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Original article

Time-dependent gene expression analysis after mouse skeletal muscle contusion

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Abstract

Background: Though the mechanisms of skeletal muscle regeneration are deeply understood, those involved in muscle contusion, one of the most common muscle injuries in sports medicine clinics, are not. The objective of this study is to explore the mechanisms involved in muscle regeneration after contusion injury.

Methods: In this study, a total of 72 mice were used. Eight of them were randomly chosen for the control group, while the rest were subjected to muscle contusion. Subsequently, their gastrocnemius muscles were harvested at different time points. The changes in muscle morphology were assessed by hematoxylin and eosin (HE) stain. In addition, the gene expression was analyzed by real-time polymerase chain reaction.

Results: The data showed that the expression of many genes, i.e., specific markers of immune cells and satellite cells, regulatory factors for muscle regeneration, cytokines, and chemokines, increased in the early stages of recovery, especially in the first 3 days. Furthermore, there were strict rules in the expression of these genes. However, almost all the genes returned to normal at 14 days post-injury.

Conclusion: The sequence of immune cells invaded after muscle contusion was neutrophils, M1 macrophages and M2 macrophages. Some *CC* (*CCL2*, *CCL3*, and *CCL4*) and *CXC* (*CXCL10*) chemokines may be involved in the chemotaxis of these immune cells. *HGF* may be the primary factor to activate the satellite cells after muscle contusion. Moreover, 2 weeks are needed to recover when acute contusion happens as used in this study.

Keywords: Chemokines; Contusion; Cytokines; Gene; Macrophages; Satellite cells; Skeletal muscle

1. Introduction

Muscle injuries are common musculoskeletal problems. The mechanisms of skeletal muscle recovery are becoming clearer recently. The healing process of injured skeletal muscle consists of three distinct phases: degeneration and inflammation, regeneration, and fibrosis.[1–3] The first phase is characterized by local swelling at the injury site, formation of hematoma, necrosis of muscle tissue, degeneration, and inflammatory response. The second phase includes phagocytosis of the damaged tissue and regeneration of the injured muscle. And the final phase is characterized by scar tissue formation if the injury is serious.

The first phase usually consists of the infiltration of activated macrophages and neutrophils into the injured tissue. Many chemokines may play important roles in the chemotaxis of these immune cells.[4,5] In the regeneration phase, many growth factors can regulate the activation, proliferation, and differentiation of satellite cells, which are necessary for the regeneration of injured skeletal muscles.[6]

As we mentioned above, there is an appreciable understanding of the mechanisms of skeletal muscle regeneration among researchers. However, our understanding in this domain has been limited to the injury models of toxicant injection, freeze-induced injury, burn, disuse muscle atrophy, mdx mice, *etc*.[7–10] As a result, the mechanisms involved in muscle contusion, one of the most common muscle injuries in sports medicine clinics, are still not fully understood. Therefore, in the present study, we construct a model of skeletal muscle contusion with the objective of exploring the mechanisms involved in muscle regeneration. At different time points post-injury, we studied many genes expression

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such as the specific markers of neutrophils (*MPO*),[11] M1 macrophages (*CD68*) and M2 macrophages (*CD163*),[3,12] proliferation (*MyoD*), and differentiation (*myogenin*)[12–14] of satellite cells. Moreover, we examined the mRNA levels of inflammatory cytokines (i.e., *TNF-α*, *IL-1β*, *IL-6*, and *IL-10*) and chemokines (i.e., *CCL2*, *CCL3*, *CCL4*, and *CCL8*; *CXCL9*, *CXCL10*, and *CXCL12*).[3,5] We also analyzed the transcript levels of some regulatory factors which play important roles in satellite cell activation and muscle regeneration (i.e., *HGF*, *uPA*, *IGF-I*, *MGF*, and *myostatin*).[15–19]

2. Methods

2.1. Mice

Seventy-two C57BL/6 male mice (weighing 18.2–22.9 g, purchased from Shanghai Lab. Animal Research Center, Shanghai, China) were provided food and water ad libitum and maintained on a 12 h:12 h light–dark cycle. Eight mice were randomly chosen for the uninjured control group ($n = 8$), while the rest were subjected to muscle contusion ($n = 64$). In preparing the mice for muscle injury induction, they were anesthetized with 400 mg/kg chloral hydrate administered intraperitoneally. The study was approved by the Ethics Review Committee for Animal Experimentation of Shanghai University of Sport.

