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Regulatory T-cells and IL-5 mediate pain outcomes in a preclinical model of chronic muscle pain

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Abstract

Fibromyalgia (FM) is a chronic musculoskeletal pain disorder primarily diagnosed in women. Historically, clinical literature focusing on cytokines and immune cells has been inconsistent. However, recent key studies show several layers of immune system dysfunction in FM. Preclinically, studies of the immune system have focused on monocytes with little focus on other immune cells. Importantly, T-cells are implicated in the development and resolution of chronic pain states, particularly in females. Our previous work showed that monocytes from women with FM produced more interleukin 5 (IL-5) and systemic treatment of IL-5 reversed mechanical hypersensitivity in a preclinical model of FM. Typically, IL-5 is produced by T_{H2}-cells, so in this study we assessed T-cell populations and cytokine production in female mice using the acid-induced chronic muscle pain model of FM before and after treatment with IL-5. Two unilateral injections of pH4.0 saline, five days apart, into the gastrocnemius muscle induce long-lasting widespread pain. We found that peripheral (blood) regulatory T_{helper}-cells ($CD4^+$ FOXP3+) are downregulated in pH4.0-injected mice, with no differences in tissue (lymph nodes) or $CD8^+$ T-cell populations. We tested the analgesic properties of IL-5 using a battery of spontaneous and evoked pain measures. Interestingly, IL-5 treatment induced place preference in mice previously injected with pH4.0 saline. Mice treated with IL-5 show limited changes in T-cell populations compared to controls, with a rescue in regulatory T-cells which positively correlates with improved mechanical hypersensitivity. The experiments in this study provide novel evidence that downregulation of regulatory T-cells play a role in chronic muscle pain pathology in the acidic saline model of FM and that IL-5 signaling is a promising target for future development of therapeutics.

Keywords

Chronic muscle pain, T-cells, IL-5, neuroimmune, female, fibromyalgia

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Introduction

Fibromyalgia (FM) is a chronic pain disorder characterized by widespread muscle pain, fatigue, and cognitive symptoms.[1,2] Immune dysfunction may play an important role in the etiology of FM, as recent key studies have indicated that peripheral immune cell function is altered in women with FM.[3–6] A major theory of FM etiology is the autoimmune hypothesis, in which immune system dysfunction contributes to chronic pain and fatigue symptoms via interactions with sensory neurons.[7–10] Patients with FM often have comorbid autoimmune disorders[11] and the prevalence of

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FM in patients with autoimmune disorders is much greater than the general population[12,13]; however, literature on immune cell function and cytokines in FM patients is inconsistent and the study of immune cells in preclinical models of FM has been limited to monocytes.[6,14–16]

We have previously shown that monocytes isolated from female patients with FM produce significantly more interleukin (IL)-5, a cytokine typically produced by anti-inflammatory TH_2 cells alongside IL-4.[17,18] Production of IL-5 by peripheral monocytes was highly correlated with resting pain, movement-evoked pain, and fatigue in patients with FM. Furthermore, using a preclinical model of chronic widespread pain, we found that female mice given systemic intravenous (I.V.) injections of IL-5 showed analgesic action and polarized monocytes towards an anti-inflammatory phenotype.[17] There is not currently much known about IL-5 and its role in pain; however, IL-5 has been shown to activate vagal neurons and influence cytokine production by immune cells in the lung in a model of allergic airway inflammation.[19] Therefore, continuing to examine immune dysfunction and the role of IL-5 in chronic muscle pain represents a promising target for future therapeutic development.

IL-5 is primarily produced by T-cells; however, eosinophils, monocytes, and mast cells are also potential sources of IL-5.[6,20,21] Like T-cells, eosinophils produce IL-5 and utilize IL-5 signaling as a mechanism for differentiation and expansion.[20,22] Induction of IL-5 production in monocytes seems to occur primarily in disease states, such as during infection or immunodeficiency, as they do not produce IL-5 during naïve or resting states.[21,23] Because prototypic IL-5 production occurs in T-cells, we sought to follow-up our monocyte study by assessing T-cell populations in a preclinical model of FM. T-cells have been shown to contribute to both the onset and resolution of chronic pain and are a source of female biased mechanisms in neuroimmune signaling.[24–28] Several studies have shown alterations in the levels of T-cell derived cytokines in peripheral blood of patients with FM.[5,29,30] For example, IL-4, IL-2, and IL-10 levels are increased in patients with FM; However, it is important to note that these studies assessed serum cytokine levels and were not able to assess which cell types were responsible.[5,29–31]

The goal of this study was to assess the phenotypes of T-cell populations in a preclinical model of FM to understand how they might play a role in promoting chronic muscle pain and to assess the effects of IL-5 treatment on analgesia, T-cell polarization, and anti-inflammatory cytokine production. After the onset of chronic muscle pain, we isolated T-cells from draining lymph nodes (popliteal and inguinal) and peripheral blood mononuclear cells (PBMCs) and used flow cytometry to assess differences in T-cell populations. We then tested the analgesic effects of IL-5 using conditioned place preference (CPP) and other behavioral assays. Finally, we isolated T-cells from lymph nodes and PBMCs after IL-5 treatment at the peak of analgesia to understand how IL-5 shifts T-cell populations and influences their production of other anti-inflammatory cytokines. We hypothesized that T-cell populations would shift towards a pro-inflammatory profile in pH4.0 injected mice and that treatment with IL-5 would be analgesic and shift T-cell populations towards an anti-inflammatory profile.

Materials and Methods

Animals

Female C57BL/6J mice (2-5 months old, 20-25g) were used for all experiments to match the major patient population in fibromyalgia. C57BL/6J mice (Stock no. 000664) were purchased from Jackson Laboratories and used to establish our in-house breeding colony. Animals born from this in-house breeding colony were used in all experiments. Animals were group housed (4-5 animals per cage) in polypropylene cages maintained at a room temperature of 21 ± 2°C under a 12 h light cycle (lights on from 6AM-6PM) with *ad libitum* access to water and standard rodent chow. All procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and in accordance with ARRIVE guidelines and approved by the University of Texas at Dallas Institutional Animal Care and Use Committee.

Induction of chronic musculoskeletal pain

Chronic musculoskeletal pain was induced as described previously.[17,32,33] In brief, mice were anesthetized with 4% isoflurane and the left gastrocnemius muscle (ipsilateral) was injected twice with either 20 µL of acidic saline (0.9%, pH4.0 ± 0.1) or physiological saline (pH7.4 ± 0.1) on days 0, following baseline measures, and day 5 of each experiment. Mice were randomly assigned to receive either pH4.0 or 7.4 saline following baseline measures by an independent experimenter and each cage contained mice in both groups.

