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Tumor necrosis factor-α promotes the accumulation of neutrophils and macrophages in skeletal muscle

Jennifer M. Peterson, Kevin D. Feeback, Joel H. Baas, and Francis X. Pizza
Department of Kinesiology, The University of Toledo, Toledo, Ohio

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Peterson, Jennifer M., Kevin D. Feeback, Joel H. Baas, and Francis X. Pizza. Tumor necrosis factor-α promotes the accumulation of neutrophils and macrophages in skeletal muscle. *J Appl Physiol* 101: 1394–1399, 2006. First published July 13, 2006; doi:10.1152/japplphysiol.01453.2005.—Tumor necrosis factor-α (TNF-α) has been associated with cachexia and is known to regulate multiple inflammatory cell (neutrophil and macrophage) responses. We tested the hypothesis that neutrophils and macrophages accumulate in the extensor digitorum longus (EDL) and soleus muscles of mice after chronic TNF-α administration. Murine recombinant TNF-α (~100 $\mu g \cdot kg^{-1} \cdot day^{-1}$) in vehicle solution or vehicle solution alone (sham) was administered to C57BL/6 mice for 7 days via osmotic minipumps. In EDL muscles from TNF-α-treated mice, neutrophil and macrophage concentrations were elevated seven- and threefold, respectively, compared with sham mice. Neutrophil and macrophage concentrations were also elevated five- and twofold, respectively, in solei of TNF-α- relative to sham-treated mice. Treatment with TNF-α elevated ubiquitin content by ~25% relative to sham values for both the EDL and soleus muscles; however, these elevations were not statistically significant. No differences were observed between TNF-α- and sham-treated mice in body weight, food consumption, muscle mass, myofiber cross-sectional area, carbonyl groups, total protein content, or relative abundance of myosin heavy chain protein. Furthermore, no overt signs of muscle injury or regeneration were observed in muscles from TNF-α-treated mice in either the EDL or soleus muscles. These observations suggest that 7 days of TNF-α administration promote muscle inflammation as indicated by the accumulation of neutrophils and macrophages without overt signs of atrophy, injury, or regeneration.

neutrophils; macrophages; cytokine

TUMOR NECROSIS FACTOR-α (TNF-α) is a pleiotropic cytokine with functions ranging from mediating shock in sepsis to causing necrosis of tumors (3). Elevated serum and/or muscle TNF-α concentrations have been linked to age-associated skeletal muscle atrophy (17), insulin resistance (37), and increased mortality rates (4). Direct evidence of a relationship between TNF-α and disease has been demonstrated in heart failure patients in whom a positive correlation between plasma levels of TNF-α and disease severity was reported (21, 22). TNF-α has been implicated as a mediator of the muscle-wasting syndrome known as cachexia. Cachexia manifests as a secondary but potentially lethal complication in chronic diseases such as cancer, heart failure, and rheumatoid arthritis (41).

In an attempt to elucidate the mechanisms by which TNF-α may promote cachexia in skeletal muscle, the majority of previous investigators have studied how TNF-α influences cultured skeletal muscle cells. These studies revealed that TNF-α increased mRNA and/or protein levels of TNF-α and other cytokines [e.g., interleukin (IL)-6 and interferon-γ (IFN-γ)] that are reported to have catabolic properties in skeletal muscle (1, 2). In addition, TNF-α has been reported to inhibit protein synthesis in skeletal muscle cells through insulin-like growth factor I- and nuclear factor-κB (NF-κB)-dependent pathways (12, 25, 44). Li et al. (26–28) reported that TNF-α-induced mitochondrial reactive oxygen species (ROS) production can activate NF-κB, which induces protein loss in skeletal muscle cells via the ubiquitin-proteasome pathway. Whether TNF-α alone is sufficient to cause muscle atrophy in vivo, however, is controversial. Accelerated muscle protein degradation or decreased protein content has been reported after TNF-α administration in some studies but not in others (10, 15, 16, 29, 33, 36).