2.2. Contusion model

A simple and reproducible muscle contusion model in mice was used.[20,21] The animals' hind limbs were positioned on a board, dorsiflexing the ankle to 90°. A 16.8 g (diameter 15.9 mm) stainless steel ball was dropped from the height of 100 cm through a tube (interior diameter of tube:16 mm) onto an impactor[20] resting with a surface of 28.26 mm^2 on the middle of the gastrocnemius muscle of the mouse. The instantaneous force delivered by a falling object with these characteristics was calculated to equal 0.58 $N{\cdot}m/cm^2$, where 1 N·m is equal to the force of an object weighing 100 g falling over a distance of 1 m.[22] The muscle contusion created by this method was a high-energy blunt injury that created a large hematoma and was followed by massive muscle regeneration,[20,23] healing processes that are very similar to those seen in humans.[24] The mice that had bone fracture (fracture rate of 2.7%) were foreclosed. The injured mice in this study had signs of unrelieved pain such as piloerection of fur, reluctance to ambulate, overgrooming of the injured limb, and abnormal gait or posture.[25] At different time points (6 h, 12 h, 1, 3, 5, 7, 14, and 21 days) post-injury, the mice were killed by cervical dislocation while under anesthesia and then gastrocnemius muscles were harvested.

2.3. Histology

At the time points of 1, 3, 7, 14, and 21 days post-injury, the right gastrocnemius muscles were collected and embedded in paraffin. Cross sections were cut 8 μm from the midbelly of each gastrocnemius muscle and were stained with hematoxylin and eosin (HE) for morphological analysis. Using a 40 lens objective, images were captured for each muscle section (Labphot-2; Nikon, New York, NY, USA).

2.4. RNA extraction and cDNA synthesis

Approximately 60 mg of tissue (from the middle of the left gastrocnemius muscle) was homogenized using an Ultra-Turrax homogenizer (IKA, Staufen, Germany) in a solution of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated using a modified guanidiniumisothiocyanate-CsCl method,[26,27] and the concentration and purity were determined by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer (NanoDrop 2000; Thermo Scientific, Wilmington, MA, USA). Total RNA was reverse transcribed into cDNA using the Revertaid™ First Strand cDNA Synthesis Kit from Fermentas (Fermentas, Vilnius, Lithuania). cDNA was synthesized using 2 μg of total RNA, 0.2 μg of random primers, 20 mmol/L dNTP mix, 5 × reaction buffer (Fermentas), 20U RiboLock™ RNase Inhibitor and 200 U of Revertaid™ M-MuLVreverse transcriptase in a total volume of 20 μL. The reaction was carried out at 25°C for 5 min followed by another 60 min at 42°C and was terminated by the deactivation of the enzyme at 70°C for 5 min. Control reactions lacking either reverse transcriptase or template were included to assess carry-over of genomic DNA and non-specific contamination.[28,29]

2.5. Real-time polymerase chain reaction (PCR)

Quantitative PCR was carried out in triplicate in reactions consisting of 12.5 μL 2 × Maxima SYBR Green/ROX qPCR Master mix (Fermentas), 1 μL cDNA, nuclease-free water and 300 nmol/L of each primer. Primer specifications are listed in Table 1. Amplifications were performed on a Rotor-Gene 3000 thermal cycler (Corbett, Sydney, Australia) with the following parameters: activation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. The threshold cycle (CT, the number of cycles to reach threshold of detection) was determined for each reaction, and the levels of the target mRNAs were quantified relatively to the level of the housekeeping gene *GAPDH* using $2^{-\triangle\triangle CT}$ method.[30]

2.6. Statistical analysis

All values are expressed as mean ± SD, and statistical significance was set at $p < 0.05$. Mean values were compared between groups by one-way ANOVA with the Bonferroni method as a *post hoc* test, or non-parametric Kruskal–Wallis test. Data were analyzed using SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Muscle morphology