IL-5 injections

Recombinant mouse IL-5 (R&D Systems, 405-ML-025) was reconstituted in sterile 1x phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA, Sigma-Aldrich, A9576-50ML, endotoxin-free). To induce analgesia, two I.V. injections of 0.1µg IL-5 in 100µL sterile 1× PBS were given 24 h apart ten days following the first saline injection, after the development of widespread muscle pain, following previously described methods.[17] Injections of IL-5 were given between 5am and 9am, unless otherwise indicated. Mice within the pH4.0 and 7.4 saline experimental groups were randomly assigned to receive either IL-5 or vehicle injections by an independent experimenter.

Flow cytometry

Tissue collection and dissociation. To isolate T-cell populations for flow experiments, mice were deeply anesthetized under

isoflurane and euthanized via decapitation on Day 10 of the experimental timeline. Whole blood was collected in BD blood collection tubes (VWR, BD367884) and draining lymph nodes (popliteal and inguinal) were collected in ice cold sterile 1× DPBS (Hyclone, Logan, UT). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Paque PLUS (VWR, 95021-205) as previously described.[6] Lymph nodes were passed through a 70-micron cell strainer using flow buffer (0.5% bovine serum albumin (Sigma, A9576) and 0.02% glucose (Sigma, G7528) made in 1x DPBS).[28]

Experiment 1: T-cell polarization during chronic muscle pain

The resultant cell suspensions were centrifuged at 600 rcf for 6 min at 4°C and resuspended in 1× DPBS. Isolated cells were washed twice with flow buffer. To block non-specific binding via the Fc receptor, cells were incubated in blocking buffer (anti-CD16/32 purified antibody diluted in flow buffer) for 10 minutes on ice. Samples were incubated with pre-conjugated extracellular flow antibodies (CD3, CD4, CD8, CD25, CD44) for 40 minutes diluted in flow buffer, on ice and protected from light. Prior to intracellular staining, samples were washed with flow buffer and incubated in Transcription Factor Fix/Perm Concentrate (Tonbo, TNB-1020-L050) diluted in Transcription Factor Fix/Perm Diluent (Tonbo, TNB-1022-L160) to a 1x solution. Samples were incubated for 60 minutes on ice, protected from light. Cells were carefully washed with Permeabilization buffer (Tonbo, TNB-1213-L150) and incubated with pre-conjugated intracellular flow antibodies (anti-FOXP3) and incubated for 40 min protected from light. Cells were washed and resuspended in flow buffer for data acquisition. Appropriate compensation controls and isotypes were used for determination and gating. Stained samples were analyzed using a BD LSR Fortessa analyzer (BD Bioscience, San Diego, CA) and analysis was performed using FlowJo software (San Carlos, CA). Experimenters were blinded to condition. For a complete list of antibodies used refer to Table 1.

Experiment 2: T-cell polarization and cytokine release after treatment with IL-5

To test the effect of systemic IL-5 treatment on T-cell polarization and anti-inflammatory cytokine production, mice were given two i.v. injections of 0.1μg IL-5 on Days 10 and 11 following the first saline injection. Tissue collection was performed two hours after the second IL-5 injection, as IL-5 treated mice have maximal analgesia at this timepoint.[17] Isolated cells were resuspended in 1mL of warmed cell culture media with 2μL of Cell Activation Cocktail (BioLegend, with Brefeldin A, Cat #423303) and incubated at 37°C, 5% CO_2 cell culture incubator for four hours. Cell culture media contained 1x DME:F12 1:1 (HyClone, SH30023.01, +2.50 mM L-Glutamine + 15 mM HEPES Buffer) supplemented with 10% fetal bovine serum (HyClone, SH30088.03) and 1% Penicillin/Streptomycin (Sigma, P4333-100mL). After activation, samples were washed using flow buffer, resuspended in blocking buffer, and incubated for 10 minutes on ice. Samples were incubated with pre-conjugated extracellular flow antibodies (CD3, CD4, CD8, CD25) for 40 minutes on ice, protected from light. Samples were washed once with flow buffer, centrifuged at 600 rcf for 6 minutes, and resuspended in Transcription Factor Fix/Perm Concentrate diluted in Transcription Factor Fix/Perm Diluent to a 1x solution. Samples were incubated for 60 minutes on ice, protected from light. Samples were washed once with Permeabilization buffer. Samples were incubated with pre-conjugated intracellular flow antibodies (FOXP3, IL-4, IL-10) diluted in Permeabilization buffer for 40 minutes on ice, protected from light. Samples were then washed once with Permeabilization buffer and resuspended in flow buffer for data acquisition. Appropriate compensation controls and isotypes were used for determination and gating. Stained samples were analyzed using a BD LSR Fortessa analyzer (BD Bioscience, San Diego, CA) and analysis was performed using FlowJo software (San Carlos, CA). Experimenters were blinded to condition. For a complete list of antibodies used refer to Table 1.

Table 1. Antibodies used in flow cytometry experiments.

Antibody	Company	Catalog Number	Working Dilution	Experiment
Anti-CD16/32 (purified)	eBioscience	16016185	1:2000	1 and 2
Anti-CD3 Alexa Fluor 700 conjugate	eBioscience	56-0032-80	1:100	1 and 2
Anti-CD4 FITC conjugate	eBioscience	48-0041-85	1:100	1 and 2
Anti-CD8 PE conjugate	eBioscience	12-0081-83	1:100	1 only
Anti-CD8 Alexa Fluor 594 conjugate	BioLegend	100758	1:100	2 only
Anti-CD25 eFluor 450 conjugate	eBioscience	48-0251-82	1:100	1 and 2
Anti-CD44 APC eFluor 780 conjugate	eBioscience	47-0441-82	1:100	1 only
Anti-FoxP3 APC conjugate	Tonbo	20-5773-SU05	1:100	1 and 2
Anti-IL-4 PE-Cyanine7 conjugate	eBioscience	25-7049-82	1:100	2 only
Anti-IL-10 PE conjugate	eBioscience	12-7101-82	1:100	2 only

Tissue collection and histology

To assess local inflammation in the gastrocnemius muscle after induction of chronic muscle pain, mice were given multiple injections of pH4.0 or pH7.4 saline as described above. On Day 10, mice were deeply anesthetized under isoflurane and decapitated per the University of Texas at Dallas IACUC approval 2 measures. The ipsilateral and contralateral gastrocnemius muscles were dissected and drop-fixed in 4% paraformaldehyde for 12 hours. Gastrocnemius muscle was processed and embedded in paraffin and sectioned at 10-micron thickness on a microtome. Gastrocnemius muscle sections were stained using Hematoxylin (Sigma, #HHS16) and Eosin (Sigma-Aldrich, #318906) (H&E). Images of stained serial sections were taken using an Olympus VS120 Virtual Slide Scanner microscope at 40x magnification. The gastrocnemius muscle was analyzed using CellSens version 3.1 (Olympus, Japan) for numbers of immune cells normalized per area. All histological analysis was performed by experimenters blinded to condition.

