volume when compared to the control group after each treatment ($p<0.05$ on the 1st and 5th days, $p<0.001$ on the 2nd and 3rd days, $p<0.01$ on the 4th day) (Fig. 3).
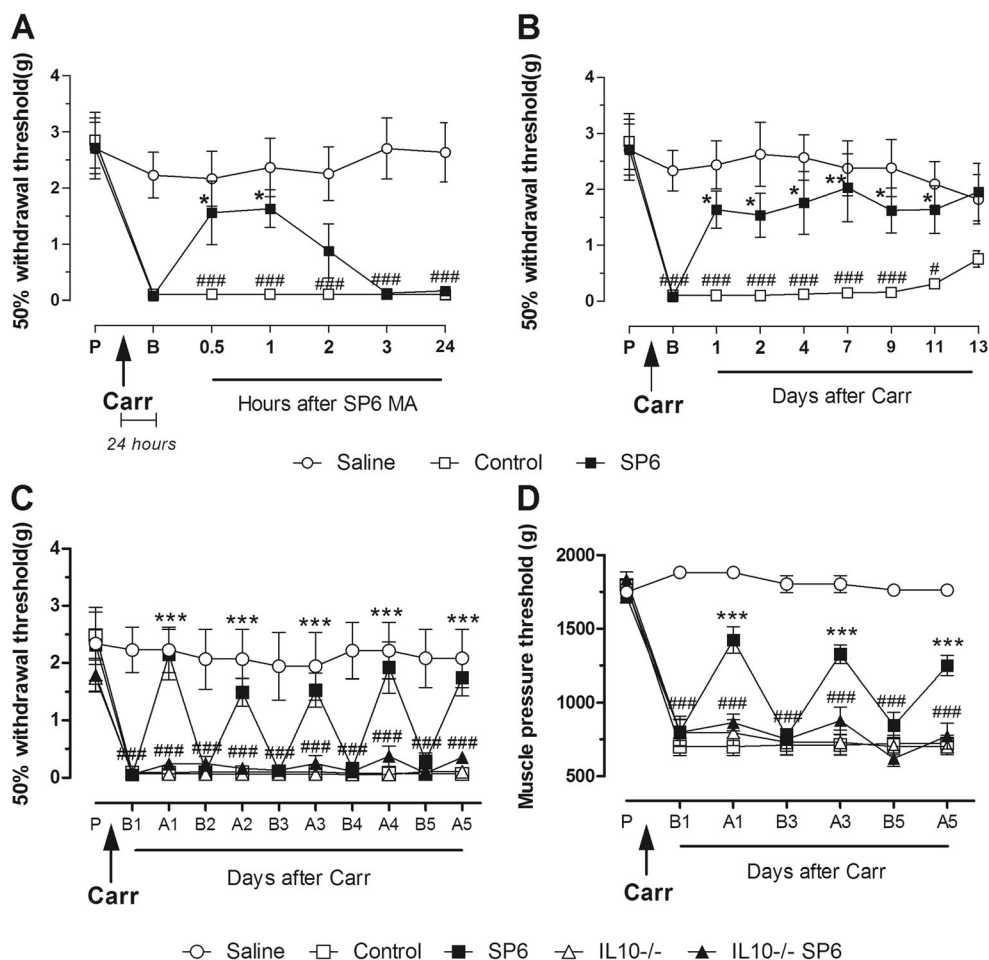
**Fig. 1** Effect of SP6 MA on carrageenan-induced mechanical hyperalgesia in mice. **a** Withdrawal responses of mouse hind paw to von Frey hairs in Swiss mice, before and after carrageenan injection or SP6 MA. Mechanical withdrawal thresholds of the paw were test for up to 24 h after SP6 MA. **b** Mechanical withdrawal thresholds of the paw for up to 13 days after the first treatment with SP6 MA in Swiss mice. **c** Withdrawal thresholds of the paw in C57BL/6 and IL-10$^{-/-}$ Mice. *Carr* =carrageenan, *P*=pre-carrageenan, *B*=the basal evaluation after carrageenan and before treatment with acupuncture, *A*=after SP6 MA. **d**

Muscle withdrawal threshold in C57BL/6 (wild-type and IL-10$^{-/-}$) mice. The animals received saline i.m. (saline group) or carrageenan i.m. (3 %, control and IL-10$^{-/-}$ groups) or carrageenan i.m. plus SP6 MA (SP6 and IL-10$^{-/-}$ SP6 groups). *Each point* represents the mean of six to eight animals with SEM. *Asterisks* denote significance levels, when compared with the inflamed group (Control), *$p<0.05$, **$p<0.01$, ***$p<0.001$. *Hash marks* denote significance levels of the inflamed group (Control) when compared with the saline group, #$p<0.05$, ###$p<0.001$