In addition to its influence on skeletal muscle cells, TNF-α influences various aspects of inflammatory and endothelial cell function to mediate inflammation. TNF-α promotes inflammation by increasing blood neutrophil concentrations (43), by elevating gene expression of adhesion molecules [e.g., intracellular adhesion molecule-1 (ICAM-1)] and chemokines [e.g., monocyte chemoattractant protein-1 (MCP-1)] by endothelial cells (40), and by increasing the adhesion of neutrophils (13) and monocytes (19) to vascular endothelium. Previous investigators have reported that TNF-α also stimulates the release of IL-1 (6) and IL-6 (6, 7) from neutrophils, which have been reported to induce catabolism in skeletal muscle (18). Finally, TNF-α-induced ROS production from adherent neutrophils may enhance TNF-α-induced activation of NF-κB and ICAM-1, suggesting a mechanism by which inflammatory cells could augment the actions of TNF-α (8, 9). Therefore, if TNF-α promotes the accumulation of neutrophils and macrophages in skeletal muscle, it is conceivable that inflammatory cells could contribute to muscle atrophy via their production of ROS and/or cytokines.

In this study, we tested the hypothesis that neutrophils and macrophages accumulate in skeletal muscles in response to chronic TNF-α administration. We also tested whether TNF-α-induced accumulation of inflammatory cells in skeletal muscle is associated with indexes of atrophy, injury, and regeneration.

METHODS

Animals. Male C57BL/6J mice (27–30 g) were commercially obtained (Jackson Laboratory, Bar Harbor, ME) and provided standard laboratory chow and water ad libitum. The animal use and care committee at The University of Toledo approved experimental procedures.

Address for reprint requests and other correspondence: F. X. Pizza, Dept. of Kinesiology, MS 119, The Univ. of Toledo, 2801 West Bancroft St., Toledo, OH 43606 (e-mail: francis.pizza@utoledo.edu).

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Pump implantation. Mice were anesthetized with an intraperitoneal injection of 2% avertin (0.015 ml/g body mass) with supplemental doses (0.1 ml of 2% avertin) given as needed. Osmotic minipumps (model 2001, Durect, Cupertino, CA) prefilled with sterile recombinant murine TNF-α (R&D Systems, Minneapolis, MN) dissolved in a vehicle solution (physiological saline with 0.1% mouse serum albumin) or vehicle alone (sham) were implanted subcutaneously on the dorsal aspect of mice through a small incision under the skin. These pumps were designed to deliver 1 μl/h of TNF-α or vehicle alone for 7 days and have been demonstrated to elicit elevated TNF-α serum concentrations (31). After the 7-day period, pumps were removed from the mice, and any remaining volume was aspirated. Preliminary data revealed that the administration of 50 $\mu g \cdot kg^{-1} \cdot day^{-1}$ of TNF-α resulted in similar neutrophil and macrophage concentrations as reported in this study but no overt muscle atrophy. We therefore chose to double the dose and administer 100 $\mu g \cdot kg^{-1} \cdot day^{-1}$ of TNF-α. The actual volume of TNF-α delivered was then calculated. The average daily delivery of TNF-α was found to be 113.2 ± 6.7 $\mu g \cdot kg^{-1} \cdot day^{-1}$. Food consumption and body weights were monitored during the study.

Tissue preparation. After 7 days of TNF-α or vehicle administration, extensor digitorum longus (EDL) and soleus muscles were dissected out, blotted dry, weighed, mounted in optimal freezing medium, rapidly frozen in isopentane cooled to the temperature of dry ice, and stored at −80°C. Control muscles were dissected from untreated mice that had normal cage activity. Anesthetized mice were then killed by cervical dislocation.