Behavior testing

The day prior to baseline measures, animals were acclimated to the testing room in their home cages for up to four hours. On testing days, mice were habituated in acrylic behavior boxes on an elevated wire mesh grid for approximately one hour. All behavioral tests occurred between 10am-2pm. Behavior racks were cleaned with a 1:3 ratio of a plant-based deodorant free cleaner (Seventh Generation^{TM}, 22719BK-5) to eliminate odor cues. Multiple baseline measures were performed on two separate days prior to the first saline injection. For CPP experiments, animals were handled daily (5 minutes each) for two weeks prior to the start of behavioral testing. All behavioral experiments were performed by experimenters blinded to condition.

Von Frey testing

Mechanical hypersensitivity of the paw was tested using the von Frey assay, described previously.[17,33,34] Paw withdrawal thresholds were assessed using calibrated von Frey hair filaments using the up-down method.[34] Filaments with logarithmically incremental stiffness (2.83, 3.22, 3.61, 3.84, 4.08, 4.17 converted to 0.07, 0.16, 0.4, 0.6, 1, 1.4 g, respectively) were applied to the plantar surface of the hind paw. A 2g cut off was applied to avoid tissue damage or unintentional agitation. A positive response was noted by paw withdrawal, licking, or shaking of the paw. Testing of the ipsilateral and contralateral paws occurred separately.

Facial grimacing

To measure spontaneous (resting) pain, facial grimacing was assessed in real time using the mouse grimace scale, in which the experimenter rates aspects of facial expression (orbital, cheeks, nose, whiskers, and ears) on a scale of 0-2, which are averaged to give the mean grimace score (MGS).[35,36] Each aspect was rated as follows: a score of "0" indicates not present, a score of "1" indicates moderately present, a score of "2" indicates obviously present. Animals were acclimated in acrylic behavior boxes for one hour prior to the assessment of facial grimacing, which occurred prior to any other behavioral tests.[33]

Conditioned place preference

We used the conditioned place preference (CPP) paradigm to assess whether treatment with IL-5 alleviated persistent pain via analgesic-induced place preference. We previously showed that clonidine, which has been used to treat chronic pain, induces place preference in the acidic saline model.[33] All CPP experiments were conducted during the dark cycle, between 6PM and 11PM. CPP was conducted in a dark room with a single low lumens light source (Maia 69E120-WH). The CPP apparatus contained three chambers: one black chamber, one white chamber, and a striped middle chamber, which was brightly lit to discourage animals spending time in this chamber. The two end chambers were differentiated by scent (chapstick applied to the ceiling) and floor texture.

Animals were handled daily for five minutes each for two weeks prior to the start of CPP experiments. Prior to pre-conditioning, animals were tested for mechanical sensitivity and facial grimacing (Day 9). During pre-conditioning, mice freely explored the middle and black (non-paired) chambers for ten minutes each. Conditioning occurred daily from Days 10-13. On conditioning days, mice were either given IL-5 (0.1μg/100μL in sterile 1x PBS, I.V.), clonidine (Sigma-Aldrich, C7897-100MG, 10μg/5μL in sterile 1x PBS, intrathecal) or vehicle (100μL sterile 1x PBS, I.V., or 5μL sterile 1x PBS, intrathecal). For intrathecal injections, mice were deeply anesthetized with isoflurane (5% induction, 2.5% maintenance) and injected intrathecally with clonidine or vehicle. One hour following injection, animals were confined to the white chamber (drug-paired) for 30 minutes. On testing day (Day 14), each mouse was placed in the middle chamber with unrestricted access to all chambers and recorded for ten minutes.

Data are presented as a proportion of time spent in the drug-paired chamber over time spent in the non-paired chamber. Locomotor behavior, measured as the number of crossings between chambers, was used as an exclusionary criterion via outlier analysis. All data was scored by two independent experimenters blinded to condition.

Thermal sensitivity

To test thermal sensitivity, mice were tested for withdrawal latency to a noxious temperature.[33] Mice were placed in an acrylic box with a temperature-controlled metal plate that was heated to 52°C (IITC). The latency (in seconds) for the mouse to withdraw its hind paw was recorded. Response latency was