### Acupuncture Swiss Mice Show Increases in IL-10 in Muscle Compared to Inflammation Controls

To examine if IL-10 was increased in muscle in response to SP6 MA, we performed ELISA of the inflamed muscles after a single carrageenan injection compared (control group) to muscle without inflammation (saline group) in Swiss mice. Interestingly, levels of IL-10 were significantly lower in the inflamed muscle when compared to the uninflamed saline controls (Fig. 4a, b). A single SP6 MA in Swiss mice with muscle inflammation significantly increased or reestablished IL-10 levels when compared to animals with muscle inflammation not treated with acupuncture ($p<0.05$) (Fig. 4a). Similarly, five SP6 MA treatments in animals with muscle inflammation significantly increased IL-10 levels when compared to

animals with muscle inflammation not treated with MA ($p<0.05$) (Fig. 4b).

### IL-10$^{-/-}$ Mice Do Not Show Reductions in Nociceptive Behaviors by SP6 MA

To test if IL-10 plays a role in the analgesia produced by SP6 MA, we examined the effects of MA in IL-10$^{-/-}$ Mice. Since IL-10$^{-/-}$ were congenic on C57Bl/6 strain, we initially confirmed the effects of acupuncture on paw withdrawal thresholds, muscle withdrawal thresholds, and edema. Similar effects were observed in C57Bl/6 mice when compared to Swiss mice, i.e., SP6 MA increased paw and muscle withdrawal threshold and decreased edema. Both wild-type and IL-10$^{-/-}$ Mice show significant and similar decreases in the
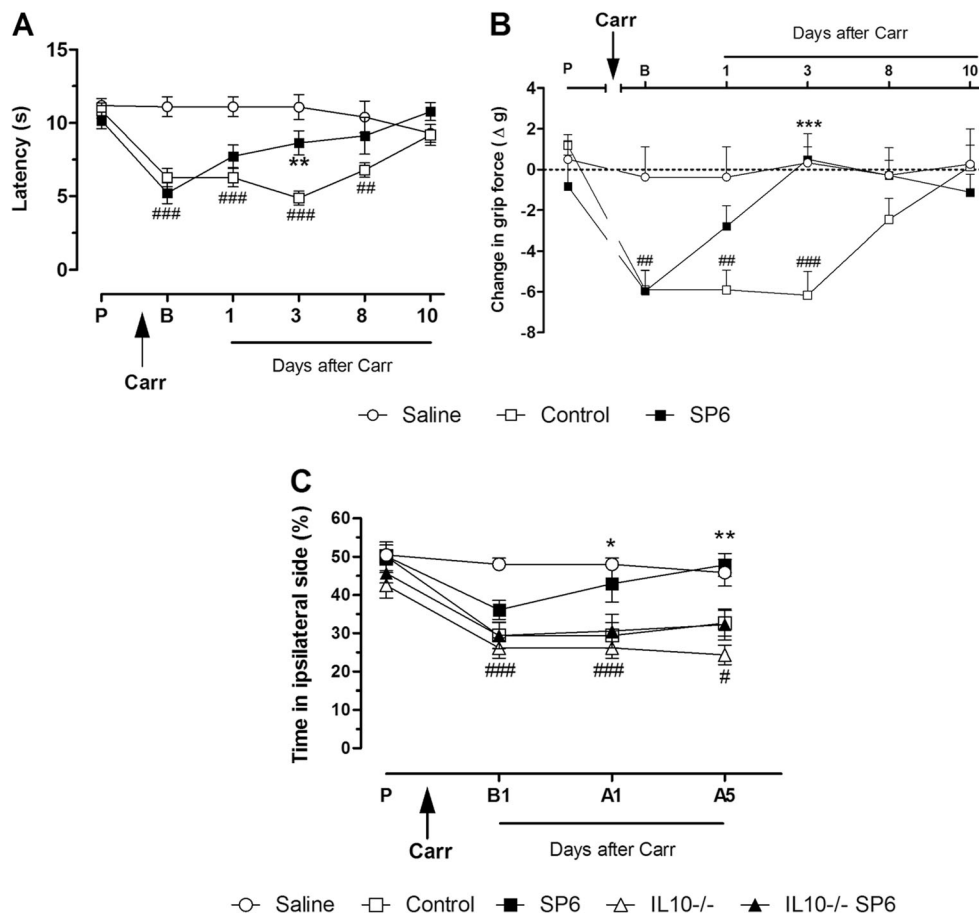
**Fig. 2** Effect of SP6 MA on carrageenan-induced thermal and mechanical hyperalgesia and cortical involvement in nociception in mice. **a** Withdrawal latency to heat in Swiss mice before and after carrageenan injection or SP6 MA. **b** Grip force in Swiss mice before and after carrageenan injection or SP6 MA. Panel C shows results of the escape/avoidance test and represents the time spent on the side of the chamber when the ipsilateral paw was stimulated in C57BL/6 (wild-type and IL-10$^{-/-}$) mice before and after carrageenan injection or SP6 MA. Carr represents the injection of carrageenan, P represents the pre-carrageenan, B represents the basal evaluation after carrageenan and before treatment with acupuncture and A represents after SP6 MA. The first evaluation after B is the first assessment after treatment with SP6 MA. The animals received saline i.m. (saline group) or carrageenan i.m. (3 %, control and IL-10$^{-/-}$ groups) or carrageenan i.m. plus SP6 MA (SP6 and IL-10$^{-/-}$SP6 groups). *Each point* represents the mean of six to eight animals with SEM. *Asterisks* denote significance levels, when compared with the inflamed group (control), *$p<0.05$, **$p<0.01$, ***$p<0.001$. *Hash marks* denote significance levels of the inflamed group (control) when compared with the saline group, #$p<0.05$, ##$p<0.01$, ###$p<0.001$