Inflammatory cells. Transverse sections were cut (10 μm) from the midbelly of the muscles on a cryostat (Leica, Bannockburn, IL) and prepared for immunohistochemistry as previously described (39). Neutrophils and macrophages were identified using an anti-mouse Ly6G antibody [clone RB6-8C5; 1:100 in phosphate-buffered saline (PBS); PharMingen, Franklin Lake, NJ] and an anti-mouse F4/80 antibody (clone CI:A3-1; 1:100 in PBS; Serotec, Raleigh, NC), respectively. Slides serving as negative controls received PBS instead of primary antibody. After a 2-h incubation at room temperature with the primary antibody, sections were washed in PBS, incubated with biotinylated mouse absorbed anti-rat IgG (1:200 in PBS; Vector Laboratories, Burlingame, CA) followed by avidin D horseradish peroxidase (1:1000 in PBS; Vector). Sections were then developed with a 3-amino-9-ethylcarbozyle kit (Vector).

Sections were viewed with a light microscope (Olympus IX-70, Melville, NY) with Nomarski optics. The number of labeled inflammatory cells within two entire sections for each muscle was manually counted, and the total area of each section was assessed using a calibrated square grid. The volume of muscle sampled was calculated as the product of the section area and thickness (10 μm). Inflammatory cells were expressed as number of positive cells per cubic millimeter (mm^3).

Histology. Transverse sections were cut (10 μm) from the midbelly of the muscles and stained with hematoxylin and eosin. Myofibers that had a pale or discontinuous cytoplasmic staining, were substantially swollen in appearance, or were invaded by cells were considered injured (20). The number of central nucleated myofibers was determined and used as a marker of muscle regeneration (39). The number of injured and central nucleated myofibers in two entire sections for each muscle were manually counted and then expressed as a percentage of the total number of myofibers within each section.

Cross-sectional area (CSA) of myofibers was determined and used as an indicator of muscle atrophy. Multiple digital images (×400) were captured (SPOT RT KE; Diagnostics, Sterling Heights, MI) of one entire section for each muscle. Images were then analyzed for CSA using Image Pro Plus software (version 5.1, Media Cybernetics, Silver Spring, MD).

Localization of carbonyl groups. Immunohistochemistry was performed on transverse sections to qualitatively assess oxidative damage to muscle fibers after TNF-α administration as previously described (39). Briefly, muscle sections were fixed in acetone, treated with 0.2% H_2O_2, covered with 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl for 1 h, and blocked with 3% bovine albumin, 5% Tween 20, and 2% gelatin. Sections were rinsed with PBS between each of these steps. Rabbit anti-dinitrophenyl (1:100 in PBS; Sigma) was then applied to each section and incubated for 16 h at 4°C. Slides serving as negative controls received PBS instead of primary antibody. Sections were then washed in PBS, and then they were incubated with biotinylated anti-rabbit IgG (1:200 in PBS; Vector) followed by avidin D horseradish peroxidase (1:1,000 in PBS). Sections were developed with 3-amino-9-ethylcarbozyle (Vector).

Protein content and myosin heavy chain. Muscles were homogenized manually in reducing sample buffer on ice as previously described (39). Homogenates were centrifuged at 4°C and the amount of protein in supernatants was determined and expressed as micrograms per milligram of muscle mass (34).

Muscles were then boiled and equal amounts of protein (1 μg) were separated on 8% gels via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Hoefer mini VE, Amersham, Piscataway, NJ) at 20 mA. Proteins were transferred to nitrocellulose membranes overnight (200 mA; Hoefer TE series Tank Transphor unit, Amersham) and stained with Ponceau S to confirm equal loading. Membranes were then blocked in 3% powdered milk for 1 h and subsequently incubated with sarcomeric myosin heavy chain (MHC; MF20; 1:100 in blocking buffer; Developmental Studies Hybridoma Bank, Iowa City, IA; see ACKNOWLEDGMENTS) for 1 h. Unbound antibody was washed off, and membranes were incubated with alkaline phosphatase (AP) sheep anti-mouse IgG (1:5,000 in blocking buffer; Jackson ImmunoResearch, West Grove, PA) for 1 h. After additional washing, MHC was detected using an AP conjugate substrate kit (Bio-Rad, Hercules, CA). Images of the ∼200 kDa sarcomeric MHC band were captured digitally (Olympus C-5060 equipped with DOC-ItLS image acquisition software, UVP, Upland, CA). Densitometry was performed using Image Pro Plus software, and densities were expressed as percentages of control values.

