Original Article
IL-6 improves myogenesis in long-term skeletal muscle atrophy via the JAK/STAT3 signalling pathway

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Abstract: Objectives: To investigate the roles of macrophages and interleukin-6 (IL-6) in the regeneration of atrophic muscles. Results: In vitro, Satellite cells (SCs) cultured with DMEM, IL-6, IL-6+AG490, which is an inhibitor of IL-6 by JAK/STAT3 pathway, or co-cultured with macrophages. In vivo, the denervated gastrocnemius muscles were injected with DMED, IL-6, IL-6+AG490 or macrophages. The administration of IL-6 up-regulated the expression of myogenic markers during muscle regeneration both in vitro and in vivo, whereas this function was remarkably inhibited by the application of AG490. The expression of myogenic markers was low in the SCs when co-cultured with macrophages in vitro. However, when macrophages were transplanted into atrophic muscles, the expression of the myogenic markers in the SCs was enhanced. Conclusions: IL-6 improves myogenesis in long-term skeletal muscle atrophy, at least in part, via the JAK/STAT3 signalling pathway. Macrophages might stimulate muscle regeneration in vivo through an indirect approach.

Keywords: Interleukin-6 (IL-6), macrophage, satellite cells, signal transducer and activator of transcription-3 (STAT3), skeletal muscle atrophy

Introduction
Muscle atrophy is a common clinical problem that is often associated with three conditions: chronic diseases (cachexia), disuse conditions (atrophy), and aging (sarcopenia) [1]. Muscle atrophy is characterized by a substantial decrease in muscle mass, fibre cross-section area, myonuclear number, protein content, and power output [2-5]. Beyond a poor functional status and quality of life, muscle atrophy also increases the risk of death by exacerbation of primary illness and increases the risk of developing secondary medical complications [6].

As an important target of peripheral nerves, muscles undergo atrophy after peripheral nerve injury via a complex and multifactorial pathophysiological process [7]. The regeneration of skeletal muscles relies on satellite cells (SCs) that are located beneath the basal lamina of the muscle fibre membrane [8, 9]. After skeletal muscle injury, SCs are activated and proliferate to form a new myofibre or fuse with existing myofibres to support growth [10-12]. During the early phase after denervation, although the mass and weight of the muscle is significantly reduced, the number of SCs in the muscle is increased in both rats and humans [13-15]. Long-term denervation is harmful to the regeneration of skeletal muscles due to progressive muscle atrophy, fibrosis and irreversible pathological changes [16]. Furthermore, during long-lasting denervation, persistent activation of the SCs involved in muscle regeneration may lead to a decrease in and even exhaustion of SCs [13].

Recently, many studies have demonstrated that various inflammatory cells and inflammatory cytokines are associated with muscular regeneration, including neutrophils, macrophages, transforming growth factor-beta (TGF-β), interleukin-10 (IL-10) and interleukin-6 (IL-6) [5, 17, 18]. IL-6 is a multifunctional cytokine that is produced at local tissue sites and released into the circulation [19, 20]. Unlike other cytokines, IL-6 is involved not only in pro-inflammatory but also in regenerative processes and the regulation of metabolism [21, 22]. Moreover, an increasing number of studies have shown that the muscle cells are a source of IL-6. After
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30 min of exercise, the IL-6 in skeletal muscle is up-regulated [23, 24]. Begue and colleagues found that the IL-6/STAT1/STAT3 signalling pathway plays an important role in regulating the SC pool and muscle hypertrophy after resistance exercise [25]. IL-6-deficient mice exhibit abrogated SC proliferation and myonuclear accretion in pre-existing myoblasts due to reduced activation of STAT3 [26]. After muscle injury, the level of STAT3, a downstream effector of IL-6, was elevated, and muscle wasting was noted [27, 28].