HE stain was used to assess changes in muscle morphology after injury. On Day 1 post-injury, cross sections of gastrocnemius muscles showed substantial fiber damage and edema, and a large number of inflammatory cell infiltration. On Day 3 post-injury, a small quantity of centronucleated myofibers were observed. On Days 7 and 14 post-injury, central nucleation phenomenon became more pronounced. On Day 21 post-injury, central nucleation almost disappeared. Since centrally nucleated myofibers are a sign of regeneration in injured muscle,[19] it means

Table 1
Primers used for real-time polymerase chain reaction.

Target gene	Forward primer sequence	Reverse primer sequence
MyoD	5'-GAGCGCATCTCCACAGACAG-3'	5'-AAATCGCATTGGGGTTTGAG-3'
myogenin	5'-CCAGTACATTGAGCGCCTAC-3'	5'-ACCGAACTCCAGTGCATTGC-3'
MPO	5'-CTGAATCTGTTGTCCGTGTCA-3'	5'-GTGATGGTGCGATACTTGTCAT-3'
CD68	5'-CAAAGCTTCTGCTGTGGAAAT-3'	5'-GACTGGTCACGGTTGCAAG-3'
CD163	5'-GCAAAAACTGGCAGTGGG-3'	5'-GTCAAAATCACAGACGGAGC-3'
HGF	5'-AGGAACAGGGGCTTTACGTT-3'	5'-GCTGCCTCCTTTACCAATGA-3'
uPA	5'-AGTGTGGCCAGAAGGCTCTA-3'	5'-GCTGCTCCACCTCAAACTTC-3'
IGF-I	5'-GCTTGCTCACCTTTACCAGC-3'	5'-AAATGTACTTCCTTCTGGGTCT-3'
MGF	5'-GCTTGCTCACCTTTACCAGC-3'	5'-AAATGTACTTCCTTTCCTTCTC-3'
myostatin	5'-TGCAAAATTGGCTCAAA-CAG-3'	5'-GCAGTCAAGCCCAAAGTCTC-3'
TNF-α	5'-CTTCTGTCTACTGAACTTCGGG-3'	5'-CACTTGGTGGTTTGCTACGAC-3'
IL-1β	5'-TGACGTTCCCATTAGACAACTG -3'	5'-CCGTCTTTCATTACACAGGACA-3'
IL-6	5'-GAACAACGATGATGCACTTGC-3'	5'-CTTCATGTACTCCAGGTAGCTATGGT-3'
IL10	5'-CAAGGAGCATTTGAATTCCC-3'	5'-GGCCTTGTAGACACCTTGGTC-3'
CCL2	5'-GCTCAGCCAGATGCAGTTAAC-3'	5'-CTCTCTCTTGAGCTTGGTGAC-3'
CCL3	5'-ACCATGACACTCTGCAACCA-3'	5'-CCCAGGTCTCTTTGGAGTCA-3'
CCL4	5'-CCACTTCCTGCTGTTTCTCTTA-3'	5'-CTGTCTGCCTCTTTTGGTCAG-3'
CCL8	5'-CTTCTTTGCCTGCTGCTCATAG-3'	5'-CACTTCTGTGTGGGGTCTACA-3'
CXCL9	5'-CTCCTTGCTTGCTTACCACTTT-3'	5'-CCAGCCTTGTCTACTTTGAGAG-3'
CXCL10	5'-CCTCATCCTGCTGGGTCTG-3'	5'-GTGGCAATGATCTCAACACG-3'
CXCL12	5'-ACGGAAGAACCAAAGAGAAAGA-3'	5'-CTCAGACAGCGAGGCACAT-3'
GAPDH	5'-ACTCCACTCACGGCAAATTC-3'	5'-TCTCCATGGTGGTGAAGACA-3'

that muscle regeneration was substantially completed on Day 21 (Fig. 1).

