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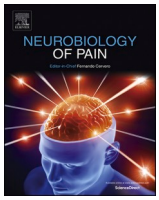

Original Research

# B-cell and plasma cell activation in a mouse model of chronic muscle pain

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ABSTRACT

Fibromyalgia (FM) is a complex chronic musculoskeletal pain disorder with an elusive pathogenesis, with a strong implication of immune interactions. We recently found that IL-5 and the adaptive immune system mediates pain outcomes in fibromyalgia (FM) patients and preclinical models of FM-like chronic widespread pain (CWP). However, there is an active debate if FM/CWP has an autoimmune etiology. Preclinical models of CWP utilize a repeated insult paradigm, which resembles a primary, then secondary response similarly observed in the antibody response, in which the subsequent event causes a potentiated pain response. Recent translational studies have implicated immunoglobulins (Ig) and B-cells in FM/CWP pathophysiology. To understand if these are involved in preclinical models of CWP, we performed comprehensive B-cell phenotyping in the bone marrow, circulation, and popliteal (draining) lymph nodes in the two-hit acidic saline model of CWP. We found increased MHC class II-expressing B-cells in peripheral blood, increased activated plasma cells in peripheral blood, and increased memory B-cells in the bone marrow. Interestingly, acidic pH (4.0) injected mice have reduced levels of IgG1, independent of treatment with IL-5. We have demonstrated that the acidic saline model of CWP induces T-cell mediated activation of B-cells, increased active plasma cells, and increased memory B-cells in female mice.

## Introduction

Chronic widespread musculoskeletal pain is the primary symptom of fibromyalgia (FM) (Arnold et al., 2019). The acidic saline rodent model utilizes repeated insults to the gastrocnemius muscle to induce a chronic, widespread pain (CWP) phenotype (Gregory et al., 2013). While the first injection of low pH saline results in ipsilateral muscular and hind paw hypersensitivity, the second injection causes hypersensitivity to extend contralaterally (Sluka et al., 2001). This paradigm results in a pain response pattern that mirrors primary versus secondary adaptive immune responses in which the second immune exposure results in a significantly stronger response that is sustained for weeks to months (Natoli and Ostuni, 2019, Nutt et al., 2015; Parker, 1993; Rajewsky, 1996). However, a ctivation of the adaptive immune response in the acidic saline model has not been previously demonstrated.

Peripheral immune function is altered in both women with fibromyalgia (Goebel et al., 2021, Merriwether et al., 2021, O'Mahony et al., 2021, Fineschi et al., 2022) and in preclinical models of chronic muscle pain (Merriwether et al., 2021, Lenert et al., 2023, Lesnak et al., 2023, Caxaria et al., 2023, Bussulo et al., 2021); however, preclinical studies have focused on the innate immune system. We have previously shown reductions in circulating regulatory T-cells (Tregs) in the acidic saline model using female mice (Lenert et al., 2023), which is a feature of autoimmune pathology. Autoimmunity and Tregs have been linked to the development and resolution, respectively, of multiple chronic pain states in female mice (Austin et al., 2012, Kuhn et al., 2021, Rosen et al., 2017, Lee et al., 2022, Lenert et al., 2023). These two adaptive immune system components are tightly linked: Tregs inhibit the overactivation of antibodies and prevent autoantibody generation (Georgiev et al., 2019; BUCKNER, 2010). Moreover, IL-5 stimulates B-cells and increases immunoglobulin production (Takaki et al., 1990, Hitoshi et al., 1990). Antibody generation and maintenance is a dynamic process that occurs across several tissue locations: bone marrow (initial B-cell development and quiescent plasma cell storage), lymph nodes (antigen presentation and B-cell activation), and circulation (surveilling functions and antibody production) (Akkaya et al., 2020, Katikaneni and Jin, 2019, Cancro and Tomayko, 2021, Arpin et al., 1995). Dysfunction in either the B-cell selection process or somatic hypermutation of memory B-cells can lead

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to autoantibody production (Wu et al., 2011, Goodnow et al., 2010). Autoantibodies have been shown to directly activate nociceptors via Fc receptors (Lacagnina et al., 2021, Lee et al., 2022, Andoh and Kuraishi, 2004, Jurczak et al., 2023) and can directly cause pain-like behaviors in animals models (Jurczak et al., 2022). Increased immunoglobulin (IgG) levels and or functionality may be related to pain symptomology in patients with FM (Fanton et al., 2023, Krock et al., 2023) and immunoglobulins from patients with FM may induce chronic widespread pain when transferred to naïve mice (Goebel et al., 2021); however, changes in B-cell and immunoglobulin phenotypes in preclinical models of chronic muscle pain have not been previously assessed.

The goal of this study was to assess the phenotypes of B-cell populations in a preclinical model of CWP to understand how they might play a role in promoting chronic muscle pain. We administered two unilateral injections of pH4.0 saline, five days apart, into the gastrocnemius muscle of mice to induce persistent widespread pain. Based on our postulation that a repeated muscle insult model would mirror a primary to secondary response, we chose to assess B-cell and plasma cell phenotypes at Day 10 (peak mechanical sensitivity at five days after the second saline injection) of the experimental timeline and anticipated to see the most robust population shifts at this timepoint. B-cells and plasma cells were isolated from peripheral blood, draining popliteal lymph nodes, and the bone marrow (femur and tibia) to ascertain a comprehensive understanding of B-cell and plasma cell populations at each of their primary tissue locations. Within each of these, we analyzed B-cell ($CD19^{+}B220^{+}$) and plasma cell ($CD19^{+}B220^{-}$) populations for expression of CD27 (memory B-cells), CD38 (upregulated with activation and differentiation), and MHC-II (required for T-cell dependent activation) (Wu et al., 2011; Piedra-Quintero et al., 2020; Parker, 1993). We posited that pH4.0 injected mice (acidic saline model) would have an increase in activated B-cell phenotypes and increased immunoglobulin levels compared to controls associated with increased evoked pain behaviors, and that IL-5 treatment would attenuate this effect. These hypotheses are based on our previous studies l showing that IL-5 treatment reduces mechanical pain sensitivity, increases anti-inflammatory monocyte polarization, and reduces effector T-cell populations (Lenert et al., 2023; Merriwether et al., 2021). Additionally, we hypothesized that B-cell populations would have increased markers of B-cell activation after two pH4.0 injections.