In addition, an increasing number of studies have reported the emergence of multiple properties of macrophages, showing that they are essential regulators of development, tissue homeostasis, and remodelling [29]. However, macrophages are also present during the process of tissue repair and/or regeneration [30, 31]. After acute skeletal muscle injury macrophages infiltrate the site of injury, whereas muscle regeneration is delayed due to impaired macrophage functions in CX3CR1-deficient mice [32-34].

In the present study, we explored the function of IL-6 and macrophages during muscle regeneration in long-term denervated skeletal muscle.

Materials and methods

Animals and denervation model

Male SD rats (4 weeks) were used for the denervation models. All animal experiments were approved by the Experimental Animal Center of Tongji Medical School, Huazhong University of Science and Technology. Denervation was induced by transection of the sciatic nerve. The cut nerve end was diverted into the gluteus muscle to ensure that the nerve stumps did not re-innervate. After the operation, the animals were housed for 8 weeks with littermates and were maintained at 22°C under 12-h light/12-h dark cycles with ad libitum access to chow diet and water. At 2 months post-operation, the gastrocnemius muscles of the rats were injected with Dulbecco's Modified Eagle’s Medium (DMEM; Gibco, CA, USA), IL-6 (1 M, 20 μl; PEPTRech, USA), AG490 (2 M, 10 μl; Sigma, Santa Clara, CA, USA) with IL-6 (1 M, 10 μl), or macrophages (5×10⁶). According to the different injections, the rats were divided into four groups as follows: the control group (DMEM), IL-6 group (IL-6), AG490 group (IL-6+ AG490), and macrophage group (macrophages). After the injections, the rats resumed normal activities for 3 days until the experiment was suspended.

Cell culture

The SCs were isolated from neonatal SD rats as previously described with slight modifications [35]. The forelimbs and hind limbs were removed from the neonatal rats, and the bones were isolated by dissection. The remaining muscle mass was minced into 1-mm³ pieces using razor blades. The minced muscle was dissociated with 0.2% collagenase (Collagenase Type II, Sigma, Santa Clara, CA, USA) and 0.4% dispase (Roche, Basel, CH) for 60 min in a 37°C water bath. The supernatant was first passed through a 150-μm filter (Millipore, MA, USA) and then through a 38-μm filter (Merck-Millipore, MA, USA). Subsequently, the tissue fragments were collected by centrifugation at 1000 rpm for 5 min and resuspended in DMEM (Gibco, CA, USA) containing 10% foetal bovine serum (FBS) (Gibco, CA, USA) and 100 U/ml penicillin-streptomycin (Thermo Scientific, Waltham, MA, USA). Preplate (PP) 1 represents a population of muscle-derived cells that adhered during the first hour after the isolation, PP2 in the next 2 h, PP3 in the next 24 h, and the subsequent preplates were obtained at 24-h intervals (PPs 4-6). The myogenic population in each flask was evaluated by desmin staining.

Peritoneal suspensions were obtained 3 days after injection using 2 ml of enriched thioglycollate broth (Merck-Millipore, MA, USA). Briefly, 10 ml of DMEM was infected i.p., and then

| Table 1. Primer sequences used for the T-qPCR assays |
|----------|-------------------------|-------------------------|
| Gene     | Forward primer (5'-3')  | Reverse primer (5'-3')  |
| MyoD     | AACGCCATCGCTACATTG      | GCACCCTCCCTGGTGCTGG     |
| Pax7     | CTGTAAGCAGCTGTCAGTC     | GCCAGAAGGTGTTGAAAGG     |
| Desmin   | CTAACCTGGGAGATGTGAC     | CGATGTGTCTGAGTAACCATC   |
| Myosin   | CACCCCGCTCAACACCCA      | TCTCCACCGCTGGTCCTCAT    |
| F4/80    | CAAGGATACGAGGTGCTGAC    | TGAAGGCTGTGATAGTGTGGA   |
| CD206    | GGTAGGGACGGAAGCAAGTC    | TATGTCCTGAGCATGAAGT    |
| INOS     | TTAAAGCGAGTTGAGTTGATGT  | GTGAGGCCCTGCTGAGTGA     |
| β-actin  | GTCACCAGCAAATGCTTCTA    | TGCCTGACCTCCTACCGTTC    |
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The abdomen was massaged and peritoneal exudate cells, consisting of lymphocytes and macrophages, were collected, with 90-95% recovery of the injected volume. Macrophages, which were identified based on morphology and CD11b staining, were counted and adjusted to 5×10⁵ cells/ml in DMEM. Cellular viability was routinely measured before and after each experiment by the trypan-blue (Solarbio, Beijing, China) exclusion test. In all cases, the viability was higher than 95%. All incubations were performed at 37°C in a humidified atmosphere with 5% CO₂.

