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Physiological role of tumor necrosis factor α in traumatic muscle injury

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ABSTRACT

The degenerative and regenerative roles of tumor necrosis factor α (TNF-α), a pro-inflammatory cytokine with pleiotropic functions, were investigated by using TNF receptor 1 and 2 double knockout (TNFR-DKO) and TNF-α antibody neutralized mice following traumatic freeze injury to the tibialis anterior muscle. In wild-type control mice, TNF-α mRNA transcripts and protein increased following injury and gradually returned to control (uninjured) levels by 13 days. A reduction in MyoD mRNA expression occurred in TNF–α-deficient mice, although there were no visible differences in MyoD immunostaining or histological characteristics in regenerating muscles. At 5 days post-injury, the reductions in isometric strength in TNFR-DKO and TNF–α-depleted mice did not differ from that of wild-type mice but by 13 days after injury, the TNFR-DKO and TNF–α-depleted mice exhibited strength deficits twice that of wild-type mice (i.e., 27–31% vs 13%). Muscle injury was also accompanied by increased expression of interleukin-6 (IL-6), but IL–6-deficient mice demonstrated MyoD expression and recovery of isometric strength similar to that of wild-type mice. These data indicate that TNF-α is involved in the recovery of muscle function after traumatic muscle injury, and this effect might be associated with modulation of muscle regulatory genes, including MyoD.

Key words: muscle function • inflammation • inflammatory cytokines

Traumatic skeletal muscle injuries, such as those induced by extreme cold, crushing, or toxins, can result in profound histopathological changes and loss of muscle function (i.e., >40% loss of strength) that does not recover after 2 weeks or more (1–3). Recovery from these injuries requires that severely injured myofibers be degraded and replaced via the orderly maturation of satellite cells. The timing of the onset and the extent of infiltrating inflammatory cells, principally macrophages, can affect both the degeneration and regeneration processes (4–7). Damage to bystander cells due to excess mitochondrial production of oxygen radicals generated at the semiquinone site is responsible primarily for degenerative processes from excessive inflammation in most tissue/organs studied, reviewed in (8). The relationship of inflammation to regeneration has been less studied, but it is believed to stem from the ability of inflammatory mediators to stimulate

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production of growth factors, such as transforming growth factor (TGF)-α, hepatocyte growth factor (HGF), and interleukin (IL)-6; reviewed in (9). In muscle, the role of inflammatory cells in regeneration appears to be causal in nature, as enhanced formation of differentiated myotubes from muscle precursor cells occurs when the cells are co-cultured with macrophages (10, 11).

As tumor necrosis factor (TNF)-α is a major product of activated macrophages, as well as a mediator of diverse functions in damaged or inflamed tissue, several investigations have examined the role of TNF-α in muscle cell metabolism and catabolism (12). TNF-α, which is produced primarily by macrophages and monocytes, is expressed as a 26 kDa integral transmembrane precursor protein from which a 17 kDa subunit is released after proteolytic cleavage (13). Originally identified as causing a muscle-wasting syndrome in tumor-bearing mice and a reduction in the size of some tumors, TNF-α is now recognized as having pleiotropic functions. In addition to mediating inflammatory and cytotoxic/apoptotic responses, TNF-α modulates growth and differentiation for many cell types. In skeletal muscle metabolism (reviewed in 12), as in other organ systems, such as the skin, lung, and central nervous system, (8; and reviewed in 9), TNF-α contributes to both degeneration and regeneration processes. For example, direct administration of TNF-α to experimental animals inhibits contractile function (14), possibly through platelet activating factor (PAF)-dependent synthesis of nitric oxide (NO) (15). TNF-α may also mediate muscle atrophy by a NF–κB-dependent loss of protein as observed in skeletal muscle myocytes exposed to TNF-α (16). This may reflect TNF–α-induced NF–κB-dependent inhibition of MyoD expression in differentiating myocytes (17). In contrast to catabolic effects in mature myotubes, other *in vitro* studies have shown that TNF-α promotes early differentiation of C2C12 murine myoblasts in an autocrine fashion; reviewed in (18). That TNF-α is important in muscle development was also suggested earlier in studies with TNF/dystrophin deficient transgenic mice (TNF^{-}/mdx^{-}), as these mice have significantly less muscle mass than TNF^{+}/mdx^{-} mice (19).

Our major objective of this study was to elucidate the role of TNF-α in degenerative and regenerative processes following muscle injury. We used two murine models to modulate TNF-α response, transgenic mice deficient in TNF receptors I and II (double knockout; TNFR–DKO), and wild-type mice-administered neutralizing antibodies to TNF-α. At specified times after freeze injury of the tibialis anterior (TA) muscle, strength was measured in control and deficient mice and was compared with local expression of TNF-α and myogenic factors, including MyoD, myogenin, and Myf6. Additionally, to evaluate whether the TNF-α response in the muscle is mediated indirectly through IL-6, local expression of IL-6 mRNA and muscle strength were measured following freeze injury in IL-6 knockout and control mice. The results from these studies suggest that TNF-α plays a moderate but significant role in recovery from traumatic muscle injury, and this response is not mediated by IL-6.

MATERIALS AND METHODS

Animals

C57BL/6 wild-type mice (The Jackson Laboratory, Bar Harbor, ME), transgenic TNFR-DKO mice on a C57BL/6xL29 background (a gift from Larry Schook, University of Minnesota, through Immunex Corp., Seattle, WA), and IL-6 KO (C57BL/6J-IL-6^{tmKopf}, graciously provided by H. Bluethmann, F. Hoffmann-La Roche, Switzerland) were provided food and water ad libitum and

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were maintained on a 12-h light/dark cycle. Experimental animal procedures, including death by CO_2 asphyxiation, were conducted in accordance with criteria outlined in the "PHS Policy on Humane Care and Use of Laboratory Animals" and the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23, 1996). Polyclonal antiserum to murine TNF-α was prepared and characterized for specificity and titer as previously described (20). An intraperitoneal injection (0.2 ml) of prefiltered, nonimmune, or immune serum was used before muscle injury to neutralized TNF-α.