## Materials and methods

### *Animals*

Female C57BL/6J mice (2–5 months old, 20–25 g) were used for all experiments to match the major patient population in fibromyalgia/chronic widespread pain (CWP). 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 (National Research Council Committee for the Update of the Guide for the and Use of Laboratory, 2011, Percie du Sert et al., 2020).

### *Induction of chronic musculoskeletal pain*

Chronic musculoskeletal pain was induced as described previously (Sluka et al., 2001, Lenert et al., 2023, Merriwether et al., 2021, Lenert et al., 2022). In brief, mice were anesthetized with 4 % isoflurane to immobilize the mice during injection, and the left gastrocnemius muscle (ipsilateral) was injected with 20 μL of acidic saline (0.9 %, pH 4.0 ± 0.1) or physiological saline (pH 7.4 ± 0.1) on days 0, following baseline measures, and again at day 5 of each experiment. Mice were randomly assigned using a random number generator to groups by an independent experimenter and each cage contained mice in both groups.

### *Behavior assessment*

Prior to baseline measures, animals were acclimated to the testing room in their home cages for up to two hours. On testing days, animals were habituated in acrylic behavior boxes on an elevated wire mesh grid for one hour. All behavioral tests occurred between 10AM-2PM. All behavior experiments were performed by experimenters blinded to condition (Lenert et al., 2023, Merriwether et al., 2021, Sluka et al., 2001, Lenert et al., 2022).

### *Von Frey testing*

To assess mechanical sensitivity, von Frey filaments were used to stimulate the hind paw of the mouse. We used 0.07-, 0.4-, and 1.0-gram filaments and measured the response frequency to 10 consecutive stimulations of the hind paw with each filament with stimulations spaced by at least 5 s (Laird et al., 2001, Pitcher et al., 2007, Inyang et al., 2019). The response frequency for each filament force was recorded and graphed as a percentage. For the up-down method, filaments of logarithmically increasing stiffness of 2.83, 3.22, 3.61, 3.84, 4.08, and 4.17 (converted to the 0.07, 0.16, 0.4, 0.6, 1, and 1.4 g, respectively) were applied to the plantar surface of the hindpaw for 2–3 s (Chaplan et al., 1994). The mechanical withdrawal threshold was calculated as the weight at which the animals responded 50 % of the time.

### *Cold hypersensitivity*

To evaluate cold hypersensitivity, mice were tested for withdrawal latency to a noxious temperature (one trial per timepoint) (Zhao et al., 2012). Mice were placed in an acrylic box with a temperature-controlled metal plate that was cooled to 0 °C (IITC). The latency (in seconds) for the mouse to withdraw its hindpaw was recorded. A cut-off of 30 s was used to prevent tissue injury.

### *Hargreaves*

Thermal hypersensitivity was measured using the Hargreaves heat plantar device (IITC) (Mody et al., 2020). Following von Frey, mice were placed in an acrylic box on a glass surface heated evenly to 29 °C. Withdrawal latencies were measured after animals received a laser beam directed to the ipsilateral and contralateral hind paw. To obtain baseline withdrawal measures no less than 10 s, we used the laser at an active intensity of 29 %. A cut-off of 30 s was used to prevent tissue injury. A minimum of three trials of withdrawal latencies were recorded at each time point.

## Flow cytometry

### *Tissue collection and Dissociation*

To isolate B-cell populations for flow experiments, mice were deeply anesthetized under isoflurane and euthanized via decapitation on Day 10 of the experimental timeline (two hours post the 2nd IL-5 injection). PBMCs were isolated from whole blood (~1 mL of trunk blood) collected in BD blood collection tubes with anti-coagulant (VWR, BD367884) using Ficoll-Paque PLUS (VWR, 95021–205) (Merriwether et al., 2021, Lenert et al., 2023). Draining popliteal lymph nodes were collected in ice cold sterile 1 × DPBS (Hyclone, Logan, UT). Lymph nodes were passed through a 70-μm cell strainer using flow buffer (0.5 % bovine serum

albumin (Sigma, A9576) and 0.02 % glucose (Sigma, G7528) made in 1 × DPBS) (Agalave et al., 2021, Lenert et al., 2023). Bone marrow was collected by flushing ice cold sterile 1x DPBS through the femur and tibial bones using a 30G needle and 10 mL syringe.

### *B-cell phenotyping*

Cell suspensions were centrifuged at 600 × rcf for 6 min at 4 °C, resuspended in 1 × DPBS, and washed twice with flow buffer. To block non-specific binding via the Fc receptor, cells were incubated in blocking buffer (1:2000 CD16/32 in flow buffer) for 10 min on ice. Samples were incubated with pre-conjugated extracellular flow antibodies (CD19, B220, CD38, CD27, MHC-II) for 60 min diluted in flow buffer, on ice and protected from light. Cells were washed and fixed using ice cold fresh 4 % paraformaldehyde (PFA) diluted in 1 × PBS for 15 min. Cells were washed once and resuspended in flow buffer for data acquisition. Appropriate compensation controls were used for determination and gating. B-cells and plasma cells were identified using unstained and single-color controls with gating for $CD19^{+}B220^{+}$ and $CD19^{+}B220^{-}$, respectively, and were further gated to identify MHC-II, CD38, and CD27. Samples were analyzed using a BD LSR Fortessa analyzer (BD Biosciences, San Diego, CA) and analysis was performed from a sampling of minimally 50,000 cells using FlowJo software (San Carlos, CA). Experimenters were blinded to condition. For a complete list of antibodies, refer to Table 1.