mechanical withdrawal thresholds of the paw and muscle after carrageenan-induced inflammation (Fig. 1c, d) and increased escape/avoidance behaviors when compared to uninflamed (saline) animals (Fig. 2c). IL-10$^{-/-}$Mice did not develop analgesia in response to SP6 MA and displayed significantly lower mechanical thresholds for paw and muscle withdrawal thresholds and increased escape/avoidance behaviors when compared to IL-10$^{+/+}$mice with SP6 MA.

### IL-10$^{-/-}$Mice Do Not Show Reductions in Edema by SP6 MA

To test if IL-10 played a role in the reduction in muscle edema by SP6 MA, we examined lower limb volume in IL-10$^{-/-}$Mice treated with SP6 MA and compared to IL-10$^{+/+}$mice. Lower limb volume in IL-10$^{-/-}$Mice without SP6 MA showed similar increases in limb volume to wild-type mice. SP6 MA-induced decreases in limb volume did not occur in IL-10$^{-/-}$Mice; limb volumes were significantly higher in IL-10$^{-/-}$Mice treated with SP6 MA when compared to IL-10$^{+/+}$mice treated with SP6 MA (Fig. 3).

### SP6 MA Induces a Phenotypic Switch from M1 to M2 Macrophages

M2 macrophages play a significant role in the production and release IL-10 and are located within inflamed muscle [6, 7, 25]. Thus, we tested if there were changes in macrophage numbers and phenotype using immunohistochemistry of muscle. To determine the phenotype of macrophages at the site of inflammation, we co-labeled macrophages (F4/80) with a macrophage marker and either an M1 (CD11c) or M2 ($\alpha$CD206) marker and counted the relative number of
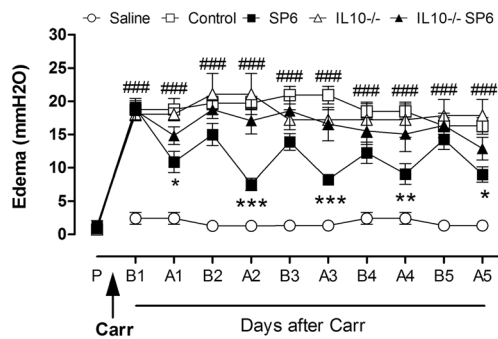
**Fig. 3** Effect of SP6 MA on carrageenan-induced muscular edema in C57BL/6 mice. The data represent edema daily for a period of 5 days in C57BL/6 (wild-type and IL-10$^{-/-}$) mice. Carr represents the injection of carrageenan, B represents the basal evaluation after carrageenan and before treatment with SP6 MA, and A represents after SP6 MA. The animals received saline i.m. (saline group) or carrageenan i.m. (3 %, control and IL-10$^{-/-}$ groups) or carrageenan i.m. plus SP6 MA (SP6 and IL-10$^{-/-}$ SP6 groups). *Each point* represents the mean of six to eight animals and SEM. *Asterisks* denote significance levels, when compared with the control group, *$p<0.05$, **$p<0.01$, ***$p<0.001$. *Hash marks* denote significance levels of Control group when compared with the saline group, ###$p<0.001$

