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Alterations of macrophage and neutrophil content in skeletal muscle of aged versus young mice

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Abstract

Background: Skeletal muscle inflammation and oxidative stress are associated with aging-related loss of muscle mass and may be attributable to alterations in the number and types of leukocytes in skeletal muscle. Here, we tested the hypothesis that aging changes the number and composition of leukocyte subsets in skeletal muscle tissue.

Methods: Skeletal muscle was sampled from 4-mo-old (young) and 27-mo-old (old) C57BL/6J mice. Mononuclear cells of the gastrocnemius muscle were isolated, and flow cytometry was used to characterize the number and types of immune cells.

Results: The number of neutrophils and Ly-6C+ inflammatory macrophages in the skeletal muscle was significantly higher in old mice than in young mice. Inflammation and oxidative stress (measured using the markers phosphorylated JNK and nitrotyrosine) were also higher in the skeletal muscle of old mice than in that of young mice.

Conclusions: Increasing age promotes skeletal muscle inflammation and oxidative stress, as well as infiltration of inflammatory macrophages and neutrophils.

KEYWORDS
increasing age, inflammation, loss of muscle mass, macrophage, oxidative stress

1 | INTRODUCTION

Aging induces loss of skeletal muscle mass and function in a process known as sarcopenia.[1] The ubiquitin-proteasome system is associated with muscle protein degradation due to denervation and disuse.[2] Aging has been shown to increase the expression levels of ubiquitin ligases (atrogin-1 and MuRF1) in the skeletal muscle of humans and animals.[3-5] There has been increasing evidence from various models of disease suggesting that inflammation and oxidative stress play an important role in the loss of skeletal muscle mass.[6,7] mRNA expression levels of inflammatory cytokines in skeletal muscle[8] and H_2O_2 levels in skeletal muscle[9] have been reported to be higher in old mice than in young mice. It is known that immune cells, such as macrophages and neutrophils, secrete inflammatory cytokines and H_2O_2, and that immune cells localized to skeletal muscle tissue regulate muscle inflammation.[10] We propose, therefore, that muscle-localized inflammatory macrophages and neutrophils may play an important role in the development of aging-related loss of skeletal muscle mass.

Abbreviations: CCR2, chemokine receptor 2; CD, cluster of differentiation; CX3CR1, CX3C chemokine receptor 1; DMEM, Dulbecco modified Eagle's medium; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Ig, immunoglobulin; IL, interleukin; JNK, Jun N-terminal kinase; MCP-1, monocyte chemoattractant protein-1; MuRF1, muscle ring-finger protein-1; NADPH, nicotinamide adenine dinucleotide phosphate; PE, phycoerythrin; TNF, tumor necrosis factor.

Using immunohistochemical analysis, recent studies have shown that the proportion of cells positive for macrophage markers (CD68 and F4/80) increases in skeletal muscle of elderly humans and old mice.[11-15] However, the effects of inflammatory macrophages and neutrophils infiltration with increasing age remain incompletely understood. From the analytic methods used in previous studies, it is difficult to determine the amount and subsets of immune cells present in the skeletal muscle. This is because immunohistochemical analysis is used only for a portion of skeletal muscle, and immunohistochemical analysis limits the number of fluorescently labeled antibodies that can be simultaneously stained in a single cell. Importantly, flow cytometry analysis can be used to define immune cell subsets, as well as to count the number of each immune cell subset present in the skeletal muscle by staining multiple fluorescently labeled antibodies per single isolated cell. In this study, we used flow cytometry analysis to test the hypothesis that increasing age alters the number and subset composition of immune cells in skeletal muscle tissue.

2 | METHODS

2.1 | Animals

Two-month-old male C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in a controlled environment with a 12 h light-dark cycle. The young mice (n = 5) were 4 mo of age, and the old mice (n = 5) were 27 mo of age when they were sacrificed using anesthetic isoflurane, following which blood was removed by hemoperfusion. The hindlimb skeletal muscles from both legs were carefully dissected and weighed. All experimental procedures were in accordance with the Guiding Principles for the Care and Use of Animals of the Juntendo Institutional Animal Care and Use Committee.