### *IL-5 injections*

Mice were given injections of recombinant mouse IL-5 (R&D Systems, 405-ML-025) following previously described methods. In brief, IV injections of 0.1 μg IL-5 in 100 μL sterile 1 × PBS were given 24 h apart ten days following the first saline injection (Lenert et al., 2023, Merriwether et al., 2021). Injections of IL-5 were given between 5AM and 9AM. Mice were randomly assigned to receive IL-5 or sterile PBS (vehicle) by an independent experimenter.

### *IL-5 ELISA*

Plasma IL-5 levels were measured using the BioLegend ELISA MAX Deluxe Set Mouse IL-5 (BioLegend, 431204) according to manufacturer's instructions. Samples were diluted 1:4. Levels of IL-5 in serum of naïve mice are typically low, ranging from non-detectable to ~ 50 pg/mL. The lower limit of detection in the assay used here is 4 pg/mL; thus, there is most likely little to no IL-5 in those samples. Data was acquired using the BioTek Synergy HTX Multimode Plate Reader with Gen5 software. All standards and samples were run in duplicate by experimenters blinded to condition.

**Table 1**
Antibodies used.

| Antibody | Company | Catalog Number | Working Dilution | RRID |
|---|---|---|---|---|
| Anti-CD16/32 (purified) | eBioscience | 16,016,185 | 1:2000 | AB_468899 |
| Anti-CD19 APC conjugate | Tonbo | 20–0193 | 1:200 | AB_2621562 |
| Anti-B220/CD45r eFluor 450 conjugate | eBioscience | 48–0452-82 | 1:200 | AB_1548761 |
| Anti-CD38 PE conjugate | eBioscience | 12–0381-82 | 1:200 | AB_465644 |
| Anti-CD27 Alexa Fluor 700 conjugate | eBioscience | 56–0271-82 | 1:200 | AB_2815228 |
| Anti-MHC-II FITC conjugate | eBioscience | 11–5322-82 | 1:200 | AB_465235 |

### *Immunoglobulin isotyping*

Plasma immunoglobulin levels were measured using the LegendPlex Mouse Immunoglobulin Isotyping Panel (6-plex) with V-bottom plate (BioLegend, Cat #740493) following manufacturer instructions. Plasma samples were isolated using Ficoll density centrifugation and stored immediately at −80 °C. Samples were diluted 1:100000 per the assay protocol. Data acquisition was performed using a BD LSR Fortessa. Instrument setup was performed following manufacturer instructions using the Setup Beads provided in the assay kit for appropriate compensation and gating. Data was analyzed using BioLegend's LegendPlex Data Analysis Software following the assay instructions. Concentrations of each immunoglobulin were determined using the standard curve generated for each analyte. All standards and samples were run in duplicate by experimenters blinded to condition.

### *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. Immunoglobulin isotyping data and plasma IL-5 data were analyzed using Ordinary Two-Way ANOVA with Sidak's post hoc. Simple linear regression was used to determine correlations between plasma IL-5 data and von Frey response data. Outlier analysis was performed using the ROUT method with Q = 1 %. Statistical significance for all tests was set at $p < 0.05$.

## Results (624 words)

### *Gastrocnemius injections of acidic saline alter circulating B-cell and plasma cell phenotypes*

Mice were given intramuscular injections of low pH (4.0) or physiological pH (7.4) saline on days 0 and 5 of the experimental timeline (Fig. 1A). We observed that female mice injected unilaterally with pH4.0 saline showed maximal increased mechanical hypersensitivity starting five days after the second saline injection as demonstrated previously (Lenert et al., 2022, Lenert et al., 2023, Merriwether et al., 2021); however, we did not observe any thermal sensitivity to cold or heat (data not shown).

Within PBMCs, we did not observe any differences in total percentage of B-cells ($CD19^{+}B220^{+}$) (Fig. 1C), activated B-cells (Fig. 1E), or memory B-cells (Fig. 1F). There was a significant increase in MHC-II-expressing B-cells in pH4.0-injected female mice compared to controls (Fig. 1D, $t(9) = 2.742$, $p = 0.0228$). We did not observe any differences in the total number of plasma cells ($CD19^{+}B220$-) (Fig. 1G), nor in expression of MHC-II (Fig. 1H) and CD27 (Fig. 1J) in plasma cell populations; however, there was a significant increase in $CD38^{+}$ plasma cells in female mice with CWP (pH4.0) compared to controls (pH7.4) (Fig. 1I, $t(8) = 3.818$, $p = 0.0051$).

### *Gastrocnemius injections of acidic saline do not alter lymphoid B-cell and plasma cell phenotypes*

B-cells migrate to lymph nodes after binding an antigen to present it to T-helper cells and begin the T-cell dependent B-cell activation process. After activation, B-cells undergo marked proliferation and differentiation into plasma cells and memory B-cells in specialized lymph nodes structures called germinal centers. Within popliteal lymph nodes, we did not observe any differences in the total percentage of B-cells (Fig. 2C), MHC-II-expressing B-cells (Fig. 2D) or activated B-cells (Fig. 2E). We did observe a trending increase in the percentage of memory B-cells in pH4.0-injected mice compared to controls, suggesting that the generation of memory B-cells within the germinal center may be