macrophages in each population. Figure 5 shows muscle tissue stained for macrophages (F4/80) and αCD206, and Fig. 6 shows muscle tissue stained for macrophages (F4/80)
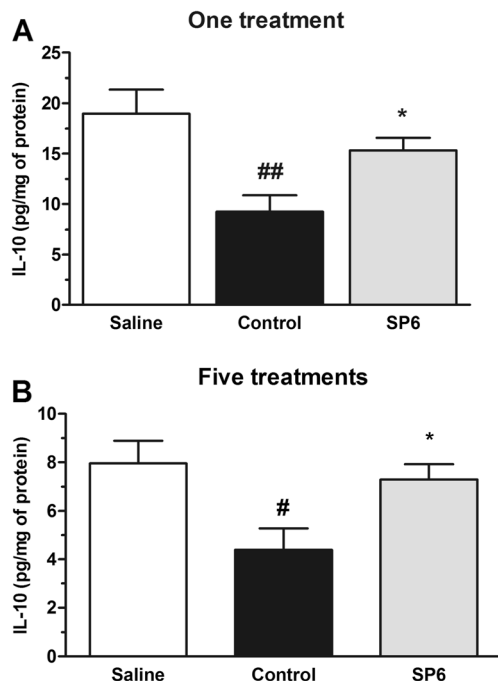


**Fig. 4** Effect of SP6 MA on carrageenan-induced decreased muscular IL-10 levels in Swiss mice. **a** Cytokine levels in gastrocnemius muscle after carrageenan injection in mice after one SP6 MA treatment. **b** Cytokine levels in gastrocnemius muscle after five SP6 MA treatments. The animals received saline i.m. (saline group) or carrageenan i.m. (3 %, control group). *Each point* represents the mean of six to eight animals and *error bars* indicate the SEM. *Asterisks* denote significance levels, when compared with the control group, *$p<0.05$. *Hash marks* denote significance levels of the control group when compared with the saline group, #$p<0.05$

and CD11c. Notice the increase in the number of macrophages in the inflamed muscle when compared to the saline-injected controls. Quantification shows that there were significantly more macrophages in the inflamed muscle when (mean 29.73 ±1.04) compared to uninflamed saline-injected animals (mean 10.95±0.46) (Fig. 7a). Further, there was a significantly greater percentage of M1-type macrophages (inflammatory-cytokine producer) in inflamed tissue (control animals) when compared to uninflamed saline animals (Fig. 7b). Interestingly, SP6 MA significantly reduced the proportion of M1 macrophages and increased the proportion of M2 macrophages when compared to animals with muscle inflammation that did not receive MA (Fig. 7b). This change in proportion of M1 and M2 phenotypes with SP6 MA did not occur in IL-10$^{-/-}$ Mice treated with SP6 MA, and there was a similar profile between IL-10$^{-/-}$ and IL-10$^{+/+}$ inflamed mice that did not receive SP6 MA. Specifically, for M1 macrophages, there were significant increases in the proportion of CD11c-positive cells in mice with muscle inflammation, IL-10$^{-/-}$ Mice with inflammation, and IL-10$^{-/-}$ Mice treated with SP6 when compared to uninflamed saline-injected controls ($p=0.003$, $p=0.016$, and $p=0.014$, respectively). For M2 macrophages, there were significant decreases in the proportion of αCD206-positive cells in saline-injected control mice and IL-10$^{-/-}$ Mice with inflammation ($p=0.039$ and $p=0.018$, respectively). Thus, inflammation increases expression of M1 and decreases expression of M2; SP6 results in a normalization of this change in phenotype to that observed in saline-injected control mice; IL-10$^{-/-}$ Mice treated with MA do not show this normalization and show a similar pattern to saline-injected control mice.

