M1 Macrophage Infiltrations Exacerbate Muscle and Bone Atrophy after Peripheral Nerve Injury

CURRENT STATUS: ACCEPTED

BMC Musculoskeletal Disorders

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DOI: 10.21203/rs.2.16511/v1

SUBJECT AREAS
Orthopedics

KEYWORDS
M1 macrophage, peripheral nerve injury, muscle atrophy, bone atrophy
Abstract

Background: Peripheral nerve injury causes limb muscle/bone atrophy, leading to chronic pain. We hypothesized that M1 macrophages, which focused on neurogenic inflammation, are important in muscle/bone atrophy after nerve injury. We investigated M1 macrophages’ influence on such atrophy after nerve injury in mice to prevent muscle/bone atrophy by suppressing M1 macrophages.

Methods: Hind limb muscle weight and total bone density were measured in chronic constriction injury (CCI) model mice. Immunohistochemistry and intravital microscopy were performed to visualize the hind limb muscle and bone, and the cells were quantitated using flow cytometry. We compared M1 macrophages’ infiltration and muscle/bone atrophy in macrophage depletion and untreated groups, and investigated these changes using administration models of anti-inflammatory drugs and drugs for neuropathic pain.

Results: Muscle weight was significantly lower 1 week after CCI compared to control groups, and total bone density significantly decreased in 5 weeks. Osteoclasts were significantly higher 1 week after CCI, compared to the control groups. M1 macrophage muscle infiltration was observed from 2 h, via intravital microscopy, and 1 week after CCI, and was significantly higher 1 week after CCI compared to the control groups. In the macrophage depletion, dexamethasone, pregabalin, and loxoprofen administration groups, M1 macrophage infiltration into muscle and bone was significantly lower, and muscle weight and bone density were significantly higher than the untreated group.

Conclusions: M1 macrophage infiltration exacerbates muscle/bone atrophy after peripheral nerve injury. By suppressing M1 macrophages at the neural injury local site, muscle/bone atrophy could be avoided.
Background

Peripheral nerve injury causes limb muscle/bone atrophy [1], which worsens patients’ functional prognosis and leads to chronic pain [2-4]. Such atrophy results from pain behavior and immobilization [2,3]. However, some studies have reported that muscle/bone atrophy after neural injury is difficult to explain only based on immobilization due to pain [5,6]; many muscle/bone atrophy cases are resistant to rehabilitation [7,8]. Pathologic significance of muscle/bone atrophy after peripheral nerve injury is unknown.

Local inflammation, associated with peripheral neuropathy (called neurogenic inflammation), exacerbates pain and involves chronic pain [9-11]. In neurogenic inflammation, after nerve injury, macrophage, neutrophil, and lymphocyte accumulation in the peripheral nervous system contributes to peripheral sensitization, which is mediated by several cytokines, such as tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), chemokine (C-C motif) ligand2 (CCL2), and C-C chemokine receptor type2 (CCR2). M1 macrophages’ importance in neurogenic inflammation and their involvement in pain amplification have been reported [12-17].

We hypothesized that M1 macrophages are important for muscle/bone atrophy after nerve injury, such as pain with neurogenic inflammation. Muscle/bone atrophy after nerve injury can be avoided by suppressing macrophages. We clarified M1 macrophages’ influence on muscle/bone atrophy after nerve injury in mice. For this purpose, we evaluated the degree of muscle/bone atrophy in neuropathic pain model mice and examined local inflammatory cell and cytokine changes focusing on M1 macrophages. We investigated whether M1 macrophage depletion by clodronate liposomes avoids muscle/bone atrophy and examined the effects of anti-inflammatory drugs and drugs used for neuropathic pain.

Methods

Study aim, design, and setting
Mice

This study adheres to the applicable Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines. The experiments were approved by the Institutional Animal Experiment Committee of Jichi Medical University (17217-01). Tables 1 and 2 show the groups (total, 34) and numbers of mice used in this study and the treatment provided to each group. Figure 1 shows the experiment time course. Six- to seven-week-old male wild-type C57BL/6J, C57BL/6-Tg (CAG-EGFP; SLC, Hamamatsu, Japan), and LysM Cre tandem TOMATO (CLEA, Tokyo, Japan) mice (19–23 g) were maintained in individual cages with a 12-h light/dark cycle at constant temperature and provided with water and food ad libitum. No mice had any abnormal health condition before the experiments. Invasive procedures were performed under general anesthesia.