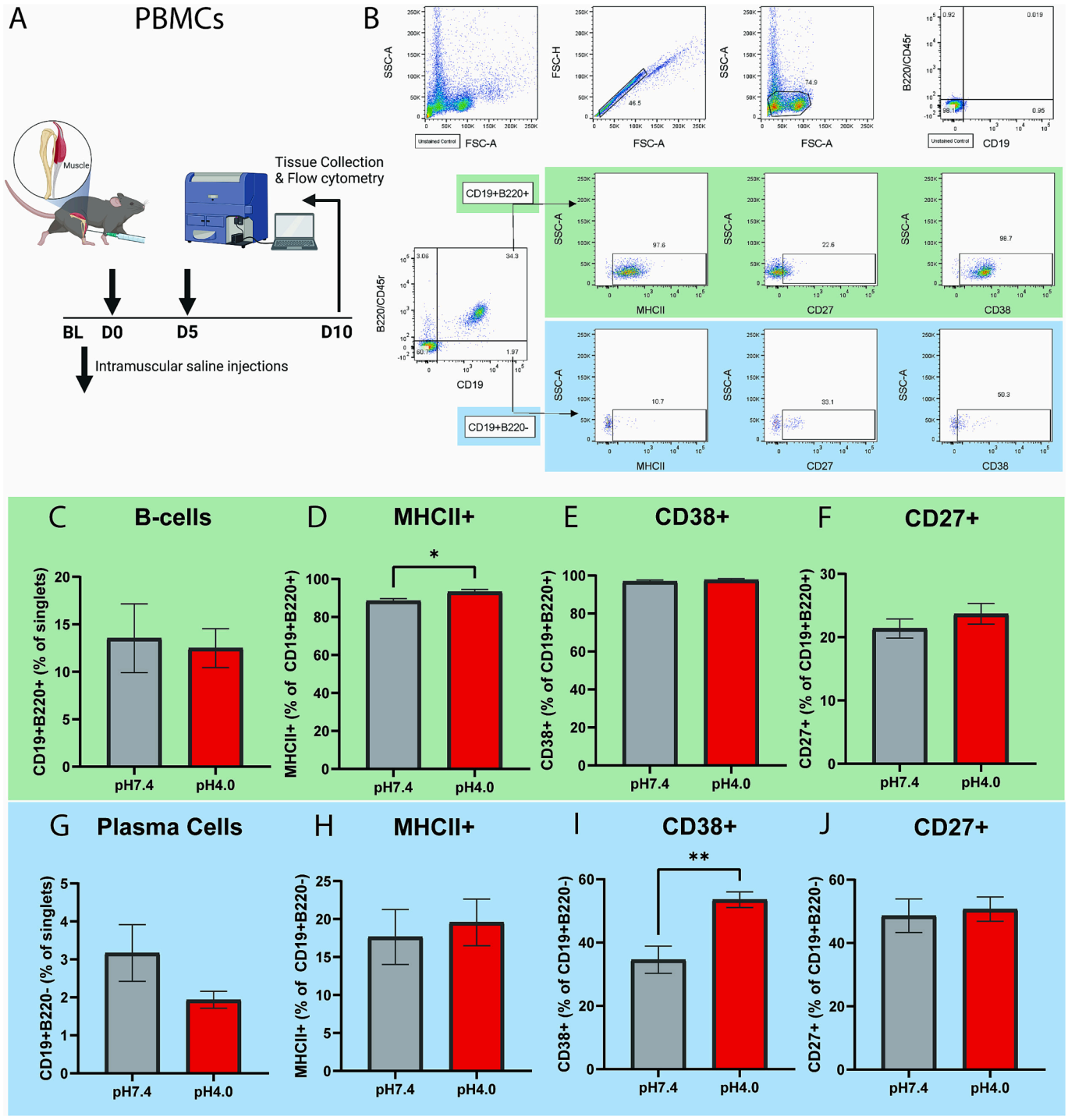



**Fig. 1.** B-cell and plasma cell phenotypes in circulation from mice with CWP. (A) Flow cytometry was used to assess B-cell and plasma cell populations of PBMCs in female mice after intramuscular injections of pH4.0 or pH7.4 saline. (B) Gating strategy to isolate B-cells ($CD19^+B220^+$) and plasma cells ($CD19^+B220$-). (C) Percentage of B-cells from PBMC isolations. Percentage of MHC-$II^+$ (D), $CD38^+$ (E), and $CD27^+$ (F) B-cells. (G) Percentage of plasma cells from PBMC isolations. Percentage of MHC-$II^+$ (H), $CD38^+$ (I), and $CD27^+$ (J) plasma cells. n = 5 for pH7.4 saline and n = 7 for pH4.0 saline. *$p < 0.05$, **$p < 0.01$.

ongoing (Fig. 2F, t(10) = 1.866, $p = 0.0916$). We did not observe an increase in the percentage of plasma cells (Fig. 2G), MHC-II-expressing plasma cells (Fig. 2H), or CD27 expression (Fig. 2J); however, there was a trending increase in the percentage of $CD38^+$ plasma cells in the popliteal lymph nodes (Fig. 2I, t(10) = 1.916, $p = 0.0843$).

*Gastrocnemius injections of acidic saline increase memory B-cells in bone marrow*

Bone marrow houses both naïve B-cells during development as well as quiescent plasma cells. Upon maturation, naïve B-cells migrate out of the bone marrow and into circulation. We did not observe any differences in the total percentage of B-cells in the bone marrow (Fig. 3C), nor in the percentage of MHC-II-expressing B-cells (Fig. 3D) or activated B-cells (Fig. 3E). Interestingly, there was an increase in the percentage of