The SCs were divided into six groups as follows: control group (SCs cultured in DMEM containing 10% FBS), IL-6 group (cultured in DMEM containing 10% FBS and 1 M IL-6), AG490 group (cultured in DMEM containing 10% FBS, 1 M IL-6 and 2 M AG490), MACR group (co-cultured with 5×10⁵ macrophages), Macrophage+IL-6 group (MI group) (co-cultured with 5×10⁵ macrophages and a culture medium containing 0.1 µM IL-6), and Macrophage+IL-6+AG490 group (MIA group) (co-cultured with 5×10⁵ macrophages and a culture medium containing 10% FBS, 0.1 µM IL-6 and 0.1 µM AG490).

RNA extraction and RT-qPCR assays

The biopsy sections were ground into a powder with a mortar pre-cooled with liquid nitrogen. Total RNA was then isolated using TRIzol reagent (Takara, Japan). The RNA concentration and purity were measured using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). The RNA was reverse-transcribed with a First Strand cDNA Synthesis Kit (Toyobo, Japan). The resulting cDNA was quantified by RT-PCR using THUNDERBIRD SYBR qPCR Mix (Takara, Japan). The pairs of oligonucleotide primers used are shown in Table 1. All procedures were conducted according to the manufacturer’s instructions.

Protein isolation and western blot analysis

The biopsy samples were homogenized in ice-cold lysis buffer (ASPEN, Wuhan, China) with 1 mM phenylmethylsulphonyl fluoride (ASPEN, Wuhan, China) and 1% (vol/vol) protease Inhibitor Cocktail (Roche, Germany). The homogenates were rotated for 30 min at 4°C and centrifuged at 12000 g for 15 min at 4°C. The protein concentration of the resulting supernatant was determined using the BCA protein assay kit (ASPEN, Wuhan, China). Next, the samples (40 µg protein/lane) were separated by SDS-PAGE and transferred onto a PVDF membrane (Merck-Millipore, MA, USA). After blocking in 7.5% non-fat milk Tris-buffered saline with Tween (ASPEN, Wuhan, China) for 2 h at room temperature, the membranes were incubated overnight at 4°C with rabbit primary antibodies against myod (1:1000, Abcam, Cambridge, UK) pax7 (1:1000, Abcam, Cambridge, UK), desmin (1:1000, CST, Boston, USA), myosin (1:2000, Abcam, Cambridge, UK)

Figure 1. The immunofluorescence staining of macrophages and muscle satellite cells. The cell nucleus was stained with DAPI (blue). A. To identify the purity of macrophages obtained from peritoneum. The macrophages were immunostained with cd11b (green). More than 95% of cells were macrophages. B. Muscle satellite cells were stained with desmin (green). The purity of the SCs was nearly 100%.
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and GAPDH (1:10000, Abcam, Cambridge, UK). The membranes were washed with TBST and incubated with species-appropriate horseradish peroxidase-conjugated secondary antibodies. The immunoreactive proteins were detected with the ECL Western blotting detection system (ASPEN, Wuhan, China), and the intensity of bands was quantified using AlphaEaseFC.