In preparation for muscle injury induction and strength testing, we anesthetized mice with isoflurane (0.75–1.5%) or by an intraperitoneal injection of 0.33 mg/kg fentanyl, 16.7 mg/kg droperidol, and 5.0 mg/kg diazepam. Isoflurane was used in experiments requiring repeated strength testing on a given animal, because it permits a more rapid recovery from the procedure. Both anesthetic regimens permit optimal *in vivo* measurement of the anterior crural muscle torque production about the ankle in the mouse (20; unpublished observations). In addition, isoflurane anesthesia was used during surgical implantation of the chronic stimulating nerve cuff.

Surgical implantation of the stimulating nerve cuff

For the experiments assessing the functional recovery from injury in the IL-6 KO mice and their controls, each mouse was implanted with a chronic stimulating nerve cuff placed on the left common peroneal nerve as previously described (21, 22). Briefly, the nerve cuff was constructed from two teflon-coated, multi-stranded, 90% platinum: 10% iridium wires (0.15-mm diameter). An incision was made through the biceps femoris muscle and the two loops, formed from 2.5-mm segments of deinsulated platinum:iridium wire, were placed around the common peroneal nerve. The proximal end of the nerve cuff was externalized in the dorsal cervical region where it could be connected to a stimulator. Mice implanted with nerve cuffs were allowed 4–5 weeks to recover before being used in the experiments.

Induction of freeze-induced muscle injury

After anesthetization, the mouse's lateral and anterior surfaces of the lower left hind limb were shaved and aseptically prepared. The TA muscle was exposed by making a 1.5-cm-long incision through the skin overlying the muscle belly. Injury was induced by applying a steel probe cooled to the temperature of dry ice to the TA muscle belly for 10 s. The probe consisted of a 6-mm-diameter rod with a rounded tip. Following injury, the skin incision was closed using a 6–0 silk suture and treated with hydrogen peroxide.

Measurement of in vivo muscle strength

Isometric strength of the left anterior crural muscles was measured before and after injury by using a miniature dynamometer as described previously (21–23). Before the first strength measurement, the lateral surface of the lower left hind limb was shaved and aseptically prepared. To electrically stimulate the muscle group, we inserted two 36-gauge needle electrodes percutaneously to a position adjacent to the nerve innervating the anterior crural muscles (i.e., the common peroneal nerve). In experiments with IL-6 KO mice and their controls, the chronically implanted nerve cuff was used for stimulation. Using 200-ms trains of 0.1-ms pulses at 300 Hz, stimulation voltage was adjusted to

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yield the maximal isometric tetanic torque of the anterior crural muscles. Injury to the TA muscle was then induced as described above. Two minutes after injury induction, maximal isometric tetanic torque of the anterior crural muscles was re-measured and the mouse was allowed to recover. Muscle strength was re-measured at either 5 or 13 days after injury induction, except in experiments with IL-6 KO mice, and their controls where strength was measured at 3, 7, and 14 days after injury. The timing of the post-injury measurements was selected based on our exercise-induced model of injury in which at 5 days post-injury, recovery of strength is just beginning, whereas by 2 weeks post-injury, the original strength loss has been halved (24). The anterior crural muscle strength measurement provides a valid assessment of TA muscle function, because the TA comprises 82% of the muscle group mass and contributes 89% of the maximal isometric tetanic torque produced by the muscle group (25; unpublished observations).

Histopathology and immunohistochemistry

Muscles processed for histology and immunohistochemistry were embedded in Tissue Tek OCT (Miles Scientific, Naperville, IL), frozen in melting isopentane, and stored at –80°C. Ten cross sections (10-m-thick) were cut at each of six levels equally spaced along the length of the TA muscle in a microtome cryostat at –20°C. Sections at each level were stained for routine hematoxylin and eosin (H&E) or were used for immunohistological detection of MyoD, myogenin, Mac-1, Mac-3, TNF-α, or desmin. For immunostaining, the sections were fixed initially for 10 min with ice-cold phosphate-buffered saline (PBS) containing 2% formaldehyde followed by a 10-min incubation in PBS with 0.3% H_2O_2 and 0.2% Triton X-100 and then blocked for 30 min using 10% goat serum. Primary antibodies against MyoD, myogenin or desmin, (Santa Cruz, Santa Cruz, CA), and TNF-α (Research Diagnostics, Minneapolis, MN) were diluted 1:100 in PBS containing 1.5% goat serum and 0.2% Tween-20 and applied to the sections for 1 h at room temperature (RT). After washing in PBS with 0.2% Tween-20, a biotinylated goat anti-rabbit secondary antibody (Histostain-Plus kit #85-9243, Zymed, San Francisco, CA) was applied for 10 min. Following application of streptavidin-biotinylated horseradish peroxidase conjugate for 10 min, the antibody-enzyme complex was visualized by using 3,3'-diaminobenzidine as the enzyme substrate. Immunostaining for Mac-1 and Mac-3 was conducted on cryosections that were acetone-fixed and blocked for 20 min in 3% bovine serum albumin. The primary antibodies (BD PharMingin, San Diego, CA) were diluted 1:200 in PBS and applied to the sections for 1 h at RT. After washing in PBS, the link antibody, rabbit anti-rat IgG (Dako, Copenhagen, Denmark) was applied at a 1:50 dilution for 30 min, followed by rat APAAP (Dako) at a dilution of 1:50 for an additional 30 min. The antibody–enzyme complex was visualized by using Fast Red as a substrate (Dako).

Gene expression studies

The whole TA muscle was collected in RNAlater^R (Qiagen, Santa Clarita, CA) and homogenized, and total RNA was extracted by using a commercial kit (Rneasy: Qiagen) following the manufacturer's protocol. RNA purity and concentration were assessed by determining A_{260}/A_{280} absorption. cDNA was synthesized from 1 μg of RNA. PCR amplification was performed as previously described, by using commercially available PCR primers for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) from Clontech Laboratories Inc. (Palo Alto, CA) or custom designed primers for MyoD (26) synthesized by Gibco, Inc. (Grand Island, NY) containing the following base sequences:

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G3PDH: sense, 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3'
antisense, 5'-CATGTGGGCCATGAGGTCCACCAC-3'
MyoD: sense, 5'-CCCCGGCGGCAGAATGGCTACG-3'
antisense, 5'-GGTCTGGGTTCCCTGTTCTGTGT-3'

The PCR products were visualized by ultraviolet illumination after electrophoresis through 2.0% agarose (Ultra-Pure, Sigma, St Louis, MO) at 60 V for 80 min and staining in Tris borate/EDTA buffer (89 mmol/L Tris, 89 mmol/L boric acid, 2.5 mmol/L EDTA, pH 8.2) containing 0.5 mg/ml ethidium bromide. The gels were analyzed by using the Eagle Eye II Image Analysis System (Stratagene) and NIH Image 1.54 software (National Institutes of Health, Bethesda, MD). The area under the curve was normalized for G3PDH content.