2.2 | Isolation of mononuclear cells from skeletal muscle tissue

Mononuclear cells were isolated from skeletal muscle tissue as previously described.[16] The sectioned fresh gastrocnemius muscle of the right leg was minced with scissors, added to 10 mL of DMEM containing 10 mg/mL collagenase type 2 (Worthington, Lakewood, NJ), and the suspension was shaken for 40 min at 37°C in a shaking incubator, following which DMEM containing 10% fetal bovine serum was added to the digested tissue. The sample was then filtered through a 70 μm mesh and centrifuged for 5 min at 800*g*. The pellet containing mononuclear cells was washed twice with staining buffer (Becton Dickinson, San Jose, CA) and the total number of cells was counted. The total number of mononuclear cells was used to normalize the number of cells contained per gram of skeletal muscle.

2.3 | Flow cytometry analysis

Mononuclear cells and blood leukocytes (2.5×10^5 cells) were blocked with anti-Fc-receptor anti-CD16/CD32 (14-0161; eBioscience, San Diego, CA) for 20 min and stained with combinations of anti-CD11b PE-Cy7 (25-0112), anti-F4/80 FITC (11-4801), anti-Ly6C PE (12-5932), anti-Ly6G PE-Cy5 (15-9668), anti-CD3e PE Cy7 (25-0031), and anti-CD45 FITC (11-0451) for 20 min. Flow cytometry was performed using an Attune® Acoustic Focusing Cytometer (Thermo Fisher Scientific) analyzer, and data analysis was performed using Attune™ NxT Software (Thermo Fisher Scientific). Flow cytometric identification of neutrophils, T cells, and macrophage subtype was validated as previously described.[16] Validation of the flow cytometric approach for the identification of macrophages, neutrophils, and T cells is shown in Supporting Information Figures S1 and S2, which are available online.

2.4 | Real-time quantitative polymerase chain reaction

The gastrocnemius muscles of the left leg were extracted from each mouse for the preparation of the homogenate and for RNA and protein isolation. The gastrocnemius muscle was pulverized cryogenically using a mortar and pestle. Total RNA was isolated from muscle tissue powder using the RNase mini kit (Qiagen). The purity of total RNA was assessed using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA (2 μg) was reverse transcribed to cDNA using a high capacity cDNA Reverse Transcription Kit (Invitrogen). Quantitative polymerase chain reacgtion (PCR) was performed using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and PowerUp SYBR® Green Master Mix (Applied Biosystems). Thermal cycling consisted of 10 min of initial denaturation at 95°C, followed by 40 cycles of 15 s denaturation time at 95°C and 1 min annealing time at 60°C. 18S ribosomal RNA was used as a reference gene control, and all data are represented as fold change relative to 18S ribosomal RNA expression (standard curve method). Sequences of all primers used are summarized in Supporting Information Table S1.

2.5 | Western blot analysis

The gastrocnemius muscle tissue powder was mechanically homogenized using polytron homogenizer in ice-cold T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche Life Science). Homogenates were centrifuged for 15 min at 14000*g*, and the supernatant was removed. The protein concentration of homogenized muscle lysates was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific). Muscle lysates were solubilized in Laemmeli sample buffer (Bio-Rad) and heat-

TABLE 1 Body mass and muscle mass in young mice and old mice

	Young (4 mo) n = 5	Old (27 mo) n = 5	*P* value
Body mass (g)	28.6 ± 0.3	32.9 ± 1.4	<.05
Skeletal muscle mass (mg)			
Soleus	10.5 ± 0.2	8.9 ± 0.3	<.01
Plantaris	19.8 ± 0.5	16.5 ± 0.3	<.01
Gastrocnemius	147.6 ± 2.1	122.9 ± 3.6	<.01
Quadriceps muscle	240.9 ± 6.1	203.2 ± 7.8	<.01
Skeletal muscle mass/body mass (mg/g)			
Soleus	0.37 ± 0.01	0.27 ± 0.02	<.01
Plantaris	0.69 ± 0.02	0.50 ± 0.02	<.01
Gastrocnemius	5.16 ± 0.05	3.76 ± 0.15	<.01
Quadriceps muscle	8.42 ± 0.15	6.18 ± 0.20	<.01

Note: Data are presented as mean ± SEM.