Cell proliferation assay

Cell viability was assessed using a Cell Counting Kit (CCK)-8 (Beyotime, Shanghai, China). Briefly, 3*10^3 SCs were seeded onto each 96-well plate, and according to the above groups, IL-6, AG490, or macrophages were added to the wells. After 3 days of incubation, the culture medium was removed, and CCK-8 reagent (10 μl in 100 μl DMEM) was added to each well and incubated for 4 h at 37°C. The optical density at 450 nm was determined in each well by a microplate reader (DR-200Bs, Diatek, Wuxi, China). Experiments were performed in triplicate and repeated at least three times.

Immunofluorescence

For immunofluorescence staining, each muscle sample was embedded in Tissue-Tek Optimum Cutting Temperature (OCT) (Sakura, USA) compound freezing medium. Serial cross-sections (10 mm thick) were labelled using indirect immunofluorescence techniques. The sections were permeabilized with 0.3% Triton X-100 (Sigma, China) in PBS, blocked with 10% FBS for 1 h and incubated with a primary antibody overnight at 4°C. On the following day, the slides were washed three times with PBS and then incubated with the secondary antibody for 1 h at 37°C. The slides were again washed three times with PBS in the dark and stained with DAPI (Roche, Germany) for 15 min to label the nuclei. PBS was substituted for the primary antibody in the same slides as a negative control. The primary antibodies included myod (Abcam, UK) pax7 (Abcam, UK), and desmin (CST, USA). The secondary antibodies were conjugated to Alexa Fluor 488 (donkey anti-mouse or donkey anti-rabbit, Invitrogen, USA). The immunofluorescence reactions were examined using a Nikon confocal microscope or OLYMPUS inverted microscope.

Statistical analysis

The data are presented as the mean ± SEM. The differences among different groups were analysed by one-way ANOVA with Scheffe's
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Results

The molecular features of SCs cultured with IL-6 and macrophages

To verify the characteristics of macrophages and SCs, the cells harvested from rats as described previously were stained with CD11b and desmin, respectively. Immunofluorescence showed that the purity of the cultured cells was greater than 95% (Figure 1).

To analyse the effect of IL-6 and macrophages on SCs, the expression levels of several markers related to SC proliferation and differentiation were first investigated at the mRNA level. The PCR results (Figure 2) showed that the expression levels of myod, desmin, and pax7 decreased in SCs co-cultured with macrophages compared to the control group. However, the expression of these myogenic markers was clearly higher in the IL-6 group than in the other groups. The protein expression levels of these markers were also detected by western blotting. Although the expression patterns revealed by the western blotting results were similar to those determined by PCR, no statistically significant differences were found among these groups (P=0.25). However, after the SCs were co-cultured with macrophages, the expression levels of Myod, desmin, pax7 and myosin were down-regulated at both the mRNA and protein levels (Figure 3A).

IL-6 promotes the proliferation of SCs via the JAK/STAT3 signalling pathway

As shown above, IL-6 promoted the proliferation of SCs. We hypothesized that the JAK2/STAT3 signalling pathway, which is an intracellular mediator of IL-6 signalling [36], might play an important role in this process. To explore the function of JAK2/STAT3 in the above results, we employed AG490 to inhibit the JAK2/STAT3 pathway. As expected, the function of IL-6 was negatively regulated by AG490. The expression levels of myod, pax7, desmin and myosin at the mRNA and protein levels were down-regulated compared to the control group (Figure 4). However, the expression levels of all markers in the AG490 group were still higher than those in the control group. This result implied that IL-6 regulated the proliferation of SCs, at least partly, via the JAK2/STAT3 pathway. The western blot results are shown in Figure 3A.

The effect of macrophages and IL-6 on SCs

After co-culture with macrophages and IL-6, the SCs displayed completely different alterations. To further explore the collective effect of macrophages and IL-6 on SCs, we added another test. The Mann-Whitney test was used to compare two groups. Statistical significance was accepted at P<0.05. All statistical tests were performed using GraphPad Prism 6 software.