Ubiquitin content. Equal amounts of protein (30 μg) were loaded into wells of 4–15% gradient gels (Jule, Milford, CT) and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes (2 h; 200 mA), stained with Ponceau S, and blocked with 3% powdered milk (1 h). Membranes were incubated overnight (4°C) with a ubiquitin mouse monoclonal antibody (1:1,000 in blocking buffer; P4D1; Cell Signaling Technology; Danvers, MA), washed to remove unbound antibody, and subsequently incubated with alkaline phosphatase sheep anti-mouse IgG(1:5,000 in blocking buffer; Jackson ImmunoResearch) for 1 h. After additional washing, ubiquitin was detected using an AP conjugate substrate kit (Bio-Rad). Images of the entire lane for each sample were captured digitally. Densitometry was performed using Image Pro Plus software and densities were expressed as percentages of control values.

Statistical analyses. Analysis of variance tests (SigmaStat, Sigma Chemical, St. Louis, MO) were performed to evaluate the effects of TNF-α administration and sham treatment on body weight, muscle mass, inflammatory cell concentrations, overt signs of injury and regeneration, CSA of myofibers, total protein, and ubiquitin content. The Newman-Keuls post hoc test was used to locate the difference between means when a significant F ratio was observed ($P \leq 0.05$). Data are reported as means ± SE.

RESULTS

Muscle concentrations of neutrophils and macrophages in the TNF-α-treated mice were elevated above sham and control mice. Neutrophil concentrations in TNF-α-treated mice were seven- and fivefold above sham concentrations in the EDL and soleus muscles, respectively (Fig. 1). Macrophage concentrations in TNF-α-treated mice were three- and twofold above sham concentrations in the EDL and soleus muscles, respec-

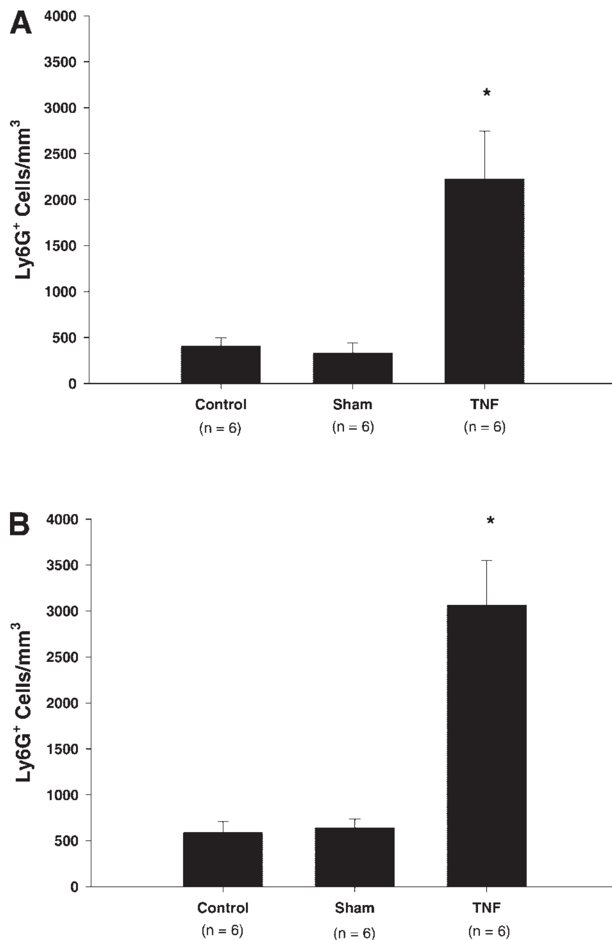

Fig. 1. Neutrophil (Ly6G$^+$ cells) concentrations in extensor digitorum longus (*A*) and soleus (*B*) muscles of mice after control, sham, and TNF-α (TNF) treatment. Values are means ± SE; *n*, no. of mice. *Significantly elevated above control and sham concentrations, $P < 0.05$.