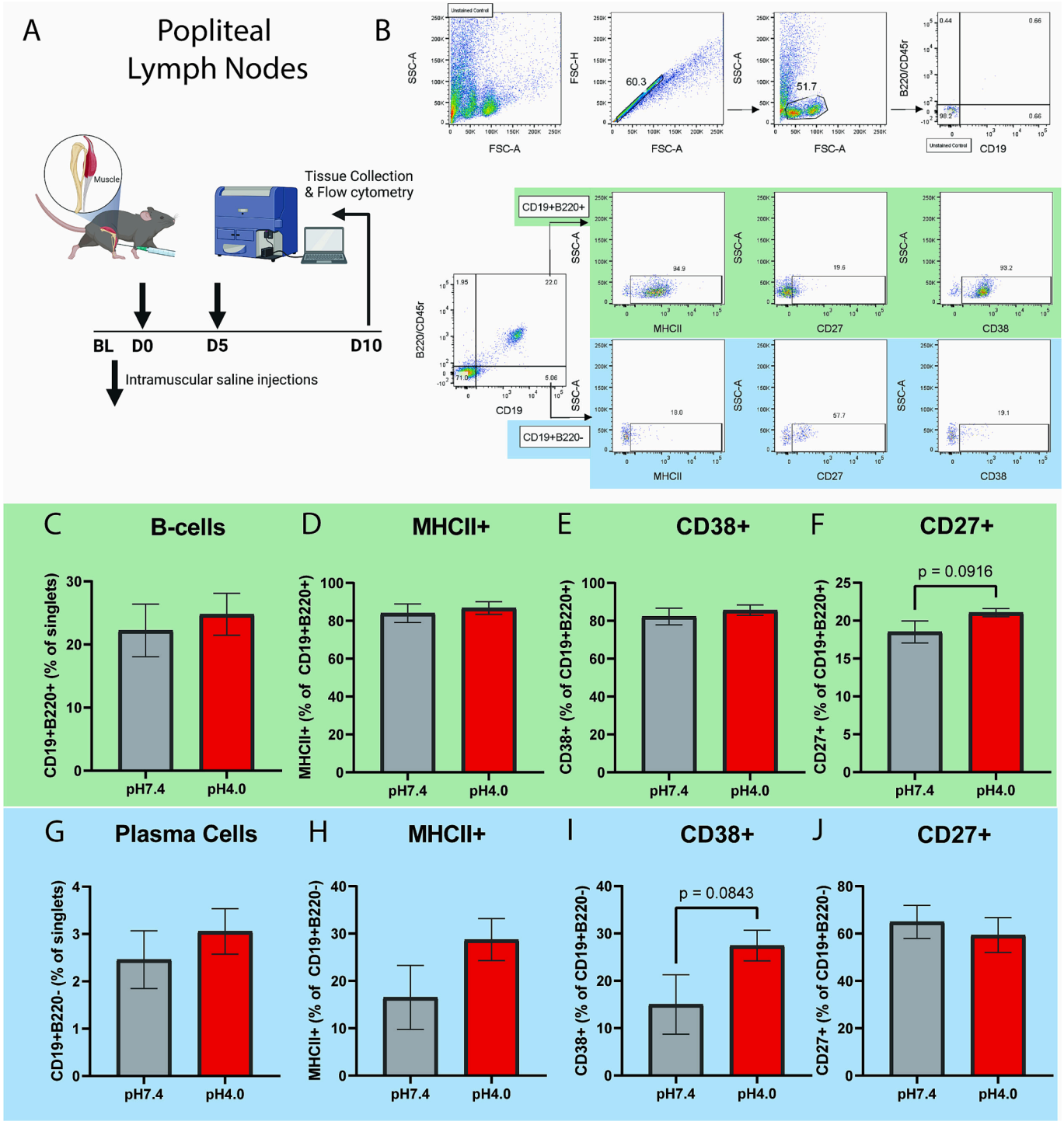



**Fig. 2.** B-cell and plasma cell phenotypes in popliteal lymph nodes (pLN) from mice with CWP. (A) Flow cytometry was used to assess B-cell and plasma cell populations in pLN in female mice after intramuscular injections of pH4.0 or pH7.4 saline. (B) Gating strategy to isolate B-cells ($CD19^+B220^+$) and plasma cells ($CD19^+B220$-). (C) Percentage of B-cells from pLN isolations. Percentage of MHC-$II^+$ (D), $CD38^+$ (E), and $CD27^+$ (F) B-cells. (G) Percentage of plasma cells from pLN isolations. Percentage of MHC-$II^+$ (H), $CD38^+$ (I), and $CD27^+$ (J) plasma cells. n = 5 for pH7.4 saline and n = 7 for pH4.0 saline.

memory B-cells in the bone marrow of mice injected with pH4.0 saline compared to controls (Fig. 3F, $t(10) = 2.706$, $p = 0.0221$). There were no differences in the total percentage of plasma cells in the bone marrow or in any of the measured subpopulations (Fig. 3G-J).

*Gastrocnemius injections of acidic saline reduce circulating IgG1 levels*

Peripheral blood was collected at two hours post IL-5 injection on Day 11 (Fig. 4A) during previously demonstrated peak of analgesia (Lenert et al., 2023, Merriwether et al., 2021). We found that there was no detectable level of IL-5 in plasma of mice that received vehicle, while IL-5 levels were significantly increased in mice that received IV IL-5 injections, independent of saline treatment (Fig. 4B, IL-5: $F(1, 22) = 9.391$, $p = 0.0057$). *Post hoc* comparisons between control (pH7.4) groups showed significantly increased IL-5 concentration in IL-5 treated mice (pH7.4, IL-5) compared to vehicle (pH7.4, vehicle) (Fig. 4B). There