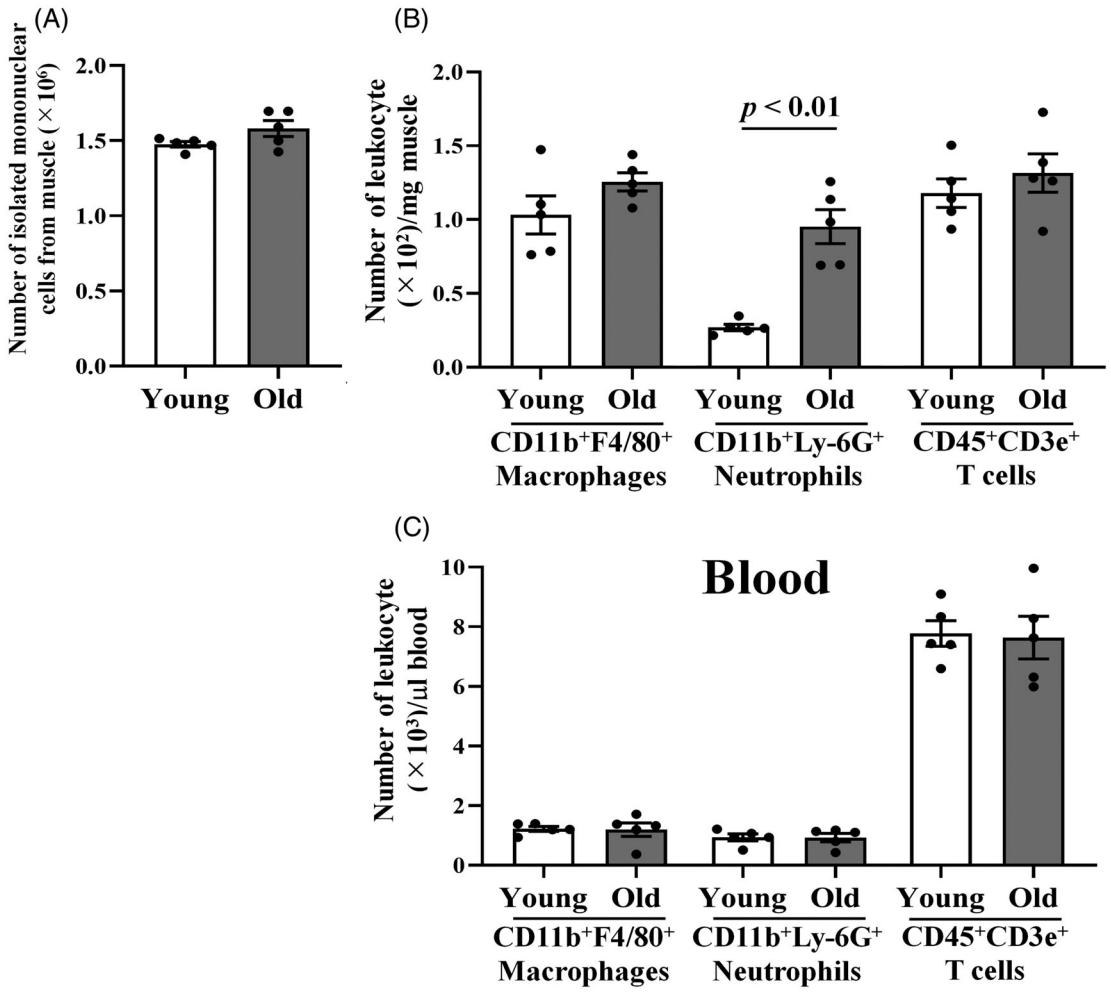

FIGURE 1 Immune cell proportions in skeletal muscle and blood. A, Number of mononuclear cells in skeletal muscle. B, Number of $CD11b^+$ $F4/80^+$ cells (macrophages), $CD11b^+$ $Ly\text{-}6G^+$ cells (neutrophils), and $CD45^+$ $CD3e^+$ cells (T cells) in skeletal muscle. C, Number of $CD11b^+$ $F4/80^+$, $CD11b^+$ $Ly\text{-}6G^+$, and $CD45^+$ $CD3e^+$ cells in blood

denatured for 5 minutes at 100°C. Protein samples (20 μg protein) were resolved using 10% or 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with PVDF Blocking Reagent (TOYOBO), and incubated overnight with an appropriate primary antibody (1:2000 dilution

for JNK [9252; Cell Signaling Technology], p-JNK [4668; CST], Atrogin-1 [AP2041; ECM Biosciences], MuRF1 [MP3401; ECM Biosciences], Nox2 [80 508; Abcam], Nox4 [13 303; Abcam], nitrotyrosine [61 392; Abcam], and 1:5000 dilution for α- tubulin [18 251; Abcam]). α-Tubulin was used as an internal loading control. Membranes were then incubated with horseradish peroxidase-linked secondary antibody (1:5000 dilution for anti-rabbit immunoglobulin [Ig]G [6741; Abcam] and anti-mouse IgG [6708; Abcam]). Blotted samples were analyzed with an EzWestLumi plus (ATTO) visualizer and images were quantified by densitometry.