Figure 3. Protein expression levels of MyoD, Pax7, desmin and myosin were assayed using western blotting. Quantification of the western blot results was performed using AlphaEaseFC, and the expression levels were normalized to the internal control GAPDH. Comparisons between two groups (A vs. B, B vs. C, C vs. D) were performed using a t test. *P<0.1, **P<0.01, ***P<0.001. A. Protein expression levels in SCs in vitro. B. The protein expression levels of SCs in atrophic muscles. C. The fold induction of SC protein expression.
two groups, the MI group and the MIA group. These two groups were compared with the MACR group; and the results are shown in Figure 5. Myosin expression did not differ significantly among the three groups. In the MI group, myod, pax7 and desmin were down-regulated. When AG490 was added to the culture medium, the expression of myod and pax7 returned to the same level observed in the MACR group. However, desmin remained lower in the MIA group than in the MACR group. The western blot results are shown in Figure 3A.

**Different groups of macrophage phenotypes**

To explore whether IL-6 could alter macrophage phenotypes, F4/80, INOS and CD206 were selected for the phenotypic analysis. The results lower in the AG490 group than in the IL-6 group, although they were significantly up-regulated compared with the control group. Interestingly, the function of macrophages in muscle differed from the in vitro experiment. When SCs were co-cultured with macrophages, specific myogenic mRNAs exhibited low expression levels. However, when macrophages were injected into long-term atrophic muscles, the expression levels of myod, pax7 and desmin were higher than in the control group, and there were no significant differences in myosin between the MACR group and the control group. The western blot results were similar to the PCR results. Myogenic protein expression was highest in the IL-6 group, followed in decreasing order by the AG490 group, the macrophage group and the control group (Figure 4B and
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Discussion

Denervation induces skeletal muscle atrophy. A number of clinical diseases can cause denervation, such as trauma, motor neuron disease, systemic diseases and infections [37-40]. However, there is currently no reliable treatment to prevent muscle atrophy or promote muscle regeneration. Therefore, effective therapies to treat muscle wasting are needed.

Inflammation can aggravate the injury and clear the tissue debris generated by the impaired tissues. However, in recent decades, a large amount of evidence has shown that inflammatory processes play a positive role in tissue regeneration. Various inflammatory cells and inflammatory cytokines are involved in the regeneration of skeletal muscles, such as neutrophils and macrophages, interleukin 4, interleukin 6 and interleukin 10. Specifically, macrophages play a major role in the maintenance of tissue homeostasis [41]. In various models, macrophages appear at the site of injury a few h after the damage occurs [32, 42, 43]. When the entry of monocytes into the injured muscle is blocked, muscle regeneration is impaired with the decreasing number of macrophages in the injured areas [44]. Macrophages have been classified as M1 and M2 macrophages according to their activation state. The M1 macrophages are associated with the early phases of acute inflammation, and the M2 phenotype is characterized by its anti-inflammatory function. M1 macrophages are activated by pro-inflammatory molecules (IL-1β, TNFα, IL-6) and chemokines (CXCL9, CXCL10). M2 macrophages are triggered by IL-4, IL-13, CCL-24 and CCL17. After skeletal muscle is injured, macrophages exhibit inflammatory profiles that promote phagocytosis and conversion to M2 macrophages to stimulate myogenesis and fibre growth [45]. An interesting study showed that M1 macrophages preferentially appear in regenerative areas containing only proliferating MPCs, whereas M2 macrophages are mainly associated with regenerative areas containing differentiating myogenic cells [46].