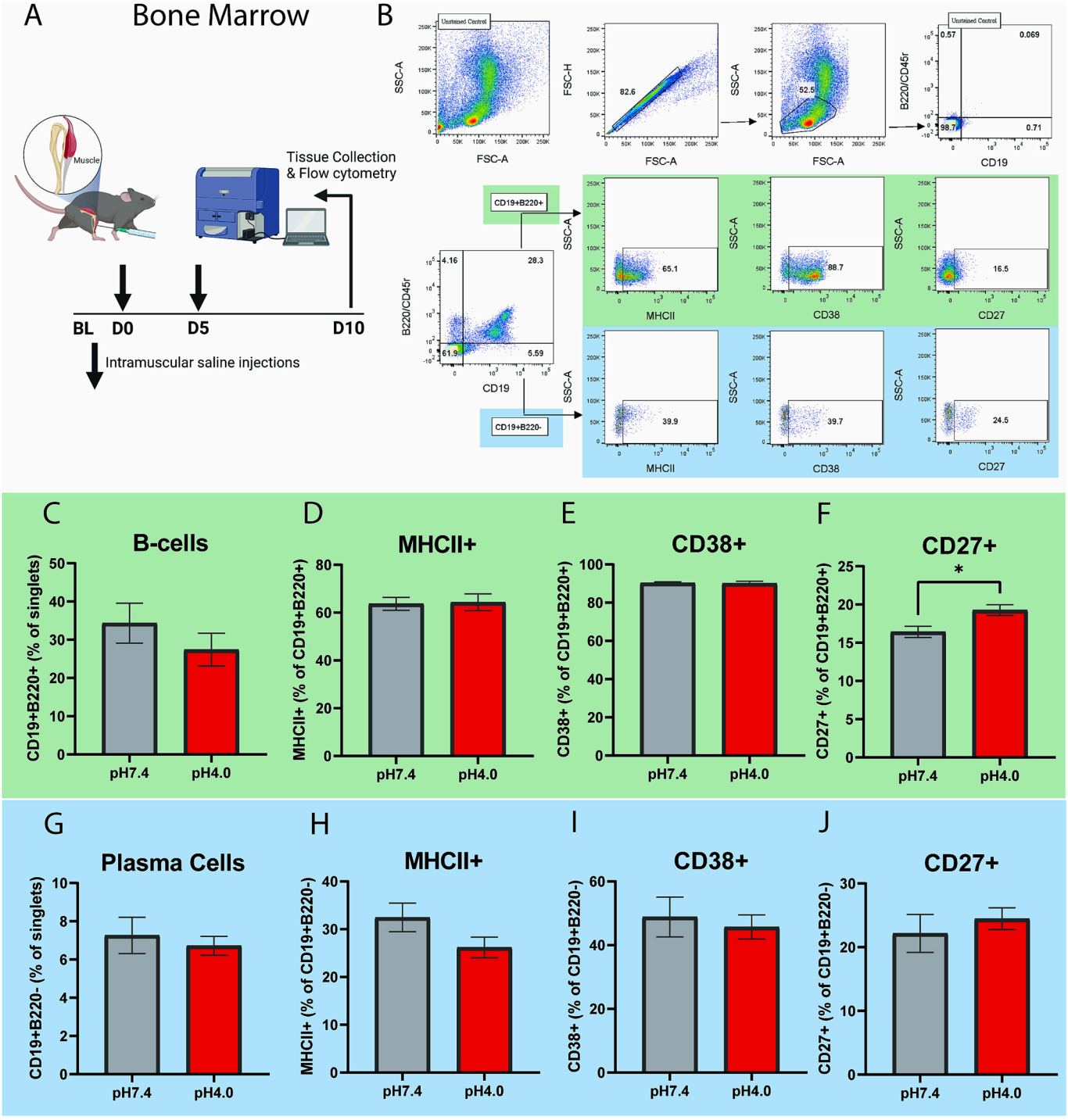
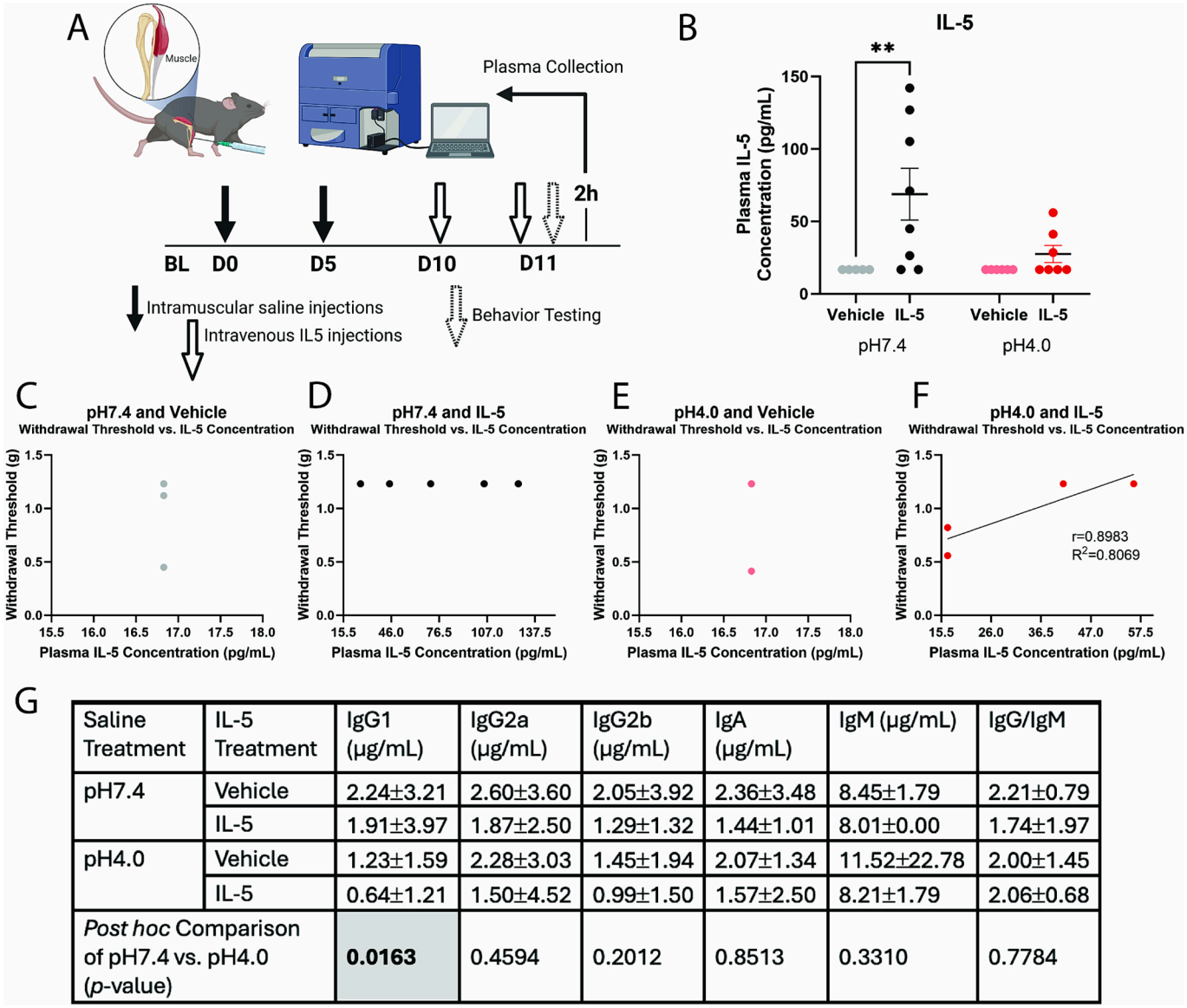