2.6 | Statistical analyses

All data are expressed as the mean ± SEM. Statistical analyses were performed using the Statistical Package for the Social Sciences (Version 18.0; SPSS Inc., Chicago, Illinois). Statistical differences between two comparable groups were determined using Student's *t* test. A value of $P < .05$ was used as a cutoff for statistical significance.

3 | RESULTS

3.1 | Body mass and tissue mass

Body mass and visceral adipose tissue mass were greater in old mice than in young mice (Table 1). In contrast, skeletal tissue mass of the soleus, plantaris, gastrocnemius, and quadriceps femoris muscles were lower in old mice than in young mice (Table 1).

3.2 | Immune cell proportions in skeletal muscle and blood

There was no significant difference between the old mice and young mice in the total number of mononuclear cells isolated from the muscle (Figure 1A). We found that old mice had a higher absolute number (2.8-fold) of $CD11b^+Ly\text{-}6G^+$ cells (neutrophils) in the gastrocnemius muscle compared to young mice (Figure 1B). However, there was no significant difference in the number of $CD11b^+F4/80^+$ cells (macrophages) and $CD45^+CD3e^+$ cells (T cells) in old and young mice

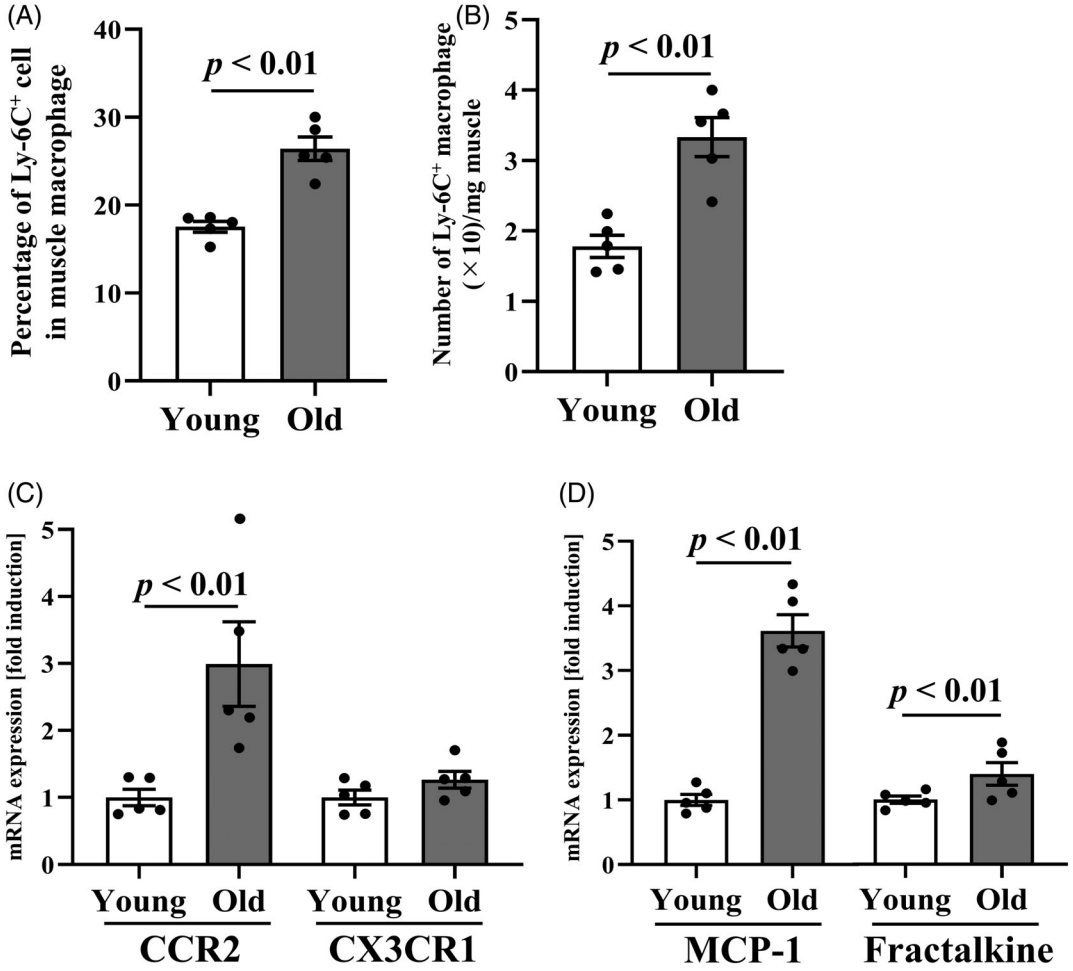

FIGURE 2 Proportions and numbers of different macrophage subtypes in skeletal muscle. A, Percentage of $Ly6C^+$ cells in macrophage. B, Number of $Ly6C^+$ macrophage in skeletal muscle. C, *CCR2* and *CX3CL1* mRNA expression levels in skeletal muscle. D, *MCP-1* and *fractalkine* mRNA expression levels in skeletal muscle