Table 1. Group allocation and testing for C57BL/6J mice

| C57BL/6J Mice          | Control | CCI Surgery |
|------------------------|---------|-------------|
|                        | No Tx   | Clod | Dex | Preg | Loxo | Neuro | Amitryp |
| Muscle weight          | 8       | 8    | 8   | 8    | 8    |   -   |   -     |
| Total bone density     | 8       | 8    | 8   | 8    | 8    |   -   |   -     |
| Immunohistochemistry   | 8       | 8    | 8   | 8    | 8    | 8    | 8      |
| Flow cytometry         | 8       | 8    | 8   | 8    | 8    | 8    | 8      |
| RT-PCR                 | 8       | 8    |   - |   -  |   -  |   -   |   -     |

A total of 30 × 8 = 240 mice were tested. Control, surgery without chronic constriction injury (CCI); No Tx, no treatment; Clod, Clodronate liposome; Dex, Dexamethasone; Preg, Pregabalin; Loxo, Loxoprofen; Neuro, Neurotropin; Amitryp, Amitriptyline.

Table 2. Intravital microscopy group allocation and testing

| Test/Drug                | Control | CCI-No Tx |
|--------------------------|---------|-----------|
| C57BL/6-Tg (CAG-EGFP)    | 8       | 8         |
| LysMCre tandem TOMATO    | 8       | 8         |

A total of 4 × 8 = 32 mice were tested. The C57BL/6-Tg (CAG-EGFP) mice were tested immediately after CCI (chronic constriction injury), whereas the LysM Cre tandem TOMATO were tested one week after CCI. No Tx = no treatment.

Animal Models

Chronic constriction injury (CCI) was performed as described previously [18]. The
mice were anesthetized with 1.5% isoflurane; the right common sciatic nerve was exposed by blunt dissection through the biceps femoris muscle. Four ligatures (5-0 Monocryl monofilament), 1 mm apart, were tied loosely around the nerve. The muscle and skin layers were closed using 5-0 silk thread. In control mice, the right sciatic nerve was exposed, but not ligated.

To investigate macrophages’ influence on muscle/bone atrophy after CCI, they were depleted by a single injection of clodronate liposomes (Xigieia Bioscience, Tokyo, Japan) into the tail veins (100 μg/mouse) immediately after CCI.

We also tried to prevent muscle/bone atrophy due to CCI using anti-inflammatory and neuropathic pain drugs. Immediately after CCI, dexamethasone (2 mg/kg subcutaneously; Fuji Pharma Co., Ltd., Toyama, Japan), pregabalin (30 mg/kg oral; Pfizer Japan, Inc., Tokyo, Japan), loxoprofen (3 mg/kg oral; Daiichi Sankyo, Ltd., Tokyo, Japan), neurotropin (60 NU/kg intraperitoneal; Nippon Zoki Pharmaceutical Co., Ltd., Osaka, Japan), and amitriptyline (10 mg/kg oral; Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan) were administrated/day for 1 week. Mice were randomly assigned to each group.

**Muscle Weight Measurement**

To evaluate muscle atrophy, 1 week after CCI, C57BL/6J mice were sacrificed via cervical dislocation. The right biceps femoris and gastrocnemius muscles were collected; muscle weights were measured.

**Total Bone Density**

To evaluate bone atrophy, total hind limb bone density was measured by computed tomography (CT) in C57BL/6J mice anesthetized with 1.5% isoflurane (Latheta Laboratory CT; Hitachi, Tokyo, Japan). CCI was performed; CT of the hind limb were obtained/week for 5 weeks. Images were analyzed using the CT scanner software. Total bone density of the femur and tibia were measured with 0.5-mm slices; their average values were compared.