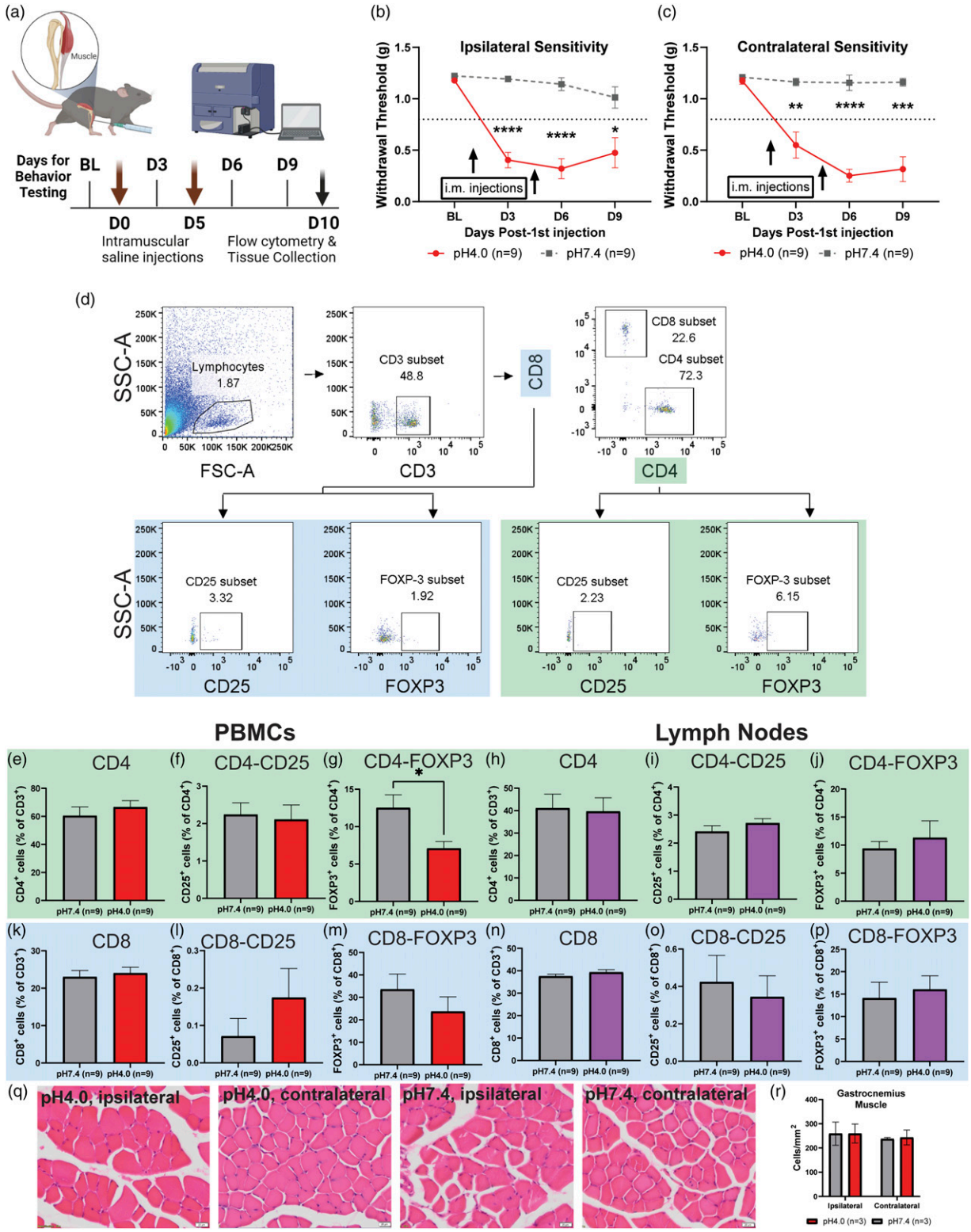

Figure 1. Gastrocnemius injections of acidic saline induces decreases in circulating $CD4^+$ Tregs. We used flow cytometry to assess T-cell populations of PBMCs and lymph nodes in female mice with CWP. (a) CWP was induced with two unilateral injections of pH4.0 saline into the left gastrocnemius muscle five days apart. (b) Mice injected with pH4.0 saline develop mechanical hypersensitivity on the ipsilateral hind paw and the (c) contralateral paw hind paw compared to controls (pH7.4 saline). (d) Gating strategy to assess T-cell populations. No differences were observed in total $CD4^+$ T-cells (e) or in $CD4^+CD25^+$ T-cells (f) from PBMCs. (g) There is a downregulation of CD4+FOXP3+ T-cells in pH4.0 saline injected mice compared to controls. No differences were observed in $CD4^+$ (h), $CD4^+CD25^+$ (i), or CD4+FOXP3+ (j) T-cells from peripheral lymph nodes. No differences were observed in $CD8^+$ (k, l), $CD8^+CD25^+$ (l, o), or CD8+FOXP3+ (m, p) from either PBMCs or lymph nodes. (q) H&E staining of gastrocnemius muscle (r) No differences were found in quantifying the number of cells in the gastrocnemius muscle on Day 10. *$p < 0.05$.

assessed at baseline, and every three days following the first injection for three weeks. A cut-off of 30 seconds was used to prevent tissue injury.

Grip strength

The grip strength assay was used as a measure of muscle pain and function.[33] Briefly, mice were suspended by the tail, allowed to grasp a wire mesh, and gently pulled backward. The maximum force (g) generated by the grip strength meter (IITC) was recorded.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 9.3.1 statistical software. All data are presented as mean ± SEM. Comparisons between pH4.0 saline and pH7.4 saline-injected mice were made using unpaired t-test with two-tailed *p*-value for each cell population. Muscle histology data was analyzed using Ordinary Two-Way ANOVA with Bonferroni's *post hoc*. CPP data was analyzed using Ordinary Two-Way ANOVA with Bonferroni's *post hoc*. Behavioral data (von Frey, grimace, thermal sensitivity, grip strength) was analyzed using Repeated Measures Two-Way ANOVA with Tukey's *post hoc*. Behavioral data is additionally represented as effect size, which is determined by calculating the cumulative difference between the value for each post-injection timepoint and the timepoint immediately preceding IL-5 injections (Day 9) to show total anti-allodynia. Effect size data was analyzed using Ordinary Two-Way ANOVA with Bonferroni's *post hoc*. Statistical significance for all tests was set at $p < 0.05$.

Results

Gastrocnemius injection of acidic saline induces decrease in regulatory T-cells in PBMCs, but not in peripheral lymph nodes

Dysregulation of T-cell subpopulations has been reported across multiple pain conditions, with T-cells playing important roles in both the promotion and resolution of pain states.[24,26,37–39] Given that the acidic saline model induces CWP after local injections into the muscle, we chose to examine T-cell subpopulations in both PBMCs and draining peripheral lymph nodes using flow cytometry: $CD4^+$ (T-helper), $CD8^+$ (cytotoxic T-cells), $CD25^+$ (activated T-cells), and FOXP3 (regulatory T-cells) (Figure 1). PBMCs and lymph nodes were collected following the development of bilateral mechanical sensitivity (Day10, Figure 1(a)-(d)). Female mice injected unilaterally with pH4.0 saline (Days 0 and 5) showed reduced mechanical withdrawal thresholds of the paw across each timepoint measured both ipsilaterally (Figure 1(b), $f(3, 48) = 10.40$, $p < 0.0001$, Repeated Measures Two-Way ANOVA) and contralaterally (Figure 1(c), $f(3, 48) = 17.38$, $p < 0.0001$, Repeated Measures Two-Way ANOVA). We did not observe differences in total $CD4^+$ or $CD8^+$ T-cell populations in either the PBMCs (Figure 1(e), (k)) or lymph nodes (Figure 1(h), (n)). Each of these populations was further analyzed for expression of CD25, a component of the IL2 receptor expressed by activated T-cells, and FOXP3, a transcription factor necessary for the immunosuppressive actions of Tregs.[40–43] During our preliminary experiments, we also stained for CD44, a marker for memory T-cells[44]; However, we saw no differences in any $CD44^+$ population and thus chose to remove it from further experiments (data not shown). We found that mice with CWP (acidic saline treated) have a significantly reduced population of CD4+FOXP3+ T-cells compared to controls in PBMCs, indicating a downregulation of circulating Tregs during CWP (Figure 1(g), $t(16) = 2.770$, $p = 0.0137$). There were no differences in $CD25^+$ subsets within $CD4^+$ populations (Figure 1(f), (i)) or $CD8^+$ populations Figure 1(l), (o) in PBMCs and lymph nodes. In summary, we observed downregulation of circulating Tregs in acidic saline treated mice compared to controls with no differences observed in activated/effector T-cells in either PBMCs or lymph nodes.