**Fig. 3.** B-cell and plasma cell phenotypes in bone marrow from mice with CWP. (A) Flow cytometry was used to assess B-cell and plasma cell populations in bone marrow in female mice after intramuscular injections of pH4.0 or pH7.4 saline. (B) Gating strategy to isolate B-cells ($CD19^+B220^+$) and plasma cells ($CD19^+B220$-). (C) Percentage of B-cells from bone marrow isolations. Percentage of MHC-$II^+$ (D), $CD38^+$ (E), and $CD27^+$ (F) B-cells. (G) Percentage of plasma cells from bone marrow isolations. Percentage of MHC-$II^+$ (H), $CD38^+$ (I), and $CD27^+$ (J) plasma cells. n = 5 for pH7.4 saline and n = 7 for pH4.0 saline. *$p < 0.05$.

were no differences observed in total IgG (IgG1 + IgG2a + IgG2b + IgG3) concentrations (Fig. 4D). Interestingly, there was a decrease in IgG1 concentration in mice with CWP (pH4.0) compared to controls (pH7.4), independent of IL-5 treatment (Fig. 4D, saline: $F(1, 22) = 6.762$, $p = 0.0163$). No *post hoc* group differences were observed. No differences were observed in IgG2a (Fig. 4D), IgG2b (Fig. 4D), IgG3 (data not shown), or IgM (Fig. 4D) concentrations, nor in total IgG: IgM ratios (Fig. 4D).

## Discussion

Adaptive immune dysfunction has been implicated in the pathogenesis of FM (Goebel et al., 2021, Merriwether et al., 2021, Banfi et al., 2020). However, the extent to which preclinical models of CWP replicate this physiological effect is not fully understood. In the current study, we found that serum IgG1 is reduced in mice receiving acidic saline compared to controls. Further, female mice injected with pH4.0 saline have increased MHC-$II^+$ B-cells and elevated $CD38^+$ plasma cells




G

| Saline Treatment | IL-5 Treatment | IgG1 (μg/mL) | IgG2a (μg/mL) | IgG2b (μg/mL) | IgA (μg/mL) | IgM (μg/mL) | IgG/IgM |
|---|---|---|---|---|---|---|---|
| pH7.4 | Vehicle | 2.24±3.21 | 2.60±3.60 | 2.05±3.92 | 2.36±3.48 | 8.45±1.79 | 2.21±0.79 |
| | IL-5 | 1.91±3.97 | 1.87±2.50 | 1.29±1.32 | 1.44±1.01 | 8.01±0.00 | 1.74±1.97 |
| pH4.0 | Vehicle | 1.23±1.59 | 2.28±3.03 | 1.45±1.94 | 2.07±1.34 | 11.52±22.78 | 2.00±1.45 |
| | IL-5 | 0.64±1.21 | 1.50±4.52 | 0.99±1.50 | 1.57±2.50 | 8.21±1.79 | 2.06±0.68 |
| *Post hoc* Comparison of pH7.4 vs. pH4.0 (*p*-value) | | **0.0163** | 0.4594 | 0.2012 | 0.8513 | 0.3310 | 0.7784 |

**Fig. 4.** Gastrocnemius injections of acidic saline reduce circulating IgG1 levels. (A) Plasma IL-5 and immunoglobulin levels were measured in female mice after intramuscular injections of pH4.0 or pH7.4 saline, with and without treatment with IL-5. (B) Plasma concentration of IL-5 (pg/mL). n = 5 for pH7.4 saline, vehicle; n = 8 for pH7.4 saline, IL-5; n = 7 for pH4.0, vehicle; n = 8 for pH4.0, IL-5. (C) Correlation of plasma concentration of IL-5 (pg/mL) and von Frey responses (g). n = 5 for pH7.4 saline, vehicle; n = 8 for pH7.4 saline, IL-5; n = 7 for pH4.0, vehicle; n = 8 for pH4.0, IL-5. (D) Concentrations of total IgG (μg/mL, IgG1 $^{+}$ IgG2a $^{+}$ IgG2b $^{+}$ IgG3), IgG1, IgG2a, IgG2b, IgA, IgM, and ratio of total IgG to IgM. n = 7 for pH7.4 saline, vehicle; n = 7 for pH7.4 saline, IL-5; n = 7 for pH4.0 saline, vehicle; n = 9 for pH4.0 saline, IL-5. *$p < 0.05$, **$p < 0.01$.

in circulation, suggesting that B-cell activation is a relevant feature of the acidic saline rodent model.

As a repeated insult model, the acidic saline paradigm may model secondary immune responses in which the secondary response is much stronger and sustained than the initial response. As the secondary immune response is mediated by the adaptive immune system, we chose to measure changes in B-cell and plasma cell populations during the peak of pain behaviors in pH4.0-injected female mice. Our chosen timepoint, five days after the second injection, should therefore reflect a robust secondary immune response (Hodgkin et al., 1996, Hoebe et al., 2004; Natoli and Ostuni, 2019; Rajewsky, 1996). We observed increases in MHC-II expression in circulating B-cells, an important component of T-cell mediated B-cell activation. Communication between T-helper cells and MHC-II$^{+}$ B-cells typically occurs in secondary lymphoid organs rather than in circulation, suggesting that activation of B-cells is still ongoing at this point. We did not observe any differences in this population within draining lymph nodes. These data suggest that at this timepoint B-cells in circulation are actively encountering antigen and making their way towards the lymph nodes for antigen presentation to T-cells (Hodgkin et al., 1996; Katikaneni and Jin, 2019). Most likely, an increase in MHC-II$^{+}$ B-cells within the draining lymph nodes would be observed at a later timepoint.