**Immunohistochemistry**

Inflammation cells and osteoclasts in the femoral bone were analyzed by immunohistochemistry. One week after CCI, C57BL/6J mice were sacrificed via cervical dislocation; the right femurs were collected. Pathologic specimen preparation and immunostaining [tartrate-resistant acid phosphatase (TRAP) staining] were outsourced (Kyodo Byori, Kobe, Japan). Images were captured on a FSX100 microscope (Olympus, Tokyo, Japan) using a ×4 (N.A. 0.1) objective and analyzed with Fiji win64 software (Rasband, W.S., ImageJ; National Institutes of Health, Bethesda, MD, USA). With a 1.5 × 2 mm view, osteoclasts were separated in a single color according to difference in staining; the osteoclast numbers were compared.
Intravital Microscopy

To visualize inflammation cell dynamics in the nerve and surrounding tissues, we used in vivo multiphoton microscopy [19]. One week after CCI, LysM Cre tandem TOMATO mice were anesthetized by urethane injection (1.5 g/kg); the hind limb skin was removed. The hind limb tissue was visualized with an inverted microscope (Eclipse Ti; Nikon). Fluorescein isothiocyanate dextran (5 mg/mouse; Merck KGaA, Darmstadt, Germany) and Hoechst 33342 (3 mg/mouse; Thermo Fisher Scientific, Waltham, MA, USA) were injected into the tail vein to visualize cell dynamics and blood flow. The tissues were excited at 920 nm, using a Ti:sapphire laser (Vision II; Coherent, Inc., Santa Clara, CA, USA), and images were captured as XY images using an A1R-MP system (Nikon). A ×40 (N.A. 1.15) water immersion objective lens (Nikon) was used. The dwelling time for one pixel was 0.1 µs. To observe the inflammatory response in the acute phase, CAG-EGFP mice were anesthetized immediately after CCI; Rhodamine B dextran (5 mg/mouse, Thermo Fisher Scientific), Hoechst 33342 (3 mg/mouse), and F4/80 Alx647 (25 µg/mouse; BioLegend, San Diego, CA, USA) were injected into the tail veins. We continuously observed the sciatic nerve and surrounding tissue for 3 h. Collected data were analyzed via an automatic algorithm in NIS-Elements and Fiji win64 software. With a 500 × 500 µm view, macrophages were separated in a single color based on the difference in fluorescence color, and their numbers were compared.

Flow Cytometry

Cell infiltrations in muscles were analyzed by flow cytometry. One week after CCI, C57BL/6J mice were sacrificed via cervical dislocation; right biceps femoris and gastrocnemius muscles were collected, minced, and treated with collagenase. Cells were isolated, washed twice with phosphate-buffered saline, incubated for 8.5 min in erythrocyte-lysing buffer, and finally suspended in phosphate-buffered saline. Isolated cells were then incubated with FcBlock antibody (BD Biosciences, Bedford, MA, USA) for 15 min on ice, labeled with dye-conjugated antibodies (CD45-FITC, 25 µg/mL; Ly6G-BV605, 10 µg/mL; F4/80-PE, 10 µg/mL; CD11b-BV711, 2.5 µg/mL; CD301-Alx647, 2.5 µg/mL; Ly6C-BV421, 2.5 µg/mL; BioLegend), and analyzed by flow cytometry using BD LSR Fortessa (BD Biosciences) and FlowJo V10 (Tomy Digital Biology, Tokyo, Japan) software. DRAQ7 (BioLegend) was used to exclude dead cells.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To investigate cytokine changes in the muscles after CCI, RT-PCR was performed. One week after CCI, C57BL/6J mice were sacrificed, the right biceps femoris and gastrocnemius muscles were collected; RNA was extracted from the muscle tissues using
Trizol (Thermo Fisher Scientific). cDNA was synthesized using primescript reverse transcriptase (Takara Bio, Kusatsu, Japan). Taqman probes of TNFα, IL1-β, CCL2, and CCR2 (Thermo Fisher Scientific) were used in RT-PCR, performed with 10 ng cDNA with a Vii A real-time PCR system (Thermo Fisher Scientific). Processing of raw data and normalizing the relative quantities were performed using the ΔΔ-Ct-method. mRNA expression levels are expressed relative to the control group.

Statistics
Statistical analysis was performed using Graphpad Prism7 software (GraphPad Software, Inc., San Diego, CA, USA). All data were of equal variance in the F test; however, in the D'Agostino and Pearson normality test, except RT-PCR data, all data were not normally distributed. Taking into account that $n = 8$ is the least number of samples detectable by the D'Agostino and Pearson test, a nonparametric test was chosen, except for RT-PCR. The Mann–Whitney $U$ test was used to compare the groups. The Kruskal–Wallis test was used to compare ≥3 groups. The Friedman test was used for repeated measurements of the same sample. The Dunn's multiple comparison test was used for post hoc tests. The results are expressed as median and interquartile range. Since RT-PCR data were normally distributed, Student’s $t$-test was chosen as the parametric test. Results were expressed as means ± SD. $P < 0.05$ was considered significant.