Gastrocnemius injections of acidic saline do not induce long-term local inflammation

We used H&E staining to assess local inflammation in the gastrocnemius muscles after acidic saline injections to determine whether inflammation in the muscles is increased long term in this model. At 10 days following the first saline injection, the gastrocnemius muscle from both the ipsilateral and contralateral sides was collected and stained with H&E Figure 1(q). Analysis of cell counts shows no differences in the number of cells in the muscle at the injection site, indicating no significant changes in local inflammation of the muscle long term in this model (Figure 1(r)).

Systemic IL-5 treatment is analgesic but does not affect physical function

In our initial study on the role of IL-5 in CWP, we found that systemic injections of 0.1μg IL-5 reduces mechanical sensitivity of the hind paw in mice with widespread musculoskeletal pain. In this study, we applied a battery of behavioral assessments following IL-5 treatment to determine whether the analgesic effects we observed apply to additional outcome domains (Figure 2(a)). First, we recapitulated the results of our previous study by testing mechanical sensitivity of the hind paws (Figure 2(b)-(e)). We again saw that I.V. injections of 0.1μg IL-5, 24 h apart, transiently reverse both ipsilateral and contralateral sensitivity back to baseline (Figure 2(b) and (d), respectively). Mechanical withdrawal thresholds of the ipsilateral paw of mice with CWP (pH4.0) were significantly increased following the second injection of IL-5 compared to mice with CWP given vehicle (Figure 2(b)): 1h-post 2nd IL-5