## Discussion

We show for the first time that SP6 MA reduces pain behaviors and edema through IL-10 concentrations in inflamed muscle. We further show that MA changes the phenotype of macrophages in the inflamed muscle, and this alteration in phenotype may be the underlying mechanism responsible for the analgesic and anti-inflammatory effects of IL-10 by MA. SP6 MA promotes reductions in reflex hind limb withdrawal responses to mechanical stimulation of the inflamed muscle, i.e., primary hyperalgesia as well as mechanical stimulation of the paw, i.e., secondary hyperalgesia. We also show changes in escape/avoidance behaviors, suggesting that SP6 MA not only affects evoked reflexive behaviors, but also reduces affective behaviors.

The current study shows, for the first time, an increase in IL-10 content in muscle during MA and that the anti-inflammatory and anti-hyperalgesic effects of MA do not occur in IL-10$^{-/-}$ Mice, thus supporting that MA produces its
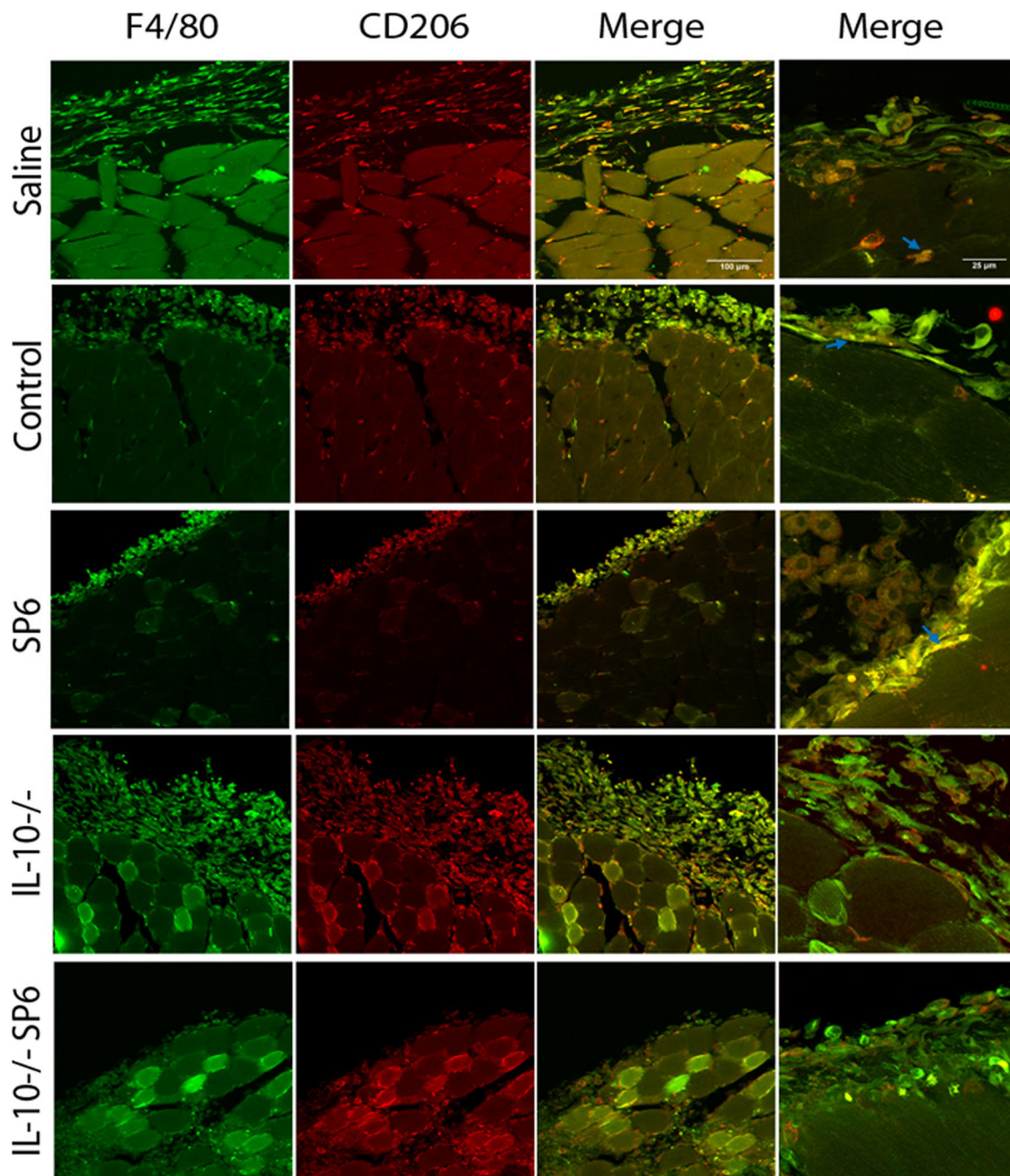
**Fig. 5** Immunohistochemistry confocal micrographs of the gastrocnemius muscle in C57BL/6 mice after carrageenan injection in the saline, inflamed (control), SP6, IL-10$^{-/-}$, and IL-10$^{-/-}$SP6 animal groups. Sections show immunohistochemical labeling for macrophages with F4/80 (*green*) and labeling for M2 macrophages with αCD206 (*red*). Merged pictures in *columns 3* and *4* show co-localization of F4/80 and αCD206 as *yellow* (*arrows*). Scale bars=100 and 25 μm