Sample sizes were calculated using GPower 3.1 software (Heinrich-Heine-University, Düsseldorf, Germany) for primary outcomes [muscle weight (control vs. CCI groups, 1 week later) and total bone density (CCI group, 0–5 weeks)] based on the pilot studies of five mice. To detect a 50 mg decrease in muscle based on a standard deviation (SD) of 30 mg, $n = 8$ in each group was considered appropriate ($\alpha = 0.05; 1 – \beta = 0.8$). To detect a 60 mg/cm$^3$ decrease in total bone density based on a SD of 50 mg/cm$^3$, $n = 8$ in each group was considered appropriate ($\alpha = 0.05; 1 – \beta = 0.8$). Therefore, $n = 8$ was chosen for each group (total groups = 34, total $n = 272$).

Results

Muscle/Bone Atrophy Occurs in an Early Phase after CCI
Recently, in neurogenic inflammation after nerve injury, M1 macrophage infiltrations to peripheral nerves have been reportedly important in pain amplification [9–17]. We investigated M1 macrophages’ influence on muscle/bone atrophy after nerve injury of mice. The results of numeric values in this study are shown in Additional file 1.
One week after CCI in C57BL/6J mice, biceps femoris and gastrocnemius muscle weights were significantly lower in the CCI group than in the control group ($P = 0.001$ and $P < 0.001$; Fig. 2A).

Total hind limb bone density was measured by CT/week for 5 weeks (Fig. 2B). Total bone density decreased significantly in the femur and tibia at 5 weeks ($P = 0.005$ and $P < 0.001$). On multiple comparisons with 0 weeks, total femoral bone density decreased significantly at 2 and 3 weeks ($P = 0.037$ and $P = 0.035$, respectively); total tibial bone density decreased significantly at 2, 3, and 5 weeks ($P = 0.025$, $P = 0.016$, and $P < 0.001$, respectively). The control group showed no significant difference in total femoral and tibial bone densities at 5 weeks ($P = 0.808$ and $P = 0.480$, respectively).

**M1 Macrophages Infiltrate into Muscles and Bones after CCI**

We investigated the molecular mechanism of muscle/bone atrophy after CCI. We focused on neurogenic inflammation involvement, especially M1 macrophages, which has recently attracted attention because of neuropathic pain [12–17].

Inflammation cells and osteoclasts in the femur were analyzed by immunohistochemistry. One week after CCI in C57BL/6J mice, TRAP staining of the right femur revealed that osteoclast numbers (Fig. 2C) were significantly higher in the CCI group than the control group ($P = 0.043$).

The nerve/muscle cell dynamics of LysM Cre tandem TOMATO mice were observed with intravital microscopy 1 week after CCI. Infiltration of lysosome-producing cells around the sciatic nerves was observed in the CCI group (Fig. 2Da); such infiltration was mild in the control group (Fig. 2Db). To observe inflammatory responses in the acute phase, hind limbs of CAG-EGFP mice were observed immediately after CCI. In the CCI group, macrophage infiltration from the blood vessels to the muscles was observed from 2 h (Fig. 2Dc); this was not observed in the control group (Fig. 2Dd).
The M1 macrophage infiltration in the hind limbs observed during intravital microscopy was quantitated by flow cytometry 1 week after CCI (Fig. 2E). In the biceps femoris and gastrocnemius muscles, M1 macrophages (Ly6C+/F4/80+, CD301−/CD11b+) were significantly greater in the CCI group than in the control groups ($P < 0.001$ and $P = 0.049$, respectively).

**TNFα, IL-1β, CCL2, and CCR2 are involved in M1 Macrophage Infiltration into Muscles and Bones after CCI**

In neuropathic pain, inflammatory cytokines are involved in neurogenic inflammation [9–11]. We thought that inflammation cytokines were also involved in M1 macrophage infiltration and muscle/bone atrophy after CCI. To investigate cytokine changes in biceps femoris and gastrocnemius muscles after CCI, RT-PCR was performed 1 week after CCI in C57BL/6J mice. mRNA expression levels were expressed relative to the control group (Fig. 2F). In the biceps femoris and gastrocnemius muscles, mRNA relative expression was significantly higher in the CCI group than in the control group for TNFα, IL-1β, CCL2, and CCR2 ($P < 0.001$ for all).