Several PCR products were analyzed by real-time PCR by using SYBR^R Green or TaqMan^R PCR method and an ABI Prism^R 7700 Sequence detector (PE Applied Biosystems, Foster City, CA). Briefly, RNA isolated as described, was subjected to DNAse I treatment according to the manufacturer's instructions (Ambion, Austin, TX). DNAse I-treated RNA ((2 mg) was reverse-transcribed by using Reverse transcriptase II^R (Life Technologies, Gaithersburg, MD). Of this mixture, 5 ml was used to conduct PCR reaction according to the manufacturer's instructions. The comparative threshold cycle (C_T) method (user Bulletin #2, ABI Prism^R 7700 Sequence detector (PE Applied Biosystems) was used to calculate the relative concentrations. This method involves obtaining C_T values for the cytokine of interest; normalizing to the housekeeping gene, including G3PDH or 18S/rRNA (TaqMan assay reagents^R, PE Applied Biosystems); and comparing the relative increases between control and experimental samples. Real-time PCR for TNF-α, IL-6, and 18S/ rRNA were performed by using pre-developed primers and probes (TaqMan assay reagents ^{R}, PE Applied Biosystems) on the ABI Prism^R 7700 Sequence detector (PE Applied Biosystems). The primers used for SYBR^R green were:

G3PDH: sense,5'-CCATCACTGCCACCCAGAAGAC-3'
antisense, 5'-ATGACCTTGCCCACAGCCTTG-3'
MyoD: sense, 5'-ACCCAGGAACTGGGATATGGA-3'
antisense, 5'-AAGTCGTCTGCTGTCTCAAA-3'
Myogenin: sense, 5'-GCACTGGAGTTCGGTCCCAA-3'
antisense, 5'-TATCCTCCACCGTGATGCTG-3'
Myf6: sense,5'-GAGATTCTGCGGAGTGCCAT-3'
antisense,5'-TTCTTGCTTGGGTTTGTAGC-3'

Statistical analysis

Experiments were performed routinely with five or more mice per group with values presented as mean ± SE. All studies were replicated with representative data shown. The strength differences among the groups of mice at various time points after injury were analyzed by two-way (group × time) repeated ANOVA measures, with time as the repeating factor. When significant interactions were found, we used single degree-of-freedom contrast as post-hoc test (25). Differences in initial strength, body weight, and PCR signals between control and treatment groups were determined by one-way ANOVA and Student-Newman-Keuls post hoc tests when applicable.

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RESULTS

Histopathology and immunohistochemistry

H&E staining indicated that approximately 60% of the TA muscle was affected by the freeze injury. Approximately three-quarters of the muscle cross section was damaged directly underneath where the steel probe had been applied, while the deepest one-quarter of the muscle cross-section appeared histologically normal. By day 2 post-injury, considerable inflammatory cell infiltration was apparent in the injured muscles (Fig. 1A). The infiltration was generally restricted to the damaged muscle region adjacent to the deep, uninjured region. The inflammatory cells stained positive for Mac-1 (or Cd11b/CD18), a member of β2-integrin family of receptors involved in leukocyte recruitment (Fig. 1C). Additionally, most of the inflammatory cells were positive for Mac-3, a marker of activated monocytes/ macrophages (Fig. 1D). In the infiltrated region, MyoD and myogenin staining of nuclei were observed infrequently. These nuclei belonged presumably to activated myoblasts. By day 3 post-injury, the number of infiltrating inflammatory cells in the damaged muscle reached near peak levels, as did the number of presumptive myoblast nuclei in the infiltrated region that were MyoD- or myogenin-positive (Fig. 1E and F). By 5–7 days post-injury, the inflammatory cells had progressed to more peripheral portions of the damaged muscle, while the presumptive myoblast activation paralleled the peripheral migration. Regenerated myofibers, identified by their central nuclei, were also apparent by this time in the deeper injured muscle region. By 13 days-post injury, the injured muscles showed minimal signs of inflammation or degenerating fibers (data not shown). Most of the muscle cross section was composed of centronucleated myofibers of near normal diameter. At this time point, most of the nuclei that were MyoD- or myogenin-positive were associated with centronucleated myofibers and exhibited low staining intensity.

Expression of TNFα and IL-6

RNA, isolated from control or injured TA muscles, was examined for TNF-α and IL-6 transcripts by TaqMan real-time PCR following muscle freeze injury. Uninjured muscle expressed low constitutive levels of TNF-α and IL-6 m RNA (Fig. 2). An increase in TNF-α expression occurred within 5 h following injury and was observed to increase maximally at 24 h after injury. The rapid increase in TNF-α mRNA transcript levels was followed by a gradual reduction over days 3–7 and a return to control levels by day 13. The time course of IL-6 expression in TA post-injury was similar to that for TNF-α expression.

Immunostaining was conducted to localize TNF-α protein within the TA muscle following freeze injury. Immunoreactive TNF-α was not detected in control muscles (Fig. 3A), while readily observed in the damaged muscle region adjacent to the deep, uninjured region. TNF-α was localized primarily within infiltrating inflammatory cells as granular cytoplasmic staining on days 2 and 3 (Fig. 3B). On days 5 and 7, inflammatory cell staining was decreased while slight cytoplasmic staining appeared in the regenerating myofibers (Fig. 3C and E). Immunostaining for desmin, an intermediate filament protein in myogenic cells, distinguished myofibers from inflammatory cells (Fig. 3D). There was no detectable TNF-α staining in the TA muscles on day 13 post-injury (data not shown).