effects through local IL-10 in muscle. The changes in IL-10 could represent a decrease in the degradation or increase in production to alter the content, nor can we determine whether there was increased release. We show that analgesia produced by SP6 MA does not occur in IL-10$^{-/-}$Mice which is consistent with prior data showing that administration of IL-10 inhibits the development of hyperalgesia in several animal models of pain, such as prodynorphin-induced allodynia, endotoxin-induced hyperalgesia, nerve injury-induced hyperalgesia, and spinal cord injury-induced spontaneous pain [26-29]. The current study interestingly shows no change in edema or nocifensive behaviors in IL-10$^{-/-}$Mice with or without injury using mechanical withdrawal thresholds of the paw, mechanical withdrawal thresholds of the muscle, or escape/avoidance tests. Prior studies using IL-10$^{-/-}$Mice have yielded mixed results in uninjured animals, and IL-10$^{-/-}$Mice have enhanced nociception as evidenced by an increase in the hot plate latency [30], the only
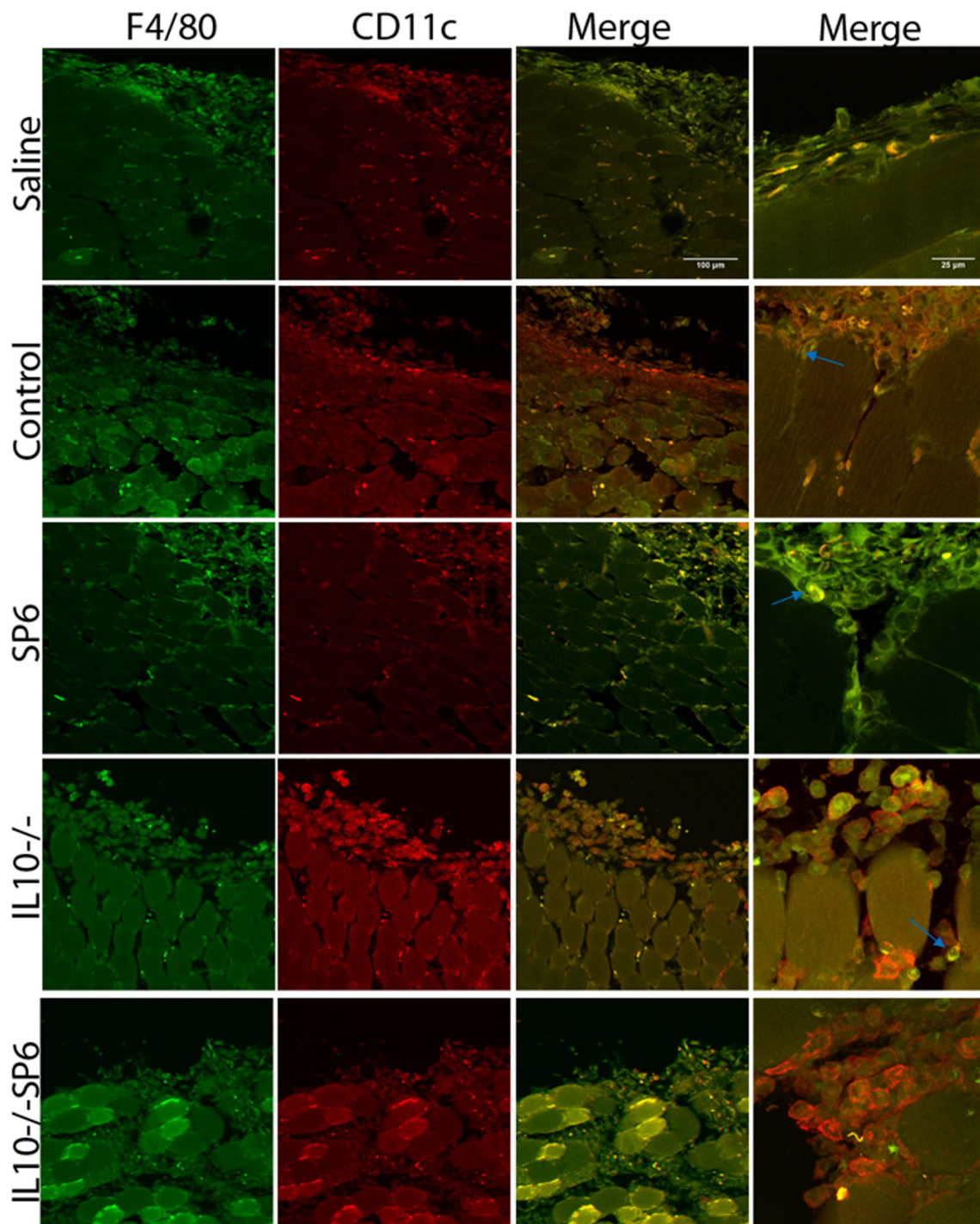
**Fig. 6** Immunohistochemistry confocal micrographs of the gastrocnemius muscle in C57BL/6 mice after carrageenan injection in the saline, inflamed (control), SP6, IL-10$^{-/-}$, and IL-10$^{-/-}$SP6 animal groups. Sections show immunohistochemical labeling for macrophages with F4/80 (green), and labeling for merged pictures in *columns 3* and *4* shows co-localization of F4/80 and CD11c as *yellow* (*arrows*). *Scale bars*=100 and 25 μm