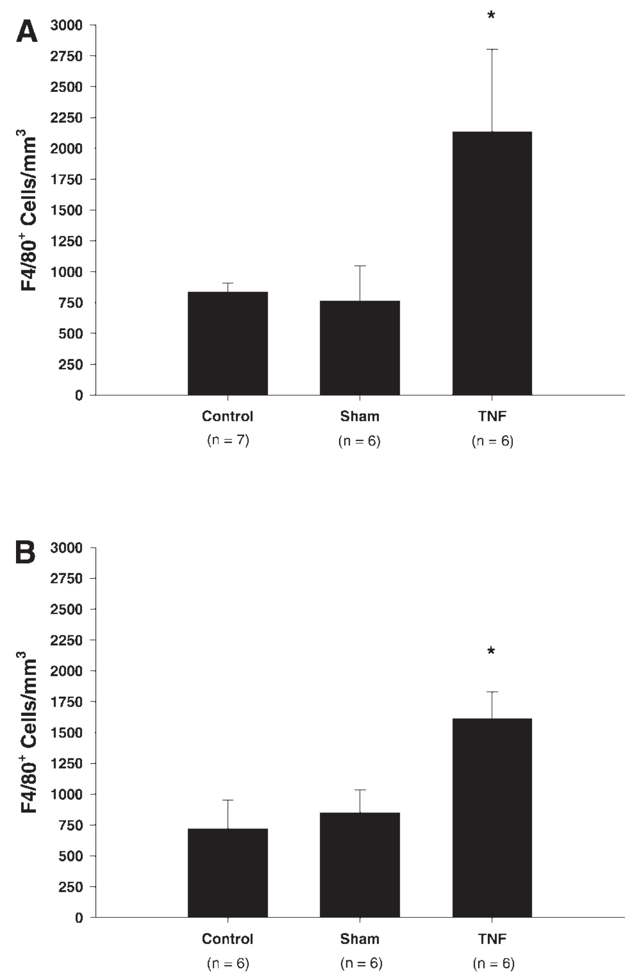

Fig. 2. Macrophage (F4/80$^+$ cells) concentrations in extensor digitorum longus (*A*) and soleus (*B*) muscles of mice after control, sham, and TNF treatment. Values are means ± SE; *n*, no. of mice. *Significantly elevated above control and sham concentrations, $P < 0.05$.

tively (Fig. 2). For sham-treated mice, neutrophil and macrophage concentrations in the EDL and soleus muscles were not elevated relative to controls (Figs. 1 and 2). The majority of inflammatory cells within muscle sections were found adjacent to muscle fibers and within blood vessels. Cells located in blood vessels were not included in the cell counts.

The accumulation of neutrophils and macrophages was not associated with indexes of anorexia, muscle atrophy, injury, or regeneration. Food consumption and body weights were similar between groups during the 7 days of treatment (data not shown). We did, however, observe reduced cage activity in the TNF-α-treated mice during the first several days of cytokine administration. Very few myofibers (~0.15%) in the EDL or soleus muscles showed overt signs of muscle injury or regeneration after sham or TNF-α treatment. Qualitative observations of carbonyl labeling, indicative of oxidative damage, did not reveal differences in the abundance or location of carbonyl groups between TNF-α- and sham-treated muscle (data not shown). Furthermore, muscle mass, CSA of myofibers, and total protein content were not different between treatment groups in the EDL or soleus muscles (Table 1).