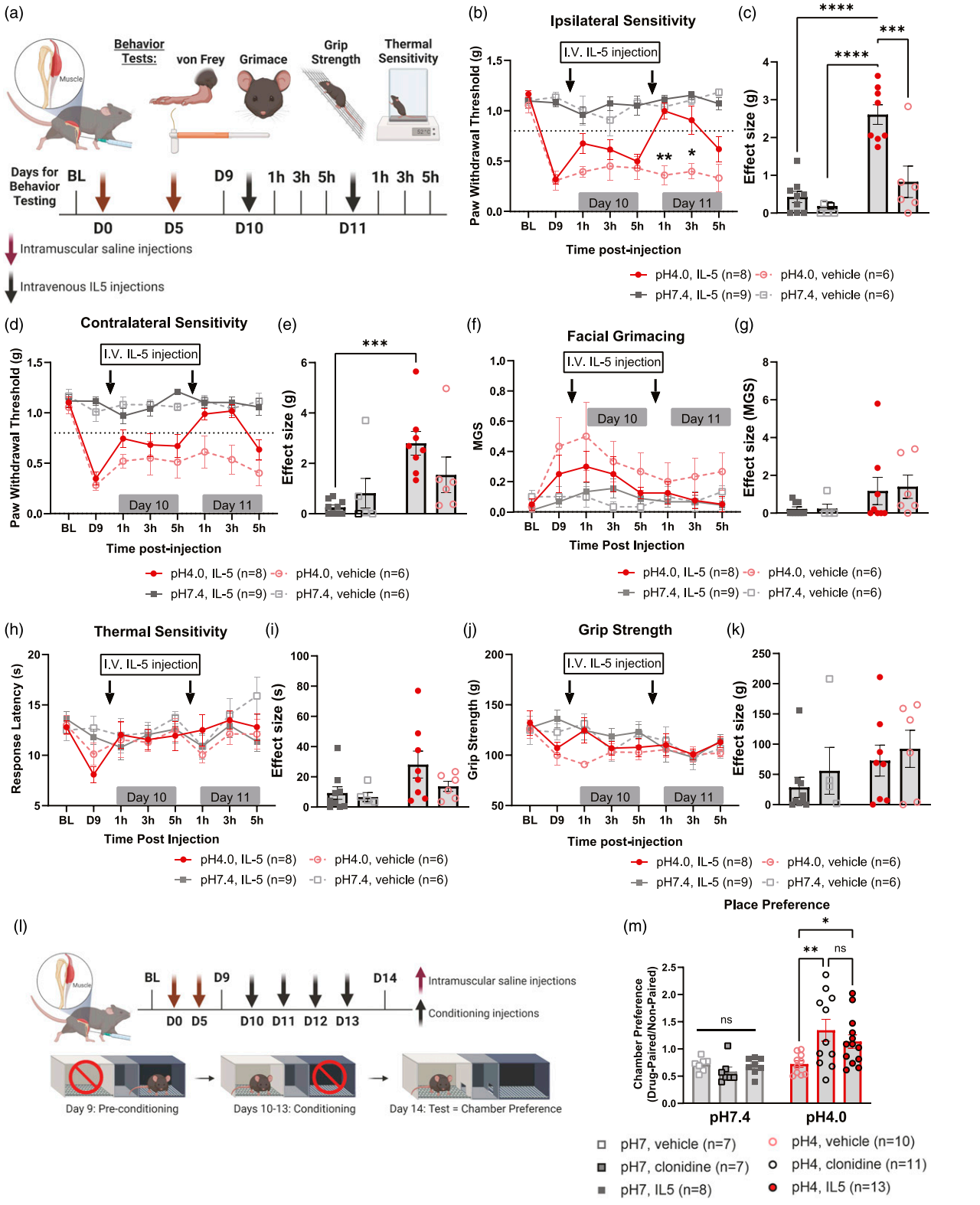

Figure 2. Systemic IL-5 treatment induces analgesia. (a) Representative timeline for behavioral experiments. Intramuscular injections of pH4.0 saline causes mechanical sensitivity of the ipsilateral (b) and contralateral (d) hind paws, which is rescued by I.V. injections of 0.1 μg IL-5. *, **$p < 0.05$, pH4.0, IL-5 vs pH4.0, vehicle. (c, e) Effect size analysis of anti-allodynia following IL-5 injections show that pH4.0 saline mice treated with IL-5 experience analgesia with no effect of IL-5 on vehicle treated mice and no-pain controls. (f) Facial grimacing is increased in the pH4.0 group compared to controls but is not rescued by treatment with IL-5. (g) Effect size analysis showed no effect of IL-5 treatment on facial grimacing. (h) No differences were observed in measures of thermal sensitivity. (i) Effect size analysis of thermal sensitivity showed no effect of IL-5 injections. (j) Intramuscular injections of pH4.0 saline reduces grip strength, which is transiently rescued by IL-5 treatment. (k) Effect size analysis of grip strength showed no effect of IL-5 injections. (l) Timeline for conditioned place preference (CPP) experiments. (m) Both clonidine and IL-5 induced place preference in mice that previously received intramuscular injections of pH4.0 saline, but not in controls. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

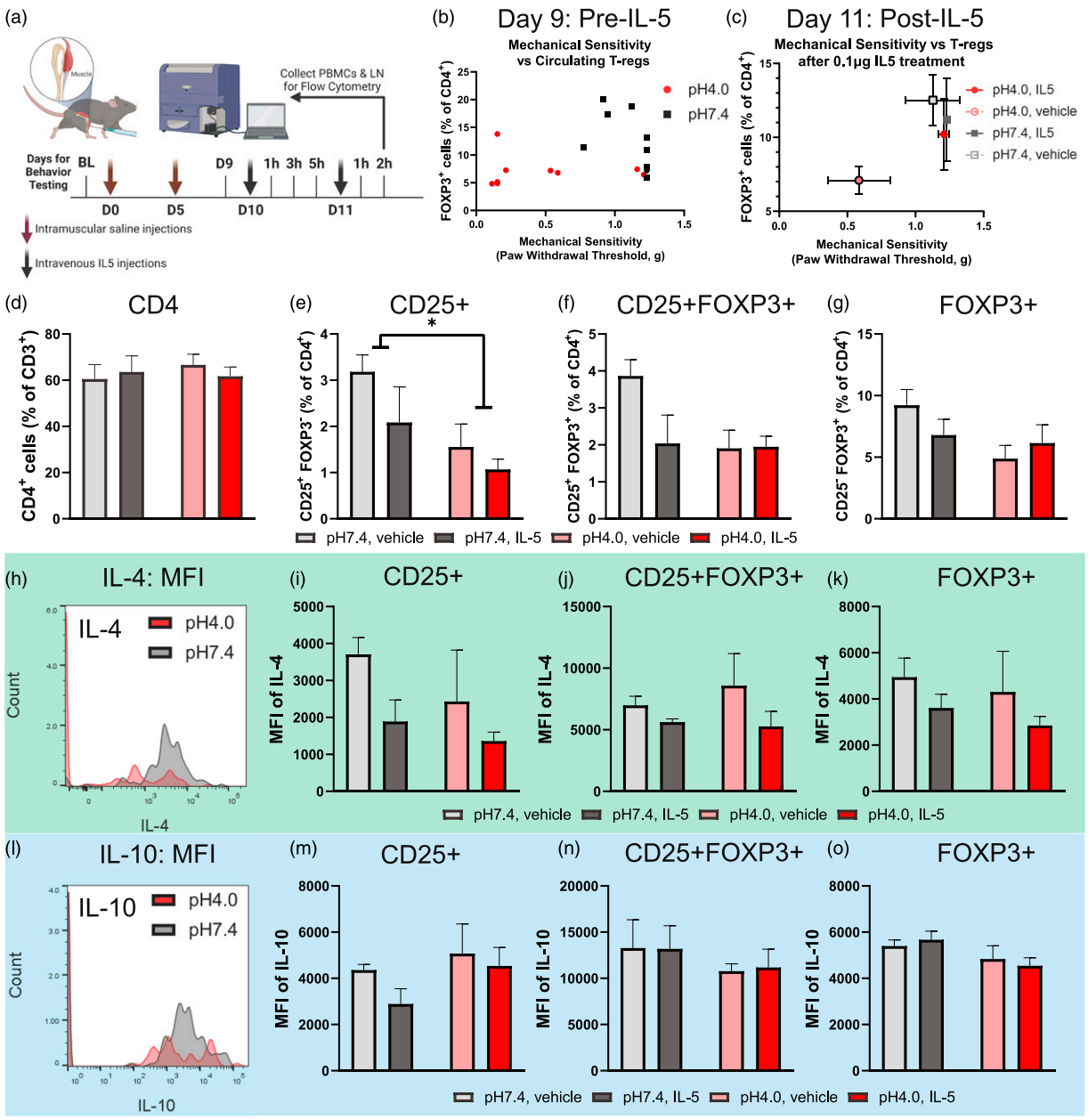

Figure 3. Systemic IL-5 treatment has limited effects on T-cell populations. (a) Timeline for injections and flow cytometry experiments. (b) Mechanical sensitivity (ipsilateral hind paw, Day 9) versus CD4+FOXP3+ T-cells from PBMCs (Pearson r = 0.2908, $p = 0.2417$). n=9/group. (c) Mechanical sensitivity (ipsilateral hind paw, 1h post IL-5) versus CD4+FOXP3+ T-cells from PBMCs. (n = 5/group for IL-5 treatment and n = 4/group for vehicle treatment). (d) Percentage of $CD4^+$ cells in PBMCs after IL-5 treatment. (e) Percentage of $CD4^+CD25+FOXP3-$ T-cells (effector T-cells) after IL-5 treatment. (f) Percentage of $CD4^+CD25+FOXP3+$ T-cells after IL-5 treatment. (g) Percentage of $CD4^+CD25-FOXP3+$ T-cells after IL-5 treatment. (h) Representative histograms of mean fluorescence intensity (MFI) for IL-4 by CD4+FOXP3+ T-cells. MFI of IL-4 produced by $CD4^+CD25+FOXP3-$ (i), $CD4^+CD25+FOXP3+$ (j), and $CD4^+CD25-FOXP3+$ (k) T-cells. Representative histograms of MFI for IL-10 by CD4+FOXP3+ T-cells. MFI of IL-10 produced by $CD4^+CD25+FOXP3-$ (m), $CD4^+CD25+FOXP3+$ (n), and $CD4^+CD25-FOXP3+$ (o) T-cells. n = 5 for pH4.0, IL-5; n = 4 for pH4.0, vehicle; n = 5 for pH7.4, IL-5; n = 4 for pH7.4, vehicle. *$p < 0.05$.

injection: pH4.0, IL-5 (n = 8) vs pH4.0, vehicle (n = 6), $p = 0.0014$); 3h-post 2nd IL-5 injection: pH4.0, IL-5 (n = 8) vs pH4.0, vehicle (n = 6), $p = 0.0371$). No differences between control mice (pH7.4) given IL-5 or vehicle were observed. Effect size analysis of post-IL-5 injection timepoints showed significantly greater ipsilateral analgesia in mice with CWP given IL-5 compared to all other groups (Figure 2(c)) and significantly greater contralateral analgesia compared to control mice given IL-5 (Figure 2(e)).