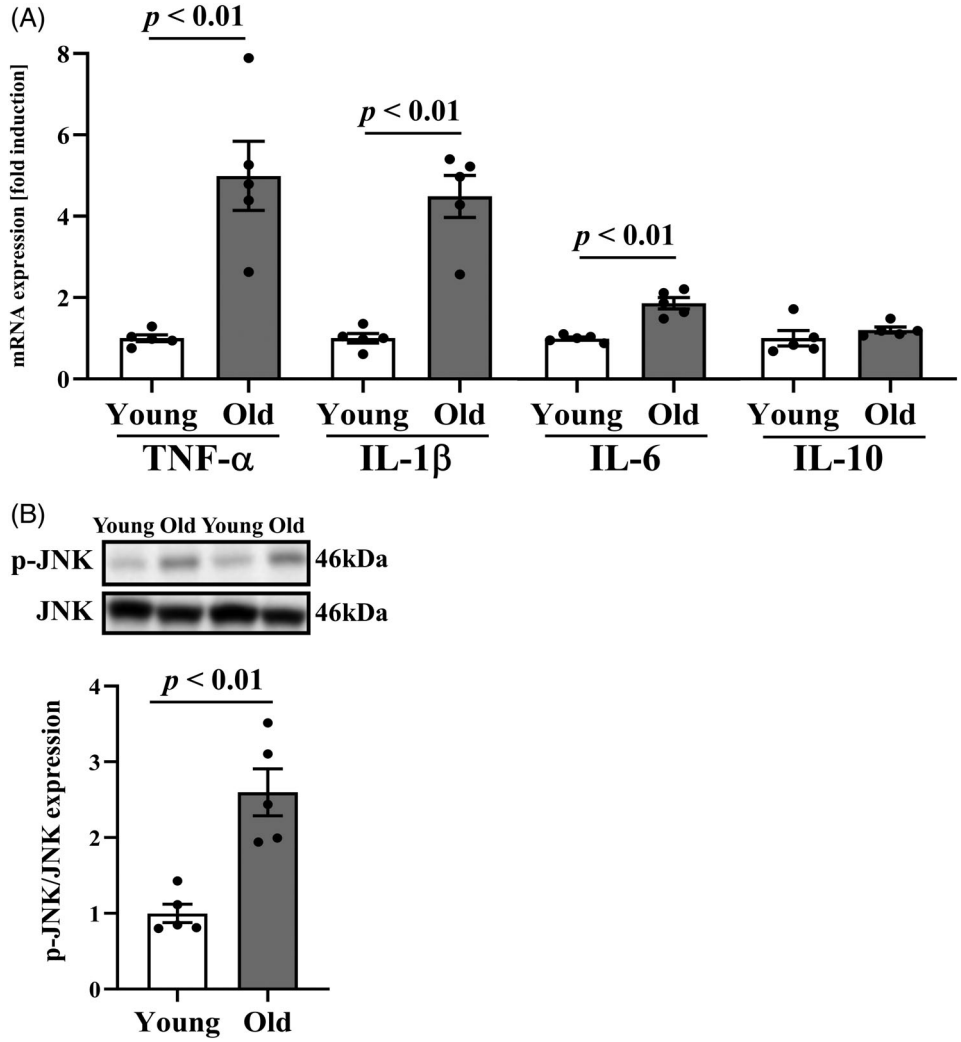

FIGURE 3 Levels of inflammation in skeletal muscle. A, mRNA expression levels of inflammatory (*TNF-α*, *IL-1β*, and IL-6) and anti-inflammatory (*IL-10*) cytokines in skeletal muscle. B, Levels of phosphorylated JNK protein in skeletal muscle

(Figure 1B). Further analysis of blood leukocyte populations indicated that the total number of circulating $CD11b^+F4/80^+$ cells (macrophages), $CD11b^+Ly\text{-}6G^+$ cells (neutrophils), and $CD45^+CD\text{-}3e^+$ cells (T cells) were not significantly affected by increasing age (Figure 1C).