**M1 Macrophage Infiltrations into Muscles and Bones are Suppressed by Clodronate Liposomes**

The second aim of this study was to clarify whether muscle/bone atrophy can be avoided by suppressing M1 macrophages. We depleted the M1 macrophages using clodronate liposomes after CCI.

One week after CCI, compared to the untreated group, the macrophage depletion group had significantly lower levels of osteoclasts on immunohistochemistry study ($P=0.049$; Fig. 3A) and of M1 macrophages on flow cytometry in the biceps femoris and gastrocnemius muscles ($P < 0.001$ and $P < 0.001$; Fig. 3B).

**M1 Macrophage Depletion Suppressed Muscle/Bone Atrophy after CCI**
Muscle weight and total bone density changes were compared in the two groups to investigate their effects on muscle/bone atrophy.

In the macrophage depletion group, the biceps femoris and gastrocnemius muscle weights were significantly higher 1 week after CCI compared to the untreated group ($P < 0.001$ and $P < 0.001$; Fig. 3C), whereas there were no significant differences in total bone density in the femur and tibia at 2 weeks ($P = 0.794$ and $P = 0.531$; Fig. 3D).

**M1 Macrophage Infiltration into Muscle/Bone after Nerve Injury was Significantly Lower in Dexamethasone, Pregabalin, and Loxoprofen Administration Groups Compared to Untreated Groups**

To clarify whether M1 macrophage infiltration and muscle/bone atrophy can be avoided by clinically usable drugs, we used dexamethasone and loxoprofen as anti-inflammatory drugs (mainly for peripheral neuropathic pain), and pregabalin, neurotropin, and amitriptyline (the latter two for central pain) as drugs for neuropathic pain.

One week after CCI, immunohistochemistry revealed that osteoclasts were significantly lower in the drug administration groups ($P = 0.003$). In multiple comparisons to the untreated group, osteoclasts were significantly lower in the dexamethasone, pregabalin, and loxoprofen groups ($P = 0.005$, $P = 0.038$, and $P = 0.042$, respectively). There were no significant differences in osteoclasts in the neurotropin and amitriptyline groups compared to the untreated group ($P > 0.99$ and $P > 0.99$; Fig. 4A).

Flow cytometry in the biceps femoris muscles 1 week after CCI showed that M1 macrophages were significantly lower in the drug administration groups ($P = 0.002$). On multiple comparisons with the untreated group, M1 macrophages were significantly lower in the dexamethasone, pregabalin, and loxoprofen groups ($P < 0.001$, $P = 0.043$, and $P = 0.034$, respectively). There were no significant differences in M1 macrophages in the neurotropin and amitriptyline groups compared to the untreated group ($P > 0.99$ and $P > 0.99$).
0.99, respectively). In the gastrocnemius muscles 1 week after CCI, M1 macrophages were significantly lower in the drug administration groups ($P = 0.033$). In multiple comparisons with the untreated group, there were no significant differences in M1 macrophages in any group (dexamethasone, $P = 0.134$; pregabalin, $P > 0.99$; loxoprofen, $P > 0.99$; neurotropin, $P > 0.99$; and amitriptyline, $P = 0.096$, Fig. 4B).

**Muscle Weight and Total Bone Density after Nerve Injury were Significantly Higher in Dexamethasone, Pregabalin, and Loxoprofen Administration Groups Compared with Untreated Groups**

Muscle weight and total bone density changes in the drug-administered groups were compared with those in the untreated group to investigate their effects on muscle/bone atrophy. These examinations were conducted only for dexamethasone, pregabalin, and loxoprofen administration groups, which had a significant difference in M1 macrophage infiltration into muscles and bones.

One week after CCI, muscle weights were significantly higher in the drug administration groups (biceps femoris, $P = 0.001$; gastrocnemius, $P = 0.002$). In multiple comparisons to the untreated group, biceps femoris muscle weights were significantly higher in the dexamethasone, pregabalin, and loxoprofen groups ($P < 0.001$, $P = 0.036$, and $P = 0.030$, respectively) and gastrocnemius muscle weights were significantly higher in the dexamethasone and pregabalin groups ($P < 0.001$ and $P = 0.046$, respectively). There were no significant differences in the loxoprofen group ($P = 0.085$; Fig. 4C).