We also tested the effects of IL-5 treatment on facial grimacing, thermal sensitivity, and grip strength to assess additional outcome domains following IL-5 treatment. Although mice that received acidic saline injections grimaced more than control mice, as reported previously, there was not a significant effect of IL-5 treatment on facial grimacing (Figure 2(f)-(g)). Furthermore, mice with CWP have reduced response latency to a noxious temperature (52°C) (Figure 2(h)), which is partially rescued by IL-5 treatment (Figure 2(i): $F(1, 24) = 4.304$, $p = 0.0489$). Finally, we tested grip strength to assess whether IL-5 could improve the loss of limb strength observed previously in this model.[33] Despite a transient increase in grip strength one hour following the first injection of IL-5, no significant differences were observed between mice with CWP treated with IL-5 and those treated with vehicle indicating that IL-5 treatment does not affect muscle strength at this dose (Figure 2(j)-(k)). Interestingly, using the conditioned place preference paradigm we found that systemic IL-5 treatment is sufficient to induce a place preference in mice with CWP treated with IL-5, but not in pain-free controls (Figure 2(m): pH4.0, IL-5 (n = 13) vs pH7.4, IL-5 (n = 8), $p = 0.0417$)). Similarly, mice with CWP treated with clonidine, but not pain-free controls, show robust place preference (Figure 2(m): pH4.0, clonidine (n = 11) vs pH7.4, clonidine (n = 7), $p = 0.0006$)). No differences in voluntary locomotion, measured as the number of chamber crossings, were observed between groups (data not shown).

Effects of Systemic IL-5 on circulating CD4$^+$ T-cell populations

To assess the effects of IL-5 treatment on T-cell populations in mice with CWP, we performed flow cytometry on female mice with CWP (pH4.0 saline) and pain-free controls (pH7.4 saline) treated with IL-5. PBMCs and peripheral lymph nodes were collected at two-hours post-IL-5 injection (Figure 3(a)), during the peak of analgesia. Because mice with CWP have a reduction in circulating Tregs (CD4+ FOXP3+) (Figure 3(b): Pearson r = 0.2417), we chose to focus on this population in the follow-up IL-5 experiments. Further, we did not observe any differences in CD8$^+$ T-cell populations in either experiment (data not shown). Interestingly, female mice with CWP treated with IL-5 have increased Tregs corresponding to increased mechanical withdrawal thresholds comparable to pain-free controls (Figure 3(c): Pearson r = 0.8575)). First, we found that treatment with IL-5 did not change overall percentages of CD4$^+$ T-cells (Figure 3(d)). Because Treg functions and immunosuppressive action can be defined by expression of CD25 in addition to FOXP3, we further separated these cells into CD25+ FOXP3- (T-effector), CD25- FOXP3+ (Treg), and CD25+ FOXP3+ (activated Treg) subpopulations. Interestingly, we found that mice with CWP, regardless of IL-5 treatment, had reduced T-effector cells compared to pain-free controls (Figure 3(e), (f) (1,14) = 6.222, $p = 0.0257$). Further, mice with CWP have a downregulation of both CD25$^+$ (Figure 3(f), $p = 0.0825$) and CD25- Tregs (Figure 3(g), $p = 0.0835$) in the absence of IL-5 treatment. For both populations, there were no differences between mice with CWP and pain-free controls following IL-5 treatment.

To assess the functionality of these cell types, we measured the IL-4 and IL-10 production using mean fluorescence intensity. IL-4 and IL-10 are both implicated in resolution of pain and inflammation. We did not observe any differences between mice with CWP and controls regarding IL-4 production in any subpopulation of CD4$^+$ cells (Figure 3(h)-(k)); however, there was a reduction in IL-4 production by CD4$^+$CD25$^+$ T-effector cells in both groups after treatment with IL-5 (Figure 3(e), $p = 0.0695$). Regarding production of IL-10, there were no IL-5 dependent differences in any subpopulation of CD4$^+$ T-cells (Figure 3L-O). There was decreased production of IL-10 by CD25- Tregs from mice with CWP compared with pain-free controls independent of IL-5 treatment (Figure 3(n), $p = 0.0529$), with no observed differences in T-effector cells or CD25$^+$ Tregs.

Discussion

The understanding of T-cells in the development and pathology of chronic muscle pain and FM is poorly understood. Thus, the purpose of the current study was to assess T-cell populations using a preclinical model of FM that recapitulates chronic muscle pain, the primary feature of FM. We also aimed to determine effects of IL-5 on nociception and functional outcomes during chronic muscle pain as a follow-up to our previous finding that exogenous IL-5 successfully relieved mechanical hypersensitivity.[6] In the current study, we found that female mice with chronic muscle pain have a reduction in Tregs (CD4+ FOXP3+), a population implicated in immunosuppression and the development of several autoimmune diseases.[45,46] Treatment with IL-5 successfully relieved persistent pain and induced robust place preference in mice with chronic muscle pain. In patients, shifts in T-cell populations have been reported, with general literature trends indicating increased CD4+/CD8+ T-cell ratio; however, there is a gap in the preclinical literature about the role of T-cells in the pathology of the chronic muscle pain characteristic of FM.[30,31,47] As there is evidence of immune dysregulation in FM,[12] understanding T-cell phenotypes and their response to potential and current therapeutics may enhance success rates of clinical trials for a wider range of patients.