Two distinct subtypes of macrophages in skeletal muscle can be identified and distinguished by characteristic cell surface marker expression. We found that muscle from old mice contained a higher proportion of inflammatory $Ly\text{-}6C^+$ macrophage cells in the total $CD11b^+$ $F4/80^+$ macrophage population than that in muscle from young mice (Figure 2A). Furthermore, old mice had a higher absolute number (1.8-fold) of inflammatory $Ly\text{-}6C^+$ macrophages in the muscle than that of young mice (Figure 2B).

The levels of *CCR2* mRNA were higher in old mice than in young mice (2.9-fold), but there was no significant difference in *CX3CRI* mRNA levels between the two groups (Figure 2C). Although levels of fractalkine (anti-inflammatory macrophage specific chemokine) were marginally higher in old mice than in young mice (1.4-fold), a much higher level of MCP-1 (inflammatory macrophage specific chemokine) mRNA was observed in old mice than in young mice (3.6-fold) (Figure 2D).

3.3 | Inflammation and oxidative stress in skeletal muscle

Old mice had higher expression levels of pro-inflammatory *TNF-α* (4.9-fold), *IL-1β* (4.4-fold), and *IL-6* (1.9-fold) in muscle than those of young mice (Figure 3A), but there was no significant difference in levels of *IL-10* expression between the two groups (Figure 3A). Levels of phosphorylated JNK, which is phosphorylated during inflammation, were higher in old mice (2.4-fold) than in young mice (Figure 3B).

The mRNA expression levels of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase isoforms *Nox2* and *Nox4* were significantly higher in old mice than in young mice (Figure 4A). NOX2 protein levels were also significantly higher in old mice (1.9-fold) than in young mice, but there was no significant difference in NOX4 protein levels between the two groups (Figure 4B). Levels of nitrotyrosine protein, which is a major marker of oxidative stress, were also higher in old mice (2.4-fold) than in young mice (Figure 4C).