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Histopathology and associated myogenic regulatory factor expression in TNF–α-deficient mice

To evaluate the role of TNF-α in muscle injury and recovery, we examined TNFR-DKO mice in parallel with wild- type mice. We found no remarkable differences between the wild-type and TNFR-DKO mice in routine histopathology at any time point examined after muscle injury (data not shown). Similarly, there appeared to be no differences between the two groups in the expression of the myogenic regulatory factors MyoD or myogenin, based on immunohistology (data not shown). However, when examined by conventional RT-PCR and semiquantitative real-time PCR SYBR Green, the expression of MyoD was moderately but consistently reduced in TNFR-DKO mice. Figure 4A, B shows MyoD expression at day 3 post-injury, the day of peak response, by using PCR and SYBR Green methods, respectively. To confirm that the effect is associated with the TNF-α response and not with differences in the genetic background between knockout and wild-type mice, we administered neutralizing antibodies to TNF-α to wild-type mice before freeze injury. Although not to the same degree as transgenics, MyoD expression was reduced by neutralizing antibodies to TNF-α (Fig. 4B). In contrast to the TNF–α-deficient mice, MyoD expression in injured TA muscles of IL- 6 KO mice was similar to that of the wild-type mice (Figure 4A). These results demonstrate that the TNF–α-deficiency leads to moderate but consistent depression of MyoD expression. Myogenin and MRF-4 expression, measured by SYBR Green, was increased in TA muscles at 3 and 5 days post-injury but to a similar degree in the wild-type, TNFR-DKO, and anti-TNF antibody-treated mice (data not shown).

In vivo muscle strength

Before injury induction, anterior crural muscle strength, as assessed by the maximal isometric tetanic torque measurements was 16–19% lower ($P<0.05$) in TNFR-DKO mice compared with the C57BL/6 wild-type mice and independent of administration of TNF–α-neutralizing antibodies (i.e., 2.12–0.07 N mm for the TNFR-DKO mice vs 2.53–0.08 and 2.63–0.11 N·mm for the C57/BL and C57/BL + TNF antibody-treated mice, respectively). These differences occurred despite the fact that total body weight was similar in all 3 groups ($p=0.67$) and are consistent with the proposed role of TNF-α in skeletal muscle development (19). Body weights for the C57BL/6, TNFR-DKO, and C57BL/6 + TNF-α antibody-treated mice were 21.3–0.2, 20.9–0.3, and 21.2–0.3 g, respectively. Two minutes after injury induction, muscle strength was reduced by 68% (Fig. 5). There were no significant differences among groups in this strength loss ($P=0.83$). By day 5 post-injury, strength had begun to recover, but there were no differences among the three groups in this recovery ($P=0.38$). At 13 days post-injury, strength for the C57BL/6 mice had recovered substantially and was only 13% lower than it was before injury. However, strength for the TNFR-DKO and TNF-α antibody-treated mice had not recovered as much by day 13 post-injury ($P=0.006$) and was still 27–31% below pre-injury levels (Fig. 5A). Muscle strength for the IL-6 KO mice recovered from injury at a rate identical to that for the C57BL/6 mice ($P=0.88$) (Fig. 5B). In these experiments, strength recovered at a slower rate in control mice compared with that observed in the TNF-α experiments, presumably because the IL-6 KO mice and their controls were older (i.e., 9–10 months vs 2 months).

DISCUSSION

Like other cytokines, TNF-α confers its signals to target cells through binding to one of two specific cell surface membrane receptors, referred to as TNFR1 and TNFR2 (27). TNFR1 is constitutively

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expressed in most cell types, including myocytes (12), and historically has been considered the main mediator for TNF responses, although a number of responses are also associated with TNFR2 (24). In the current studies we demonstrate, using real-time PCR and immunohistochemical analysis, that traumatic skeletal muscle injury-induced by freezing is accompanied by an early increase in TNF-α expression characterized by a single peak that is maximal at 24 h following injury. This finding is consistent with previous studies showing increased TNF-α levels in the rat soleus and extensor digitorum longus muscles following notexin-induced necrosis (28). In this latter model, a marked increase was observed the first day after administration of the toxin, when fibers were most necrotic and a second, but much smaller increase in TNF-α mRNA transcripts was observed at the time when newly formed muscle fibers became reinnervated. This was in contrast to the freeze-injury of the rat soleus in which a second peak was not observed.

Collins and Grounds (29), performing immunohistological assessment, established the presence of increased immunoreactive TNF-α in the TA muscle following crush injury or autograft transplant. They observed that TNF-α was localized to inflammatory cells as well as atrophic muscle fibers and muscle spindles. We observed similar TNF-α staining characteristics in mice that underwent freeze-injury. Immunostaining was localized predominantly within inflammatory cells at the early time points (2–3 days post-injury), and moderate staining was observed in regenerating myofibers at later time points. Myoblasts have been shown to express TNF-α *in-vitro* in the early stages of differentiation (18), although, compared with monocytes, the levels expressed are likely to be lower. The TNF-α response coincided with inflammatory cell infiltration, which was predominantly associated with Mac-3-expressing macrophages. Mac-3 is a surface antigen expressed on activated monocytes and macrophages (30). Macrophages are thought to serve traditionally as scavengers of tissue debris during muscle degeneration-regeneration processes; only recently has it been suggested that they are associated with enhanced satellite cell proliferation and delayed myocyte differentiation (31). TNF-α is a major regulator of inflammatory responses and influences the inflammatory cell influx, in part, by activating chemokines and adhesion molecules. In the present study the influx of inflammatory cells in TA freeze injury model was not affected significantly by blocking TNF-α's effects, suggesting a minor role, at best, in regulating monocytes/macrophages accumulation in muscle damage or that compensatory mediators are present.