Representative Western blots revealed similar MHC content between treatment groups in both the EDL and soleus muscles (data not reported). Western blots for ubiquitin content were not significantly different between treatment groups; however, TNF-α treatment resulted in ~25 and ~50% increase in ubiquitin content relative to sham and control treatments in the EDL and soleus muscles, respectively (Fig. 3).

DISCUSSION

The purpose of this study was to determine whether TNF-α caused neutrophils and/or macrophages to accumulate in skel-

Table 1. *Index of muscle atrophy*

Treatment	Weight, mg	CSA, μm^2	Total Protein, μg/mg muscle mass
EDL			
Control	10.9±0.4	1391.3±74.3	94.4±3.0
Sham	10.0±0.5	1670.4±133.4	79.3±1.2
TNF-α	10.5±0.7	1,387.7±108.3	71.8±3.8
Soleus			
Control	9.7±1.1	1,397.9±41.3	87.8±2.3
Sham	8.5±0.4	1,531.0±69.4	69.5±3.4
TNF-α	9.0±0.1	1,409.1±55.6	68.5±2.9

Values are means ± SE. CSA; cross-sectional area of myofibers, EDL, extensor digitorum longus; TNF-α: tumor necrosis factor-α.

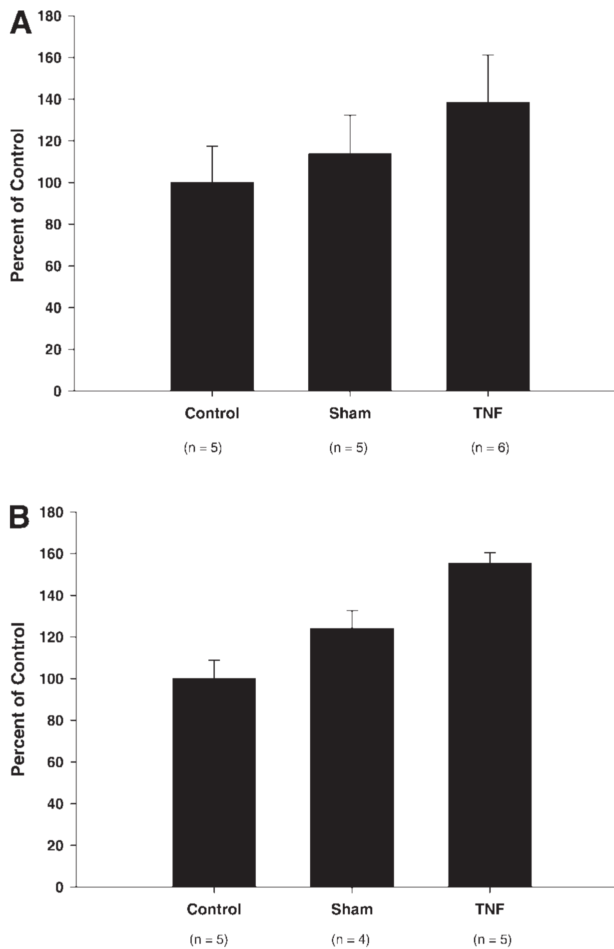

Fig. 3. Comparison of ubiquitin densities in extensor digitorum longus (*A*) and soleus (*B*) muscles of mice after control, sham, and TNF treatment. Values are means ± SE; *n*, no. of mice.

etal muscle in the presence or absence of signs of muscle atrophy, injury, or regeneration. We revealed that neutrophils and macrophages accumulate in the EDL and soleus muscles of mice as a result of TNF-α administration. Elevations in neutrophils and macrophage concentrations, however, were not associated with indexes of muscle atrophy, injury, or regeneration.