In the femur, there were no significant differences in total bone density in the dexamethasone group at 3 weeks ($P = 0.176$). Total bone density significantly decreased in the pregabalin and loxoprofen groups (pregabalin, $P = 0.002$, multiple comparisons to 0 week, significant difference at two $[P = 0.011]$ and three $[P = 0.002]$ weeks; loxoprofen, $P = 0.001$, multiple comparisons to 0 week, significant difference at two $[P = 0.006]$ and
three \( P < 0.001 \) weeks). In the tibia, there were no significant differences in total bone density in all groups at three weeks (dexamethasone, \( P = 0.789 \); pregabalin, \( P = 0.648 \); loxoprofen, \( P = 0.092 \); Fig. 4D).

**Discussion**

Our study revealed that M1 macrophage infiltrations exacerbate muscle/bone atrophy after peripheral nerve injury. To date, muscle/bone atrophy after nerve injury was thought to result from pain behavior and immobilization [2,3]. Some studies reported that muscle/bone atrophy after nerve injury is difficult to explain by immobilization alone [5,6], but these mechanisms were unknown. We focused on M1 macrophages, which have recently attracted attention because of neuropathic pain with neurogenic inflammation [12-17]. However, those studies focused only on neuropathic pain.

In neurogenic inflammation, Wallerian degeneration of peripheral nerves by M1 macrophages invading from surrounding blood vessels was reported to cause hyperalgesia [12]. Inflammatory cytokines, such as TNF\( \alpha \), IL–1\( \beta \), CCL2, and CCR2, are involved as a mechanism by which M1 macrophages are induced in injured nerves [9–11]. These cytokines were significantly increased in the muscle tissues after CCI in our study. We also observed that M1 macrophages invaded outside the surrounding blood vessels, not in the muscles and bone tissues, on intravital microscopy. From these results, as a mechanism by which cytokine-induced M1 macrophages disrupt muscles and bone tissues, we believe that the cause is not from direct phagocytosis, but from mediators such as cytokines.

Inflammatory disorders, such as chronic obstructive pulmonary disease, rheumatoid arthritis, and inflammatory myopathies, have been reported recently to display skeletal muscle atrophy [20]. In these diseases, inflammation cytokines, such as TNF\( \alpha \), interferon-\( \gamma \), interleukin–6, and interleukin–8, increase and they are thought to lead to muscle atrophy by reducing peripheral insulin action while increasing reactive oxygen species and
ischemia [21–23]. Muscle atrophy mediated by M1 macrophages, as observed in our study, also may occur via the same mechanism. In our study, muscle weight was measured for one week, and total bone density was measured for up to five weeks. In the macrophage depletion groups, total bone density was measured up to two weeks due to exacerbation of the general condition of mice. In the drug administration groups, total bone density was measured up to three weeks; because we had clear results within two to three weeks, mice were sacrificed early to avoid prolonged pain. In the future, the change in muscle and bone atrophy after nerve injury should be examined over a longer term. Further, this study should be linked to pain studies, especially chronic pain studies. In this study, we used C57BL/6J mice. Although there is a disadvantage of low bone density and altered macrophage response, C57BL/6J mice have the advantage of high Th1 reactivity and can clarify the involvement of innate and cell-mediated immunity in macrophages. C57BL/6J mice are highly sensitive to pain, are used widely for pain studies, and are often for macrophage studies as a neuropathic pain model [16]. Therefore, this study using C57BL/6J mice was considered useful for linking to future pain studies.