To determine the functional role of TNF-α in traumatic muscle injury, muscle function was monitored in freeze-injured mice genetically deficient for the two TNF-α receptors or rendered TNF–α-deficient with neutralizing antibodies. We hypothesized originally that, similar to liver injury, TNF-α would exacerbate the early degenerative process and enhance the subsequent regenerative process. For example, the hepatotoxins amantadine and actinomycin D, which function as transcriptional inhibitors, mediate liver toxicity directly through excess TNF-α activity; whereas TNF-α stimulates hepatocyte proliferation and liver repair following partial hepatectomy (32) or CCl_4-induced hepatotoxicity (33). However, we observed that only the repair process was affected by TNF-α following muscle injury. TNF-α provides growth-modulatory and differentiation activities for many cell types. Repair processes associated with TNF-α normally occur indirectly, through the ability of TNF-α to stimulate growth-promoting factors (reviewed in 9). In this respect, IL-6, which is induced by TNF-α (8), is mitogenic for both hepatocytes and keratinocytes, required for normal liver regeneration (34) and wound healing (35), respectively. To determine whether IL-6 was involved in TNF–α-associated muscle repair, we studied IL-6–deficient transgenic mice. Based on the observation of a normal strength recovery in the IL-6 KO mice, IL-6 does not appear to play a

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significant role in the repair process, although the cytokine is expressed at high levels following injury in wild-type mice.

Increasing evidence from *in vitro* studies suggests a role for TNF-α in modulating myogenesis. In contrast to the catabolic effects of TNF-α on differentiated myotubes (36, 37), recently it has been shown that TNF-α is required during the early period of myoblast differentiation (18). Consistent with these *in vitro* data, the present studies demonstrate that TNF-α plays a role in the recovery of muscle function, although this was not observed histologically. Previous histological studies using TNF–/– mice also failed to identify differences in muscle regeneration when compared with wild-type mice (29); in this respect, discrepancies between muscle function and histopathological findings have been observed (38). It is possible that moderate dysregulation in myogenic transcription factor expression or muscle metabolism might account for contractile dysfunction without significant differences in the muscle histopathology.

Nonetheless, the current studies indicate that TNF-α is involved in the recovery of muscle function after traumatic muscle injury, and this effect might be associated with modulation of muscle regulatory genes, including MyoD (Fig. 6). Effects on neuro-muscular signaling or muscle energy supplies may also contribute to TNF–α -mediated muscle strength recovery. It is important to note, however, that the absence of TNF-α only moderately affected the muscle injury process and, in the case of regeneration, only delayed recovery. This would suggest that redundant mechanisms and/or factors might exist. An important question that still remains is the role that inflammatory processes and mediators play in the repair of injured muscle.

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Fig. 1

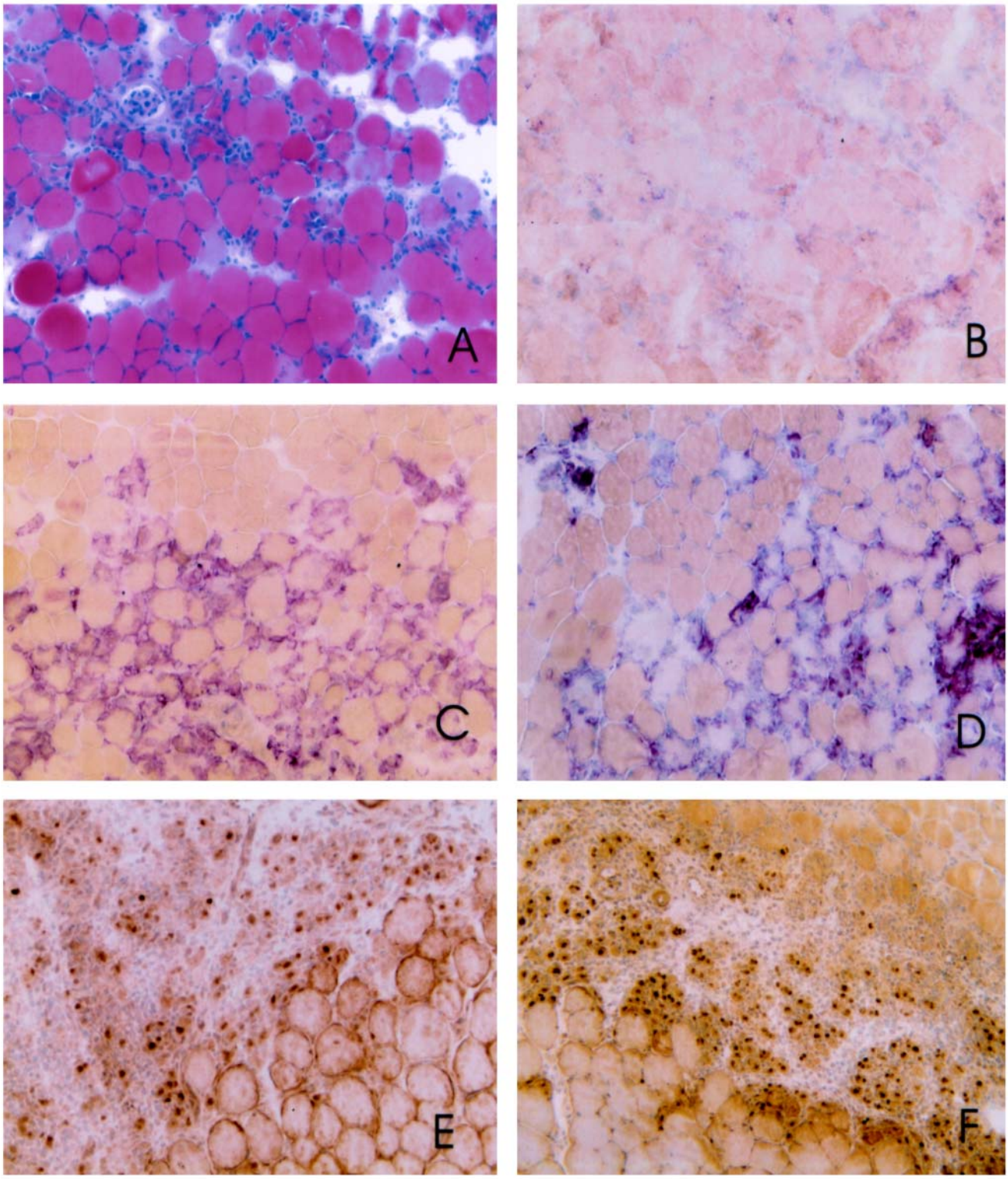

Figure 1. Microscopic appearance of TA muscle at 2 or 3 days following the freeze injury. A) H&E staining of frozen transverse sections of TA muscle at two days after injury. **B)** TA muscle section treated with normal rabbit serum used as a negative control for immunohistology. **C)** Mac-1 immunostaining on frozen transverse sections of TA muscle two days after injury. **D)** Mac-3 immunostaining of frozen transverse sections of TA muscle two days after injury. **E)** MyoD immunostaining of frozen transverse sections of TA muscle three days after injury. **F)** Myogenin immunostaining of frozen transverse sections of TA muscle three days after injury. Magnification 200×.