In the present study, the influence of macrophages on SCs was investigated in vitro and in vivo. However, the results were completely different between the in vitro and in vivo experiments. When SCs were co-cultured with macrophages, the myogenic proteins exhibited low expression levels. The addition of IL-6 to the media resulted in an increase in the expression levels of MyoD, Pax7, desmin and myosin. This was confirmed by qPCR analysis and western blotting. Additionally, the myogenic proteins exhibited low expression levels when SCs were co-cultured with macrophages. The addition of IL-6 to the media resulted in an increase in the expression levels of MyoD, Pax7, desmin and myosin. This was confirmed by qPCR analysis and western blotting.
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Figure 3. Shows that IL-6 up-regulated the expression of CD206, a specific marker of the M2 phenotype, in macrophages. These results demonstrated that M2 macrophages did not play a positive role in the proliferation of SCs. However, when macrophages were injected into atrophic muscles, the myogenic indicators were significantly up-regulated, which indicated that the macrophages improved the regeneration of atrophic muscles in other ways. These results merit further exploration in future analysis.

In this analysis, we established a denervated model by transection of the sciatic nerve. We aimed to explore the function of IL-6 and its signalling pathway in long-term muscle atrophy. Since the discovery of IL-6, many investigations have explored the role of this pleiotropic cytokine in skeletal muscle. Under different conditions, IL-6 has different functions. Some studies have shown that a sustained, systemic excess of IL-6 causes muscle atrophy [47, 48]. Serrano et al. have also reported that IL-6 blockade is a therapeutic approach for Duchenne Muscular

Figure 6. The qPCR analysis of F4/80, INOS and CD206 in the following three groups: MACR group, MI group, and MIA group. The relative transcript levels (fold change) of F4/80 (A), INOS (B) and CD206 (C) were calculated with respect to one sample from the MACR group according to the 2-ΔΔCt method. Comparisons between two groups were performed using the Mann-Whitney test. *P<0.1, **P<0.01.

Figure 7. The qPCR analysis of MyoD, Pax7, desmin and myosin in the following four groups: control group (muscle injected with DMED), IL-6 group (muscle injected with IL-6), AG490 group (muscle injected with IL-6 and AG490), and MACR group (muscle injected with macrophages). The relative transcript levels (fold change) of MyoD (A), Pax7 (B), desmin (C) and myosin (D) were calculated with respect to one sample from the control group according to the 2-ΔΔCt method. Comparisons between two groups were performed using the Mann-Whitney test. *P<0.1, **P<0.01.
Dystrophy. However, IL-6 also acts as an essential regulator of SC-mediated hypertrophy [26], and the expression of IL-6 is significantly induced by resistance exercise in human muscle and in electrically stimulated cultured human myotubes [49]. The present research showed that in long-term degenerated muscle, IL-6 up-regulated the expression of pax7, desmin, myod, and myosin. Myod expression occurs early during the activation of the SC population [50]. Desmin is expressed in undifferentiated SCs before myofibrillar proteins [51]. Pax7 is expressed in quiescent SCs and controls self-renewal [52]. Myosin is mainly expressed in differentiated myoblasts, myotubes, and regenerative myofibres [53]. Thus, these results demonstrated that IL-6 improved the proliferation of SCs.

The JAK/STAT signalling pathway, which is downstream of IL-6, has also been associated with the promotion of myoblast proliferation. Early studies have shown that proliferating SCs in regenerating muscle express activated (phosphorylated) STAT3 [54]. There have been numerous efforts to understand the role of the JAK/STAT pathway as an essential intracellular mediator of the IL-6 family of cytokines, in myogenesis [55-58]. In the present study, AG490 was used to inhibit STAT3. The results demonstrated that AG490 blocked the effect of IL-6 in long-term muscle atrophy. Thus, IL-6 improves the proliferation of SCs via the JAK/STAT3 signalling pathway. However, overall, a new approach for the treatment of muscle atrophy may be considered to restore the regeneration ability of muscle SCs via IL-6 in long-term skeletal muscle atrophy.

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Disclosure of conflict of interest

None.

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References

[1] Dutt V, Gupta S, Dabur R, Injeti E, Mittal A. Skeletal muscle atrophy: potential therapeu-


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[53] Edom F, Moul V, Barbet JP, Fiszman MY, Butler-Browne GS. Clones of human satellite...