We observed a significantly increased percentage of CD38$^{+}$ plasma cells in circulation (but not in lymph nodes or bone marrow) in pH4.0 injected mice compared to controls. CD38 upregulation is associated with inflammatory states (Piedra-Quintero et al., 2020). For example, activated plasma cells upregulate CD38 expression, drive antibody production, and increase cytokine production in T-cells (García-Rodríguez et al., 2018; Brynjolfsson et al., 2018; Viegas et al., 2011). As plasma cells can produce vast quantities of antibodies, even a slight increase in the percentage of activated and/or dysfunctional plasma cells can have profound biological effects (Nutt et al., 2015). An increase

in CD38$^{+}$ plasma cells in circulation after two exposures of intramuscular pH4.0 injections mirrors our previous findings that the acidic saline model causes reductions in circulating Tregs, further supporting the systemic inflammatory effects of this model (Lenert et al., 2022).

Autoantibodies have recently been shown to play a role in chronic pain pathology (Lacagnina et al., 2021; Linher-Melville et al., 2020; Wang et al., 2019; Bersellini Farinotti et al., 2019). The current study shows that B-cell and plasma cell activity increased in pH4.0-injected mice compared to controls; however, IgG1 levels were decreased in these mice with no changes in other immunoglobulins. Our assessment of immunoglobulin levels did not account for self-reactive antibodies or autoantibodies, so it is unclear whether this model causes an increase in autoantibodies. Peripheral sensory neurons express Fc receptors (Fcγ and Fcε) that respond to circulating antigens and immunoglobulin complexes, causing subsequent sensitization (van der Kleij et al., 2010, Liang et al., 2019, Andoh and Kuraishi, 2004, Bersellini Farinotti et al., 2019). Thus, an increase in Fc receptor expression and autoantibody binding on peripheral sensory neurons may explain the decrease in circulating IgG1, however we did not directly assess this in this study. Though patients with FM (with and without comorbid autoimmune disorders) have been reported to have increased self-reactive immunoglobulin levels, this phenotype seems to occur in patients with more severe widespread pain symptomology (Krock et al., 2023, Fanton et al., 2023). Although adoptive transfer of immunoglobulins from patients with FM to naïve mice may induce chronic muscle pain (Goebel et al., 2021), this model has not been consistently replicated (Caxaria et al., 2023). This may be because these types of studies often utilize isolated immunoglobulins from a very small patient pool (Jurczak et al., 2023, Jurczak et al., 2022). Also, the study conducted by Caxaria and colleagues did not purify and inject all IgG subclasses as seen in the study by Goebel and colleagues. Thus, there is still a need for the development of consistent and reliable measurement of immunoglobulin phenotypes in existing preclinical models to further understand the multifaceted roles immunoglobulins play in the development, maintenance, and resolution of chronic pain.

Our previous studies have implicated IL-5 as an important target for pain and inflammation in both women with FM and in the preclinical acidic saline model of CWP. We therefore wanted to examine whether the administration of IL-5 and subsequent analgesia was associated with changes in immunoglobulin production. In our study, we utilized two injections of IL-5 24 h apart and tested immunoglobulin levels two hours after the second injection of IL-5. IL-5 treatment caused only slight reductions in IgG2b and IgA levels. These findings were unexpected as IL-5 is known to promote production of IgG1, IgA, and IgE by plasma cells (Takatsu et al., 2009). The lack of observed changes in immunoglobulin levels after the second IL-5 treatment may be due to collecting plasma at too early of a timepoint (Nutt et al., 2015, Hodgkin et al., 1996). Additionally, changes in IL-5 receptor may occur during CWP. IL-5R is typically only located on eosinophils and B-cells (Shearer et al., 2003), but an increase in IL-5R expression on nociceptors and/or monocytes could explain the analgesic and anti-inflammatory effects of IL-5 we observed in mice with CWP, but not controls (Lenert et al., 2023, Merriwether et al., 2021). Understanding changes in IL-5R expression in mice with CWP is essential for further validation of IL-5 as a viable target for treatment of chronic muscle pain.

## Conclusion

In conclusion, dysregulation of the adaptive immune system may be key to the chronic, widespread nature of persistent pain signaling in this model. The current study identified increased levels of activated CD38$^{+}$ plasma cells in circulation and evidence of B-cells undergoing T-cell dependent activation. We believe this improves the translational validity of the acidic saline model, as we have observed similar adaptive immune dysfunction seen in patients. As more is uncovered about neuroimmune interactions in patients with FM, it is imperative that preclinical models are tested and validated for similar interactions. Continued translational work between patient phenotypes and preclinical models will ultimately lead to improved preclinical-to-clinical translation of newly discovered therapeutic targets.

## Author contributions

MEL and MDB conceptualized the study and designed the experiments. MEL acquired and analyzed data, drafted, and edited the manuscript, drafted the figures, and performed formal analysis/data interpretation. ARG acquired data and edited the manuscript and figures. ENM participated in study interpretation, conception, and manuscript preparation. MDB participated and supervised in all aspects of the study from conception, design, acquisition, interpretation, and manuscript preparation. All authors reviewed and approved the final manuscript.

## CRediT authorship contribution statement

**Melissa E. Lenert:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Audrey R. Green:** Writing – review & editing, Visualization, Formal analysis, Data curation. **Ericka N. Merriwether:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Michael D. Burton:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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## Data Availability Statement

This study included no data deposited in external repositories. The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Data availability

Data will be made available on request.

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