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Fig. 2

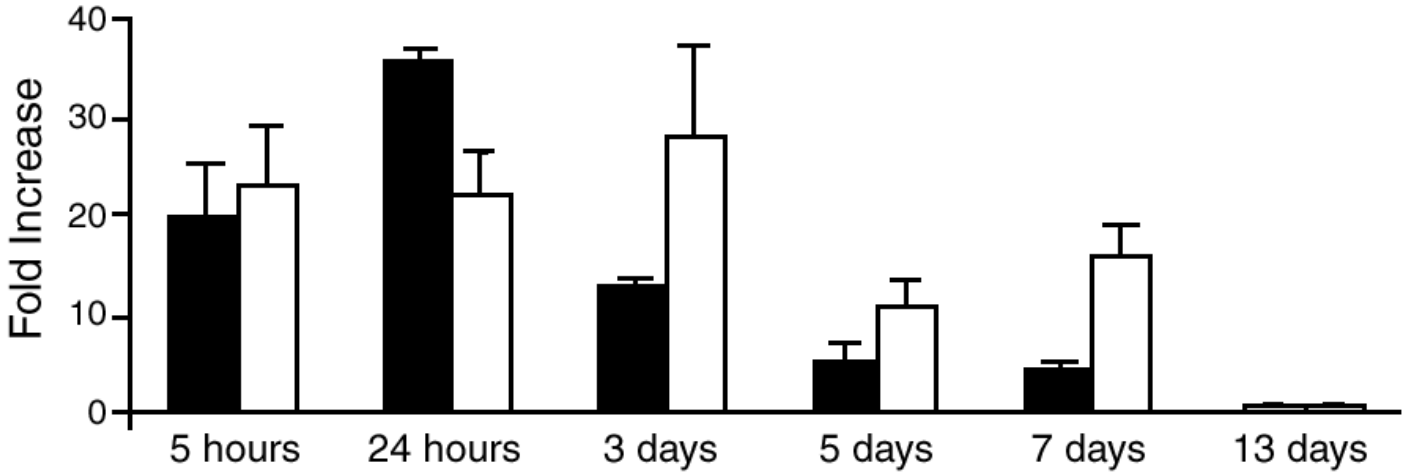

Figure 2. Time course for TNF-α (black) and IL-6 (white) expression in muscle tissue after freeze injury. The TA muscle was obtained from control and injured mice at the times indicated and was analyzed for TNF-α mRNA transcripts by using realtime PCR. TNF-α and IL-6 expression was normalized to 18S RNA samples from corresponding samples and was presented as fold-increase from controls by using 2–3 animals per time point.