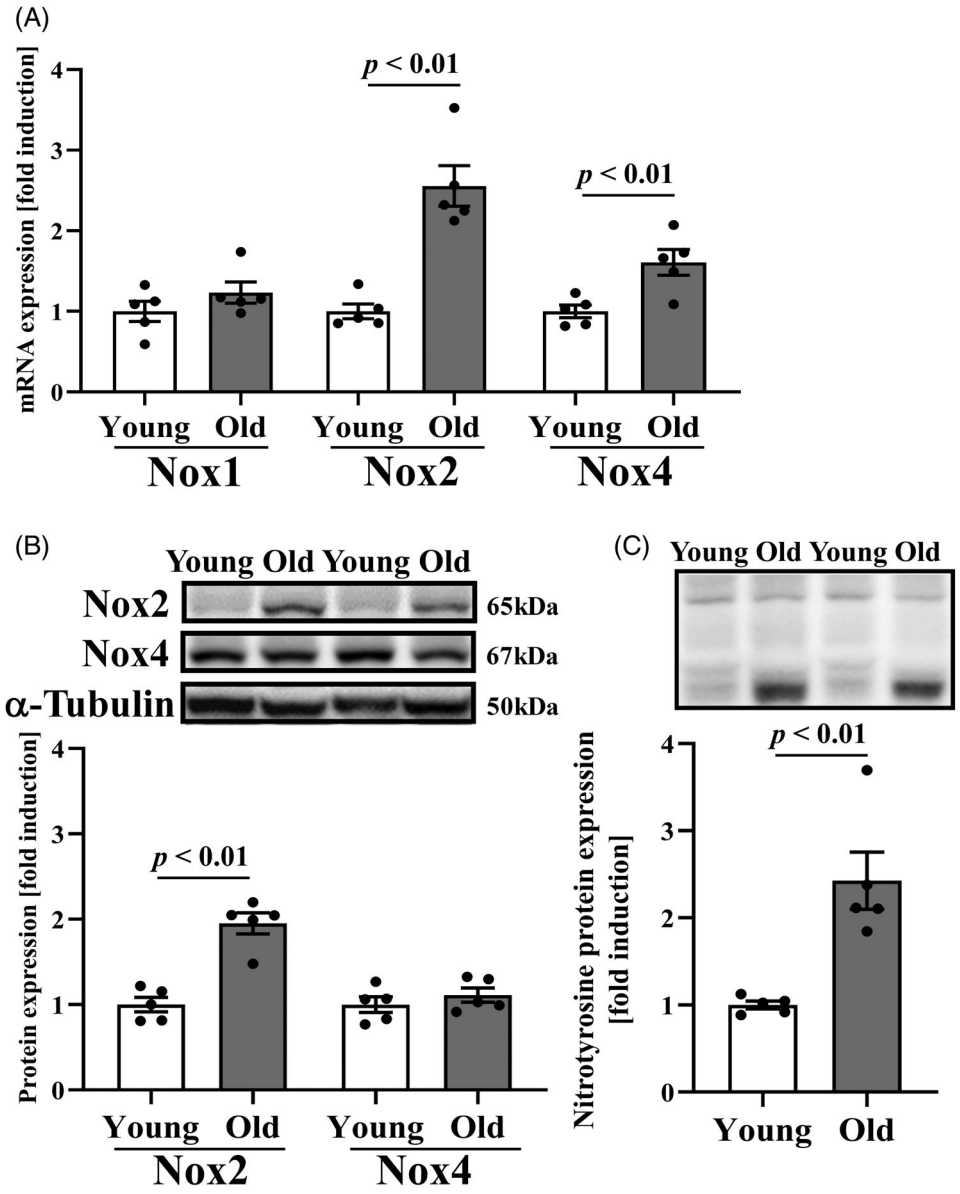

FIGURE 4 Levels of oxidative stress in skeletal muscle. A, mRNA expression levels of NOX isoforms in skeletal muscle. B, Protein expression levels of different NOX isoforms in skeletal muscle. C, Nitrotyrosine levels in skeletal muscle

3.4 | Expression levels of ubiquitin ligases in skeletal muscle

The mRNA expression levels of atrophy-related ubiquitin ligases, *atrogin-1* and *MuRF1*, were higher in old mice than in young mice (Figure 5A). Similar to the differences observed in mRNA expression levels, protein expression of atrogin-1 (1.6-fold) and MuRF1 (2.3-fold) were also higher in old mice than in young mice (Figure 5B).

4 | DISCUSSION

Muscle inflammation and oxidative stress may be important factors in aging-associated loss of skeletal muscle mass. Several studies have reported that stimulation by inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin 1-beeta (IL-1β), and hydrogen peroxide induces the expression of atrogin-1 and MuRF1 ubiquitin ligases, and subsequently results in muscle protein degradation in skeletal muscle cells.[17-19] A recent study showed that mRNA levels of inflammatory cytokines (TNF-α and IL-6) increase in the skeletal muscle of elderly humans.[20] Similar to these findings, we observed increased levels of TNF-α, IL-1-β, and IL-6 mRNA in the muscle of old mice. Phosphorylation of JNK due to inflammatory cytokine signaling is a known marker for inflammatory stress, and we also observed an increase in muscle phosphorylated JNK levels with increasing age. Our study supports the theory that increasing age is associated with an increase in muscle inflammation.

In recent years, the significant contribution of immune cells to the development of muscle inflammation has become apparent. Recent studies have shown that macrophages and neutrophils secrete pro-inflammatory cytokines, contributing to the induction of muscle inflammation during injury.[21-23] Herein, we showed that there is a marked increase in the number of neutrophils in the skeletal muscle of