In the depletion of macrophages using clodronate liposomes, mice were sacrificed within four weeks because they were weakened by infection and embolism of the removed macrophage mass. On the other hand, in the pilot study, the number of macrophages infiltrating the muscles and bones did not decrease with administration of a smaller amount of clodronate liposomes. In recent years, pain with bone metabolism abnormality after neuropathy has been reported as neuropathic bone pain, mainly in complex regional pain syndrome patients, and bisphosphonate reportedly is useful not only for bone pain, but also for neuropathic pain [24]. The dose determination of bisphosphonate necessary to prevent muscle/bone atrophy is a subject for future study. We studied dexamethasone and loxoprofen as anti-inflammatory drugs, and pregabalin, amitriptyline, and neurotropin as
drugs for neuropathic pain because they can be linked to clinical research. The reason for using drugs for neuropathic pain in addition to the anti-inflammatory drug is that pregabalin has been reported to be effective not only for neuropathic pain, but also for neurogenic inflammation [25]. In the dexamethasone, pregabalin, and loxoprofen administration groups, M1 macrophage infiltration into muscle and bone was significantly lower, and muscle weight and bone density were significantly higher, than the untreated group. These drugs might suppress muscle and bone atrophy after neuropathy via M1 macrophage suppression. Even in clinical practice, the use of steroids, nonsteroidal anti-inflammatory drugs, and antiepileptic drugs from the early stages may be effective in avoiding muscle/bone atrophy and chronic pain. From our results, steroids may be most effective, but in clinical situations, the effect of steroids on neuropathic pain is controversial in terms of effectiveness and side effects [26]. Clinical studies on the short-term use of steroids after neural injury are desired, targeting not only neuropathic pain, but also muscle/bone atrophy. On the other hand, central sensitization is reportedly involved in neurogenic inflammation and pain.[9–11]. However, contrary to our expectations, amitriptyline and neurotropin, which mainly act on the central nervous system, were not effective in suppressing M1 macrophage infiltration into the muscles and bones. Central nervous system involvement in muscle/bone atrophy was considered small since the drugs, whose analgesic pathway is mediated mainly by central nervous system, are ineffective. In the future, a study that will clear the mechanism of suppression of M1 macrophages with these drugs is desired. However, from this study alone, we cannot deny the possibility that mechanisms other than M1 macrophage suppression may be involved in muscle/atrophy suppression. Our results alone cannot be applied to clinical practice. In future clinical research, in addition to examining the avoidance of muscle/bone atrophy using medicines, muscle/bone atrophy measurement to diagnose neuropathic pain and
judge therapeutic effects are necessary. It is necessary to investigate the possibility of suppressing chronic pain by avoiding muscle/bone atrophy.

As a limitation of this study, it is undeniable that the muscle/bone atrophy following CCI might be caused by environmental factors, such as immobility due to pain. Before this study, muscle weight and bone density were observed over time in mice with hip, knee, and ankle joints fixed with ultraviolet-curing resin. Therefore, during the course of one to two weeks, muscle and bone atrophy of the fixed lower limb did not occur. The progress of muscle/bone atrophy following neuropathy in animal models has not been reported. On the other hand, in lower limb immobilization models, gastrocnemius muscle weight reportedly decreased by 22% after one week [27] and these changes are small compared to those in our study with a CCI model. In clinical practice in humans, muscle mass reduction was 0.5%/day [28] and bone loss was 3%–6%/month [6,29] after spinal injury and immobilization. It is understood that the muscle/bone atrophy in our study was due to the direct influence of neural injury.

Sufficient sample numbers could not be set for all experiments. The pilot study to determine the number of samples in this study was performed only on the main outcomes (muscle weight and total bone density). A pilot study of all experiments will require a large number of animals. In the interests of animal protection, the number of samples calculated in the main endpoint was used for other experiments. We did not adjust the analysis for multiple outcomes and for multiple comparisons of several drugs against untreated controls. Therefore, the risk for type I errors is increased, and the results should be viewed as hypothesis-generating rather than that providing conclusive evidence.

**Conclusion**

M1 macrophage infiltrations exacerbate muscle/bone atrophy after peripheral nerve injury.

By suppressing M1 macrophages at the local neural injury site, it was possible to avoid
muscle/bone atrophy.

List Of Abbreviations

CCI: chronic constriction injury

TNFα: tumor necrosis factor-α

IL-1β: interleukin-1β

CCL2: chemokine (C-C motif) ligand2

CCR2: C-C chemokine receptor type2

CT: computed tomography

TRAP: tartrate-resistant acid phosphatase

RT-PCR: reverse transcription polymerase chain reaction

SD: standard deviation

Declarations

Ethics approval and consent to participate

The experiments were approved by the Institutional Animal Experiment Committee of Jichi Medical University (17217-01).

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests
The authors declare that they have no competing interests.

Funding
This study was supported by the funding program of JMU Graduate Student Start-UP Award. We collected data on tools and drugs purchased with this fund.

Author’s contributions
NS helped design the study, conduct the study, analyze the data, and write the manuscript. AS helped design the study, conduct the study, analyze the data, and review the manuscript. TI helped design the study, and review the manuscript. MT helped design the study, and review the manuscript. SN helped design the study, conduct the study, analyze the data, and review the manuscript. All authors read and approved the final manuscript.