The development of autoimmune disorders are due to several factors, namely an imbalance between immunosuppression and effector immune cells.[46,48] Using female C57BL/6J mice, we assessed T-cell populations after induction of chronic muscle pain using the acidic saline model and found a reduction in Tregs (CD4+FOXP3+) in PBMCs, a

population implicated in immunosuppression and the development of several autoimmune diseases.[41,46,48] This reduction shifts the balance of T-effector and Tregs away from immunosuppression, which results in diminished regulation of excess inflammation and self-reactive T-cells.[45,49] By measuring changes in T-cell populations during chronic muscle pain and IL-5 mediated analgesia, our current study has provided a target population for future studies. Direct targeting of specific T-cell populations would provide strong evidence of how essential each population is for the development of chronic muscle pain. Our study and others have not found overt differences in local inflammation in the muscle or draining lymph nodes, supporting the systemic effects of the model.[50] Tregs have been linked to the resolution of multiple chronic pain states in female mice.[37–39] A recent study found that female mice develop hypersensitivity and reductions in muscle strength following peripheral injections of serum immunoglobulins isolated from women with FM, further implicating adaptive immune dysfunction in the pathology of FM[51]; however it is unclear if these cells are directly responsible for chronic muscle pain phenotypes.

We have shown previously that IL-5 reverses mechanical sensitivity, so we tested the analgesic properties of IL-5 using CPP, facial grimacing, thermal sensitivity, and grip strength. We found that IL-5 induces place preference in mice that had previously received acidic saline injections similarly to clonidine, a drug used to treat neuropathic pain.[33] Treatment with IL-5 only partially rescued thermal sensitivity and grip strength, indicating that IL-5 treatment does not fully alleviate physical function deficits at this dose. The mechanism of IL-5 on analgesia continues to be elucidated, as it is produced by mast cells, eosinophils, and monocytes in addition to Th2 T-cells.[20] Although our previous study showed that IL-5 polarizes monocytes towards an anti-inflammatory phenotype, it is likely that IL-5 action directly on sensory neurons contributes to analgesia as well.[6] One group has shown that IL-5 directly activates sensory neurons in the nodose ganglia; however, to our knowledge this has not been shown in dorsal root ganglia.[52] The focus of the current study was on the role of T-cells and IL-5 in mediating CWP, but we plan on further pursuing the mechanism of IL-5 action on sensory neurons as a follow-up to the present study.

In this study, we focused on female mice because FM is a female biased disorder; however, we acknowledge that the use of males would allow for increased generalizability of the study. At present, it is unknown whether male mice would also respond to IL-5 treatment in an analgesic manner, as our initial study was also solely conducted using female mice to sex match the female patients used in the clinical aspect of our study.[17] Previous studies have shown that males do not develop chronic muscle pain in preclinical models as often or as robustly as females and that sex hormones may play a role in this.[50,53,54] Interestingly, in models of allergic airway inflammation, ovariectomy reduces IL-5 production and eosinophil proliferation, which is IL-5 dependent, after airway sensitization, indicating that the actions of IL-5 may be at least partially dependent on sex hormones.[55] Further, FM has many symptoms in addition to pain, such as depression and anxiety, fatigue, and cognitive dysfunction.[14,56,57] The role of IL-5 in mediating these symptoms is currently unknown. As a promising target for pain relief preclinically, further research is needed to assess whether those features of FM are present in this model and whether IL-5 affects those symptoms.

Communication between T-cells and monocytes likely plays a large role in the maintenance of chronic muscle pain. A few studies have shown that T-cell and monocyte interactions in injured muscle tissue promote tissue repair, such that Tregs promote monocyte polarization towards a pro-inflammatory phenotype.[58,59] Th1 pro-inflammatory cells are robust producers of IFN-γ which increases MHC-II expression and antigen presentation by monocytes and promotes cell-mediated immune responses for pathogen defense.[60,61] Th2 anti-inflammatory cells produce IL-4 and IL-10 to promote humoral immune responses and polarize monocytes toward an anti-inflammatory phenotype.[62,63] In our current study, female mice with CWP have reduced expression of FOXP3 Th2 Tregs, the prototypical producers of IL-10; however, there were not significant differences in cytokine production by these cells. In our previous manuscript, we found that monocytes from women with FM produce significantly more IL-5, IL-4, and IL-10 from stimulated monocytes compared to no pain controls.[6] Release of these cytokines typically has anti-inflammatory functions, with IL-4 and IL-10 both acting to promote T-helper Type 2 (Th2) activation, anti-inflammatory (M2) monocyte polarization, and promote analgesia.[18,64] During T-cell dysfunction, such as with immunodeficiency, monocytes can upregulate expression of the Th2 cytokines IL-5, IL-4, and IL-13.[21] In Th2 cells, expression of the Th2 cytokines IL-5 and IL-13 are controlled by transcription factors whose expression are promoted by IL-4 signaling.[44,65] In monocytes, these same signaling mechanisms act to promote anti-inflammatory polarization.[66] Downregulation of Foxp3 Th2 regulatory T-cells in the FM preclinical model supports evidence of the monocytic IL-5 shift and immune dysfunction in the pathogenesis of chronic muscle pain.[6] We found that exogenous IL-5 treatment polarized monocytes towards an anti-inflammatory profile in the preclinical model.[6] While monocyte expression of IL-5 may be induced during disease states, whether this occurs with the IL-5 receptor is currently unknown.[21,23,67] Although we have shown that both Tregs and monocytes are dysregulated in chronic muscle pain, the specific interactions between these cells and the role of IL-5 in each continues to be elucidated.

Overall, intramuscular injections of pH4.0 saline induce long-lasting widespread pain and a downregulation of circulating regulatory T-cells (CD4+ FOXP3+) compared to controls. Treatment with exogenous IL-5 relieves mechanical

sensitivity and persistent pain, with limited effects in spontaneous pain, thermal sensitivity, and grip strength. Further, effects of IL-5 treatment on T-cell populations and anti-inflammatory cytokine production were limited. Further research involving the direct effects of IL-5 on sensory neurons as well as continuing to elucidate interactions between monocytes and T-cells during chronic muscle pain is warranted; however, IL-5 remains a promising target for the treatment of chronic muscle pain.

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Author contributions

M.E.L. designed experiments. M.E.L and T.S.P. acquired and analyzed data. M.E.L. drafted the manuscript and figures. M.E.L., and T.S.P edited the manuscript and figures. M.D.B. participated and supervised all aspects of the study from conception, design, acquisition, interpretation, and manuscript preparation. All authors reviewed and read the final manuscript.

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