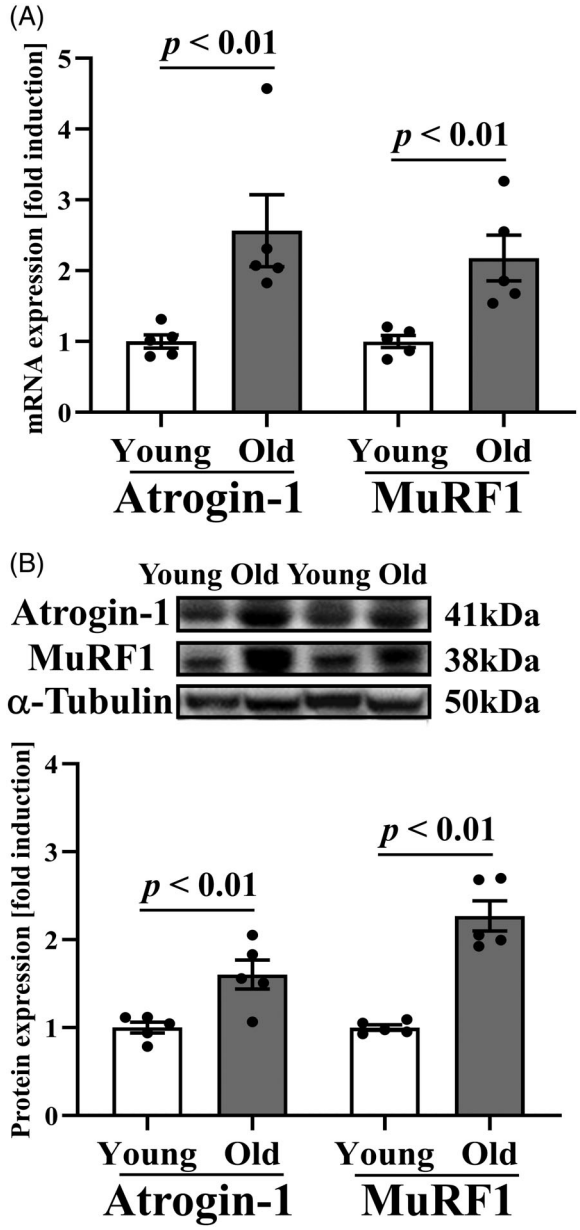

FIGURE 5 Levels of activation of the ubiquitin proteasome system in skeletal muscle. A, mRNA expression levels of *Atrogin-1* and *MuRF1* in skeletal muscle. B, Protein expression levels of Atrogin-1 and MuRF1 in skeletal muscle

old mice. Interestingly, no age-related changes were observed in the number of circulating neutrophils, suggesting that age-related changes in immune cells are not a systemic phenomenon, but rather a local phenomenon within skeletal muscle. Moreover, we observed that increasing age did not affect the total number of macrophages in skeletal muscle.

These findings in aged mice are in agreement with the results of a study examining macrophage content in the skeletal muscle of elderly humans.[24] Importantly, in addition to changes in the numbers of neutrophils and macrophages, an imbalance between the proportion of inflammatory and anti-inflammatory subtype macrophages is known to be associated with inflammatory environments within skeletal muscle during trauma.[10,11] Previous studies have shown that Ly-6C is a specific marker that can be used to distinguish between inflammatory and anti-inflammatory macrophages in skeletal muscle.[25,26] We found that increasing age increased both the proportion and absolute number of Ly-6C$^+$ inflammatory macrophages. Furthermore, we observed that increasing age increased the mRNA expression levels of MCP-1, a chemokine specific for the inflammatory subtype of macrophages. As such, our results suggest that increasing age increases the infiltration of inflammatory macrophage subtypes via the production of chemokines in skeletal muscle, which is subsequently associated with muscle inflammation. Although MCP-1 is known to be produced by immune cells and skeletal muscle cells, endothelial cells, and senescent cells, the cellular source underlying the production of chemokines in muscles of old mice is unknown. Further work is required to determine which cells produce MCP-1 and induce macrophage infiltration into the muscle tissue of old mice.

Recent evidence also shows that, apart from inflammation, oxidative stress contributes to loss of skeletal muscle mass through the induction of protein degradation in skeletal muscle cells by reactive oxygen species.[19] In this study, we found that the expression level of an oxidative stress marker, nitrotyrosine, was increased in the skeletal muscle of old mice. Oxidative stress is induced by reactive oxygen species, such as hydrogen peroxide, which is synthesized by NADPH oxidases (NOS). NOX has several isoforms that may be differentially expressed in cells, with NOX1 being expressed mainly in vascular smooth muscle cells, NOX2 in phagocytes, such as neutrophils and macrophages, and NOX4 in endothelial cells.[27] Here, we observed that the expression of NOX2 protein was increased in the muscle tissue of old mice, but there was no significant difference in the levels of NOX1 and NOX4 expression. Importantly, we also observed that nitrotyrosine expression levels in muscle correlated with the number of neutrophils ($r = 0.957$, $P < .01$) and inflammatory macrophages ($r = 0.832$, $P < .01$) in the muscle. As such, our results suggest that increased infiltration of neutrophils and inflammatory macrophages is associated with aging-related oxidative stress in muscles.

A limitation of this study was that only the gastrocnemius was examined in detail for the number and subset composition of immune cells. It is unclear whether other muscles are similarly affected by increasing age. Further work is required to examine differences in skeletal muscle tissue parts for changes in the number and subset composition of immune cells with increasing age.

In conclusion, we demonstrated that increasing age markedly enhances the loss of skeletal muscle mass, inflammation, and oxidative stress in murine skeletal muscle. We further demonstrated that increasing age increases the infiltration of inflammatory macrophages and neutrophils in skeletal muscle.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose.

ETHICAL PUBLICATION STATEMENT

We confirm that we have read the journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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