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Fig. 3

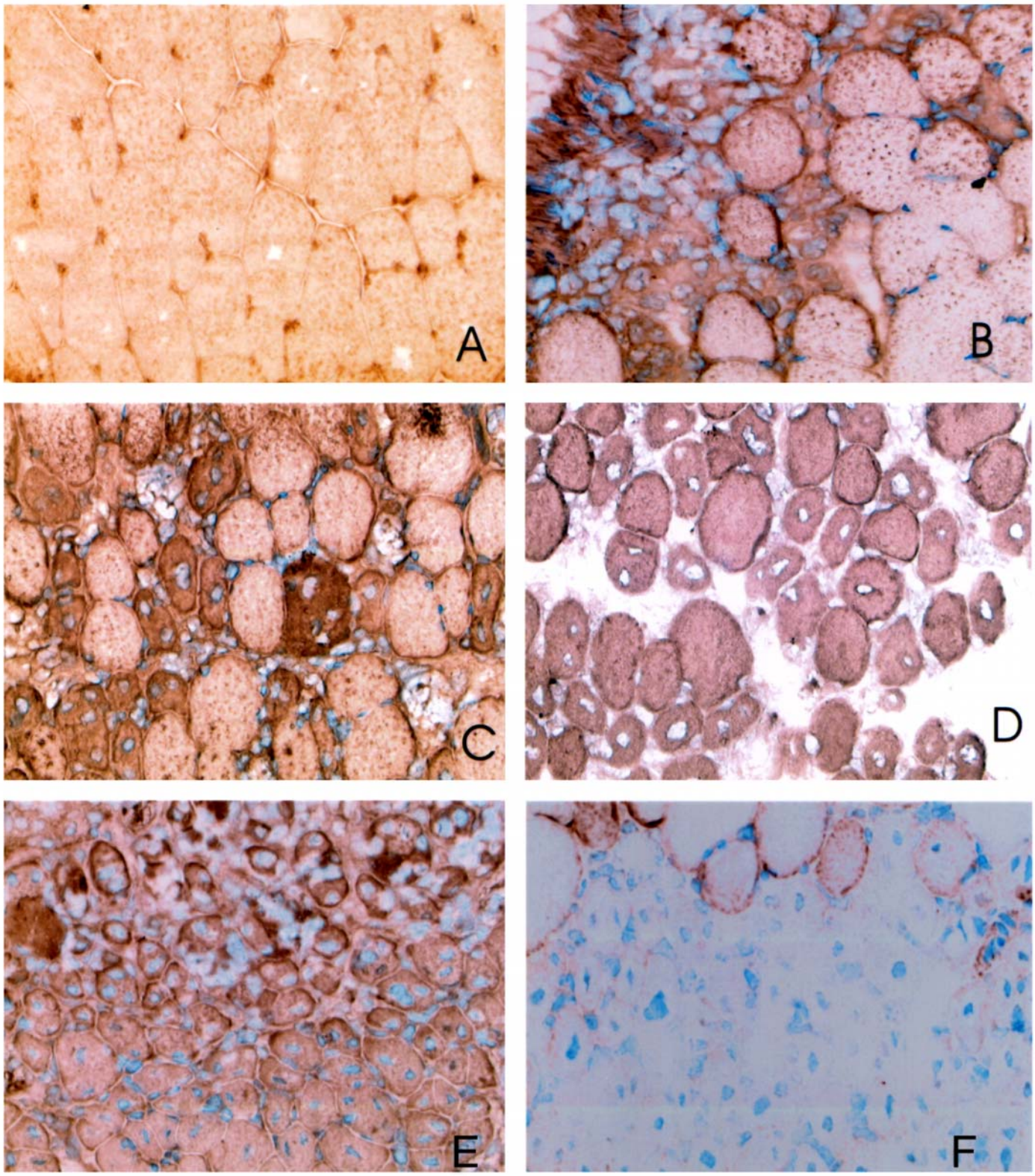

Figure 3. Time course for TNF-α immunostaining in injured TA muscle. A) Control muscle. **B)** TNF-α 3 days after injury. **C)** TNF-α 5 days after injury. **D)** Desmin immunostaining 5days after injury. **E)** TNF-α 7 days after injury. **F)** Negative control stained with normal rabbit serum. Magnification 400×.

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Fig. 4

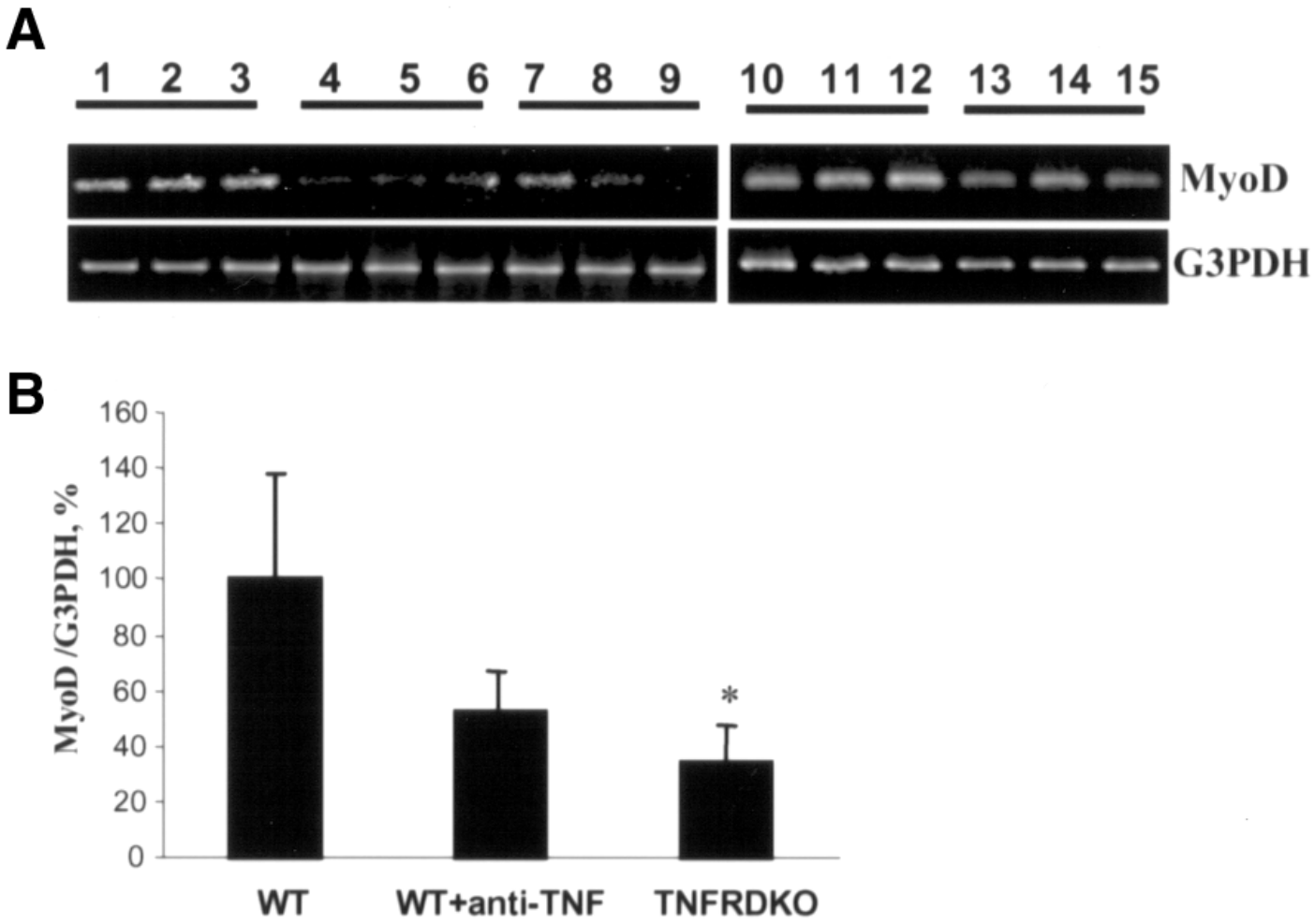

Figure 4. Expression of MyoD mRNA in TA muscle 3 days after freeze injury as assessed by RT-PCR and SYBR Green PCR methods. A) RT-PCR determination of MyoD (G3PDH expression was used for normalization) in individual mice. Lanes 1–3, wild-type mice; lanes 4–6, TNFR-DKO mice; lanes 7–9, TNF-α antibody-depleted mice; lanes 10–12, wild-type mice; lanes 13–15, IL-6 KO mice. **B)** MyoD mRNA transcripts determined by SYBR green real-time PCR analyses and presented as an inhibition from the expression in WT injured TA. Each value represents the mean of three mice per group. * Significantly different from the injured/wild-type mice at $P<0.05$.