The observed inflammatory cell accumulation after TNF-α administration is consistent with the previously reported influence of TNF-α on vascular events that precede neutrophil and monocyte dipedesis. TNF-α has been demonstrated to increase neutrophil and monocyte adhesion to vascular endothelium in vitro (13, 19). Mori et al. (35) reported that, in skin wounds, the TNF-α receptor p55 induces the expression of leukocyte adhesion molecules (E-selectin, ICAM-1, and vascular cellular adhesion molecule-1) and the release of chemokines [macrophage inflammatory protein-2 (MIP-2) and MCP-1] for neutrophils and monocytes. Furthermore, local injection of TNF-α into skeletal muscle induces rolling (23, 46), adhesion (45, 46), and extravasation (47) of leukocytes (predominately neutrophils) in cremaster muscle arterioles and venules. Zhang et al. (47) reported that the extravasation of neutrophils from cremaster venules after local injection of TNF-α was dependent on two mouse neutrophil chemoattractants, cytokine-induced neutrophil chemoattractant (KC) and MIP-2. Whether similar mechanisms are operating when TNF-α is administered systemically (present study) remains to be determined.

Previous investigators have reported catabolic changes in skeletal muscle after either acute or chronic administration of a similar concentration of TNF-α as we administered in this study. These changes include increased protein degradation (29), free and conjugated ubiquitin protein expression (14), and decreased body weight (29), food consumption (29), protein synthesis (24), muscle protein content (5), and DNA content (5). Our results differ from those of Carbo et al. (5), who reported decreased protein content in skeletal muscle, and Ling et al. (29), who reported decreased body weight and food consumption after administration of 100 $\mu g \cdot kg^{-1} \cdot day^{-1}$ of TNF-α for 8 and 6 days, respectively. Our results, however, are consistent with others who reported that higher doses of TNF-α (200–250 $\mu g \cdot kg^{-1} \cdot day^{-1}$) administered for 5–14 days did not decrease skeletal muscle protein content (14, 36), body weight (36), or food consumption (36). Finally, some authors have reported a loss in protein content (11, 42), body weight (42), and food consumption (42) only after repeatedly doubling the daily dose of TNF-α. However, neither of the authors reporting reductions in body weight reported a difference compared with pair-fed controls (29, 42). Reconciling the literature on the influence of TNF-α on markers of muscle atrophy is difficult because of the variability in methods used.

Overt signs of injury or regeneration did not accompany the accumulation of inflammatory cells after TNF-α treatment. Previous investigators using rodent exercise models have also reported moderate elevations in neutrophil and/or macrophage concentrations in skeletal muscle in the absence of signs of muscle injury or regeneration (32, 38). Interestingly, neutrophil concentrations in EDL muscles after TNF-α treatment are comparable to values we observed in mice 3 days after noninjurious isometric contractions and only one-third the levels found after injurious lengthening contractions (38). Macrophage concentrations in EDL muscles after TNF-α treatment were similar to noninjurious passive stretches and only one-sixth the levels observed after lengthening contractions (38). We do not have data on neutrophil or macrophage concentrations in mouse solei after exercise to compare inflammatory cell concentrations.

Neutrophils and macrophages are capable of producing ROS and releasing numerous proteases and cytokines (e.g., IL-6, TNF-α, and IFN-γ) (6, 30). Given that many of the inflammatory cell-derived products have been reported to induce catabolic changes in skeletal muscle, we speculated that neutrophils and macrophages would contribute to muscle atrophy after TNF-α treatment. The data, however, do not support this speculation because neutrophils and macrophages were elevated at a time when markers of muscle atrophy were unaltered. Whether neutrophils and/or macrophages are releasing factors in skeletal muscle after TNF-α treatment and whether their presence in skeletal muscle precedes atrophy remains to be determined.

Taken together, our results indicate that TNF-α administered to mice (∼100 $\mu g \cdot kg^{-1} \cdot day^{-1}$) for 7 days results in the accumulation of inflammatory cells in skeletal muscle without overt signs of muscle atrophy, injury, or regeneration. Future studies aimed at revealing the function of inflammatory cells in

skeletal muscle after TNF-α administration and in disease models of cachexia are warranted.

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