Acknowledgments
The authors would like to thank JKA for subsidizing equipment through its promotion funds from KEIRIN RACE.
The authors would also like to thank Enago (www.enago.jp) for the English language review.

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Figures

Figure 1

Experiment time course
M1 macrophages' influence on muscle/bone atrophy after nerve injury. We used a chronic constriction injury (CCI) mouse model as a nerve injury model. (a) Hind limb muscle weight was lower in the nerve injury group one week postoperatively compared to the control group (n = 8, * P < 0.05). Center lines of box plot show the medians, box limits indicate the 25th and 75th percentiles, and whiskers extend to minimum and maximum values. (b) Total hind limb bone density
decreased two weeks postoperatively in five weeks in the nerve injury group (n = 8, * P < 0.05). Dot plots show the medians and error bars indicate the 25th and 75th percentiles. (c) Osteoclasts (purple) were higher on immunohistochemistry of the femoral bone in the nerve injury group one week postoperatively compared to the control group (n = 8, * P < 0.05). (d) Lysosome-producing cells (*) increased one week postoperatively in the nerve injury group on intravital imaging of nerves and muscles of LysM Cre tandem TOMATO mice (d-a, control group: d-b; R: lysosome-producing cell, G: cytoplasm, and B: nucleus). In the acute phase, macrophage (**) infiltration from the blood vessels to the muscles was observed from 2 h on intravital imaging of the nerves and muscles of CAG-EGFP mice (d-c, control group: d-d; R: blood flow, G: cytoplasm, B: nucleus, and W: macrophage). (e) M1 macrophages were higher with flow cytometry of the muscles in the nerve injury group one week postoperatively compared to the control group (n = 8, *P < 0.05). (f) Inflammation cytokines were higher with reverse transcription polymerase chain reaction of the muscles in the nerve injury group one week postoperatively compared to the control group (n = 8, *P < 0.05).
Reduced M1 macrophages suppress M1 macrophages’ infiltration and muscle/bone atrophy after nerve injury. We used chronic constriction injury (CCI) model mice as nerve injury models. (a) Osteoclasts were lower on immunohistochemistry of the femoral bone in the macrophage depletion group one week postoperatively compared to the untreated group (n = 8, *P < 0.05). Center lines of box plot show the medians, box limits indicate the 25th and 75th percentiles, and whiskers extend to minimum and maximum values. (b) M1 macrophages were lower with flow cytometry of the muscles in the macrophage depletion group one week postoperatively compared to the untreated group (n = 8, *P < 0.05). (c) Hind limb muscle weight was higher in the macrophage depletion group than the untreated group (n = 8, *P < 0.05) one week postoperatively. (d) In the macrophage depletion group, there was no significance of the total bone density in the femur and tibia in two weeks (n = 8). Dot plots show the medians and error bars indicate the 25th and 75th percentiles.
Figure 4

Dexamethasone, pregabalin, and loxoprofen suppress M1 macrophages’ infiltration and muscle/bone atrophy after nerve injury. We used chronic constriction injury (CCI) model mice as nerve injury models. (a) Osteoclasts were significantly lower in the immunohistochemistry of the femoral bone in drug administration groups (n = 8, *P < 0.05). Center lines of box plot show the medians, box limits indicate the 25th and 75th percentiles, and whiskers extend to minimum and maximum values. (b) The biceps femoris muscles one week after CCI showed that the M1 macrophages were significantly lower with flow cytometry in the drug administration groups. In the gastrocnemius muscles one week after CCI, M1 macrophages were significantly lower in the drug administration groups (n = 8, *P < 0.05). (c) The weights of biceps femoris muscles one week after CCI were significantly higher in the drug administration groups. In multiple comparisons to the untreated group, muscle weights were significantly higher in
the dexamethasone, pregabalin, and loxoprofen groups. In the gastrocnemius muscles one week after CCI, muscle weights were significantly higher in the drug administration groups (n = 8, *P < 0.05). (d) In the femur, there were no significant differences in total bone density in the dexamethasone group at three weeks. The total bone density significantly decreased in the pregabalin and loxoprofen groups. In the tibia, there were no significant differences in total bone density in all groups at three weeks (n = 8, *P < 0.05). Dot plots show the medians and error bars indicate the 25th and 75th percentiles.

Supplementary Files

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