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Fig. 5

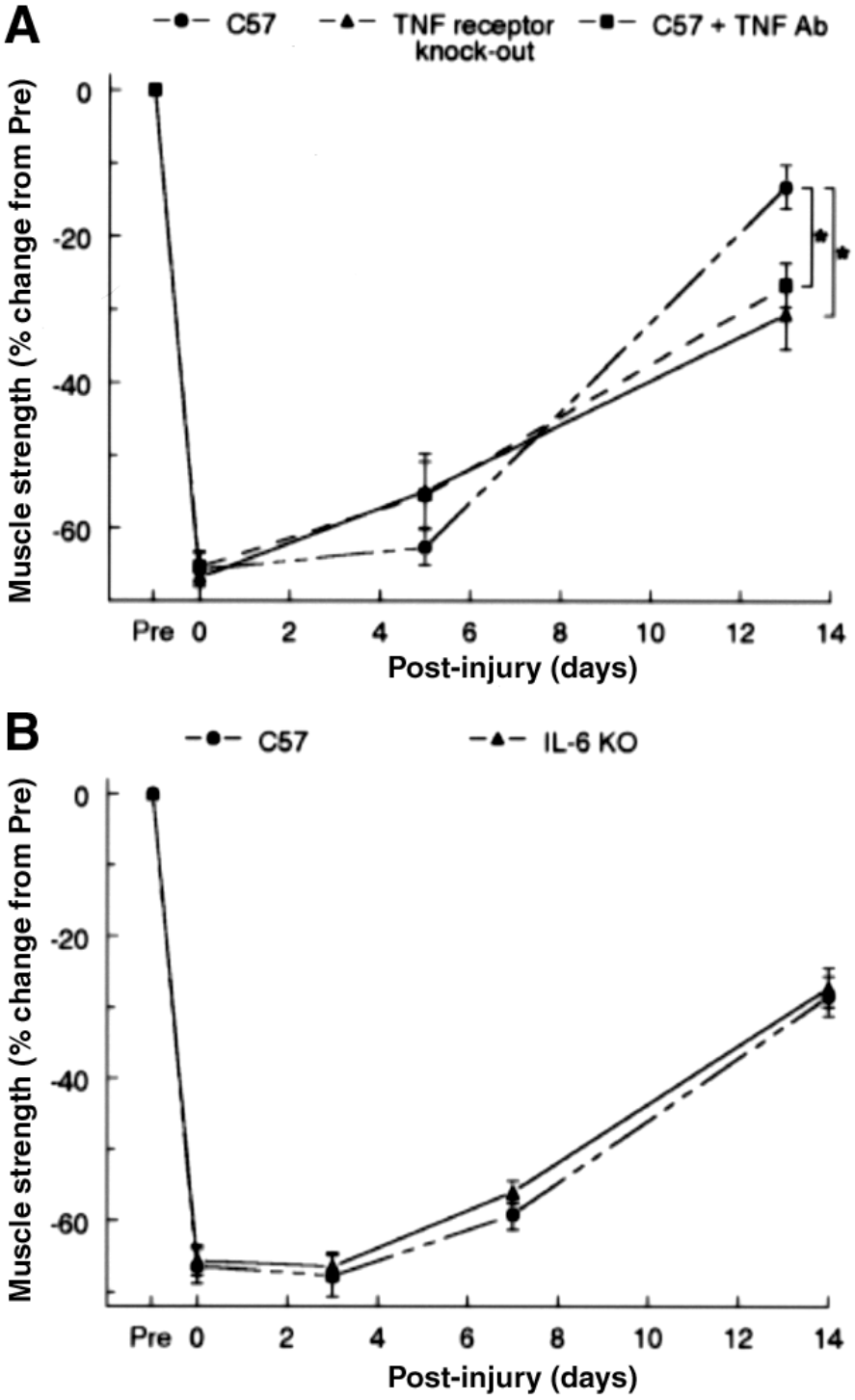

Figure 5. Effect of TNF-α (A) and IL-6 (B) deficiency on anterior crural muscle strength following freeze-injury. A) Strength of the left anterior crural muscles in C57BL/6 wild-type (●), TNFR-DKO (▲) and anti-TNF-α antibody-treated (■) mice following injury. Each value represent mean +/– SE. of 6–8 mice per group. *Significantly different from injured/wild-type mice at $P<0.05$. **B)** Strength of the left anterior crural muscles in C57BL/6 wild-type (●) (n=12) and IL-6 KO (▲) (n=10) mice following injury.

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Fig. 6

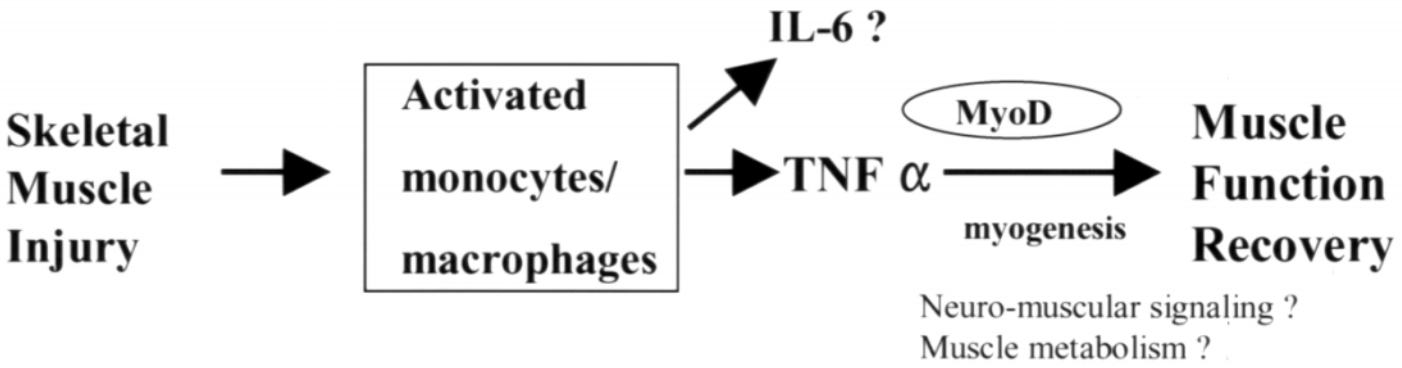

Figure 6. Schematic summary. The injury of skeletal muscle results in macrophage accumulation and expression of two inflammatory cytokines, TNF-α and IL-6. TNF-α, but not IL-6, modulates MyoD expression and muscle function recovery.

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