nociceptive test used in the study. After spinal injury, IL-10$^{-/-}$Mice have increased spontaneous pain behaviors compared to controls [31]. However, in animals with inflammation, the effects of genetic deletion of IL-10 or of peripherally administered exogenous IL-10 have not yet been tested. IL-10 could produce its effects by directly inhibiting nociceptive activity [32], and/or IL-10 may effect nociception and edema indirectly through decreasing pro-inflammatory cytokine production in M1 macrophages [30, 33-35].
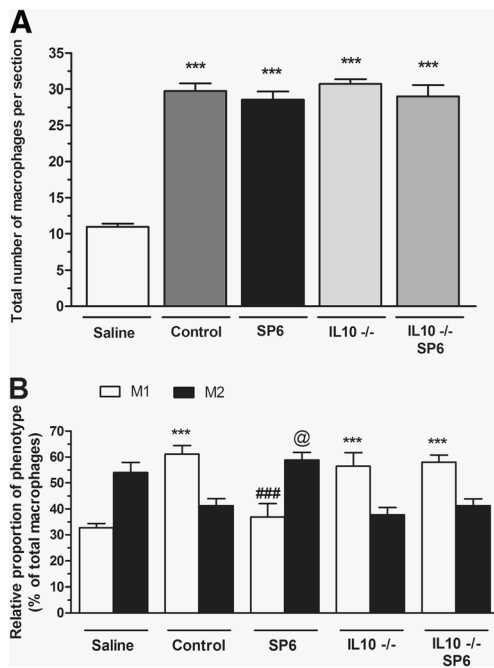
**Fig. 7** Effect of SP6 MA on total and differential macrophages in C57BL/6 mice. **a** Total number of macrophages per group. **b** Percentage of macrophage types that are labeled as M1 or M2. The animals received saline i.m. (saline group) or carrageenan i.m. (3 %, control and IL-10$^{-/-}$ groups) or carrageenan i.m. plus SP6 MA (SP6 and IL-10$^{-/-}$SP6 groups). *Each column* represents the mean of five mice and SEM. *Asterisks* denote the level of significance compared with the saline group and saline group M1 (***$p < 0.001$), *hash marks* denote the level of significance compared with the control group M1 (###$p < 0.001$), and *at symbols* denote the level of significance compared with the control group M2 (@$p < 0.05$)

We propose that macrophages are a major source of IL-10 and that macrophage phenotype is modulated by MA. Our current data supporting this view includes the following: (1) in wild-type animals with muscle inflammation, there is a greater proportion of M1 (pro-inflammatory cytokines) macrophages and less M2 (anti-inflammatory cytokines); (2) in wild-type mice with muscle inflammation treated with MA, there is reduced M1 and increased M2 macrophages; and (3) in IL-10$^{-/-}$Mice with muscle inflammation, there is increased M1 and reduced M2 macrophages (similar to wild types with muscle inflammation). Depending on the microenvironment, macrophages can acquire distinct functional phenotypes, which have been described as classically activated macrophages (M1) and alternatively activated macrophages (M2) [6, 36]. M1 macrophages secrete inflammatory cytokines and mediate host defense [6, 7], while M2 macrophages secrete anti-inflammatory cytokines like IL-10 and promote healing and recovery [8, 9]. Indeed, macrophages are stimulated to produce IL-10 by several endogenous factors including TNF-α, catecholamines, and IL-10 itself [37-43]. Further, several other cell types may also produce IL-10 including other types of immune cells (i.e., monocytes and dendritic cells) or contracting muscle [44, 45]. Since acupuncture was only able to modulate pain behaviors in animals with inflammation, and not those without inflammation, we suggest that immune cells attracted to the site of inflammation mediate the change in phenotype by MA.

Following tissue injury, the first responder macrophages usually exhibit an inflammatory phenotype and secrete pro-inflammatory mediators such as TNF-α, nitric oxide, and IL-1β, which participate in the activation of various antimicrobial mechanisms, including oxidative processes that contribute to the killing of invading organisms [6, 46]. However, macrophages are plastic and can differentiate into different subtypes including M1 and M2. Differentiation is driven by cues in the tissue microenvironment including cytokines and growth factors. These cues are thought to direct a transcriptional response that shapes the phenotype and function of the macrophages [7]. There is much evidence that distinct subpopulations of macrophages can arise in different circumstances or in response to different stimuli [3, 47, 48]. Anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 induce differentiation into alternative M2 macrophages that generate anti-inflammatory mediators such as IL-10, TGF-β, and IL-1β receptor agonist [43, 49]. We suggest that MA is associated with enhanced anti-inflammatory cytokine production, particularly IL-10 from inflammatory cells that in turn results in a change in phenotype increasing M2 macrophages, and further maintains the increased level of IL-10 in inflamed muscle. Together, the increases in IL-10 produce analgesia and reduce inflammation. Similar effects of a switch in phenotype of circulating macrophages have been found in human subjects in response to exercise with a decrease in M1 markers and an increase in M2 markers [43]. Since exercise is anti-inflammatory [43], it is possible that one endogenous inhibition mechanism that is used by nonpharmacological treatments like acupuncture and exercise involves modification of the immune system.

A number of underlying mechanisms have been proposed to mediate the analgesic and anti-inflammatory effects of acupuncture. Several studies show that both manual and electroacupuncture produce analgesia through activation of central inhibitory neural mechanisms that include the release of opioid peptides and activation of their receptors [14, 15, 50, 51]. Other studies show that acupuncture can directly modulate the immune system by increasing natural killer cell activity and changing phenotype of T cells (Th1 to Th2) [52-54]. Some of the modulation of the immune system is mediated through the hypothalamus since acupuncture activates the hypothalamus, and lesioning the hypothalamus eliminates increases in natural killer cell activity [52, 55, 56]. These mechanisms are not mutually exclusive with the role of macrophages in the anti-inflammatory and analgesic actions of acupuncture. Activation of central inhibitory pathways or hypothalamus could underlie a neuroimmune interaction that drives macrophage phenotype through activation of the autonomic nervous. Alternatively, the local release of

neurotransmitters could change the immediate microenvironment to drive macrophage phenotype. For example, previous studies show that acupuncture triggers the release of adenosine locally to reduce hyperalgesia through A1 receptors in animals with inflammatory or neuropathic pain [13]. Adenosine is converted from other purines including ATP [57] and ATP is also increased with MA [13, 58]. Increases in ATP could also activate purinergic receptors such as P2X7 that is found on immune cells [59] and, thus, further increase IL-10 concentrations in muscle from macrophages.

Although SP6 MA is effective in reducing nociception and inflammation in muscle pain, acupuncture needs further characterization. Our data clearly show that acupuncture reduced heat and mechanical hyperalgesia and decreased avoidance behaviors and edema. Additionally, we have also demonstrated that SP6 MA increases IL-10, and IL-10 is necessary for the analgesic and anti-inflammatory effect of MA. Moreover, we observed that acupuncture produced a phenotypic switch in macrophage phenotype reducing the amount of M1 and increasing M2 (IL-10 source) in inflamed muscle. Thus, acupuncture may be an alternative to medications to stimulate endogenous pathways to promote healing and reduce pain.

**Conflict of Interest** The authors declare that they have no conflict of interest.

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