3.2. Specific markers of satellite cells in muscle

MyoD and *myogenin* are the specific markers of satellite cells proliferation and differentiation, respectively. The data showed that *MyoD* mRNA increased significantly at the time points: 6 h (4.87 folds, $p = 0.000$), 12 h ($p = 0.002$), Day 1 ($p = 0.009$) and Day 3 ($p = 0.002$) post-injury (Fig. 2A). Unlike *MyoD*, *myogenin* did not increase significantly at 6 h, 12 h, and 1 day after injury ($p > 0.05$). However, a high expression of myogenin mRNA was observed at Day 3 (5.80 folds, $p = 0.000$), Day 5 (3.56 folds, $p = 0.007$), and Day 7 (3.93 folds, $p = 0.003$) post-injury (Fig. 2B).

3.3. Specific markers of immune cells in muscle

MPO, *CD68*, and *CD163* are the specific markers of neutrophils, M1 macrophages and M2 macrophages, respectively. The data showed that *MPO* mRNA increased significantly at 6 h post-injury (2.15 folds, $p = 0.000$), and then declined quickly (Fig. 2C). *CD68*, the molecule marker of M1 macrophages, increased at 6 h and 12 h, peaked at 1 day (10.15 folds, $p = 0.001$), and then declined dramatically (Fig. 2D). Unlike *CD68*, *CD163*, the molecule marker of M2 macrophages almost did not change at 6 h and 12 h post-injury. However, *CD163* mRNA level at 1 day (2.69 folds, $p = 0.036$), 3 days (2.69 folds, $p = 0.023$), and 5 days (2.86 folds, $p = 0.028$) post-injury was significantly higher than that of the control group (Fig. 2E).

3.4. Regulatory factors for muscle regeneration

We studied many regulatory factors which are involved in muscle regeneration, i.e., *HGF*, *uPA*, *IGF-1*, *MGF* (positive regulatory factors) and *myostatin* (negative regulatory factor). The data showed that *HGF* mRNA in muscle increased significantly at 6 h (2.11 folds, $p = 0.015$), 1 day (2.22 folds, $p = 0.033$), and 3 days (4.77 folds, $p = 0.002$) post-injury (Fig. 2F). *IGF-1*

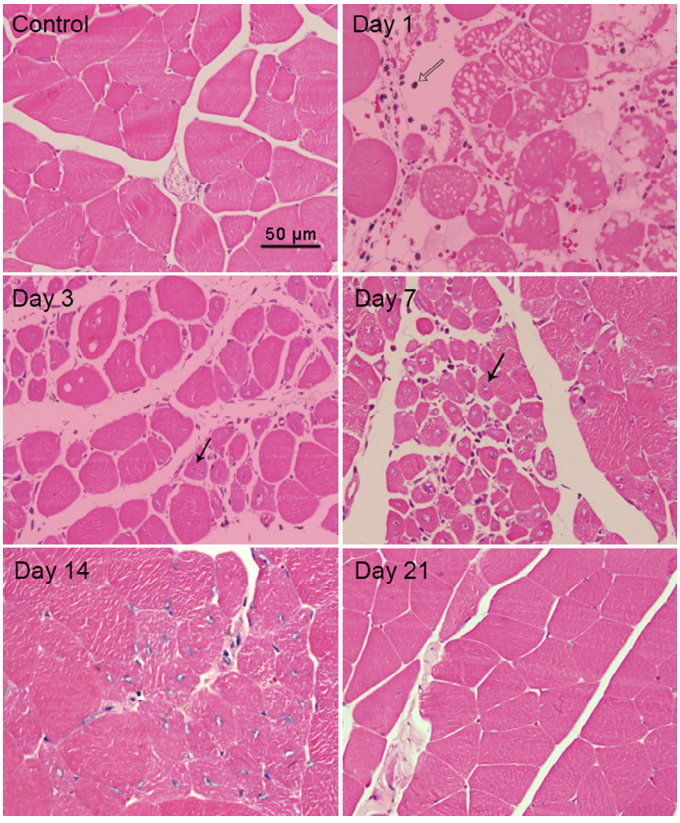

Fig. 1. Histological evaluation of the muscle healing process in gastrocnemius muscle at five time points (1, 3, 7, 14, and 21 days post-injury). ⟹ inflammatory cells; ⟶ central nucleation.

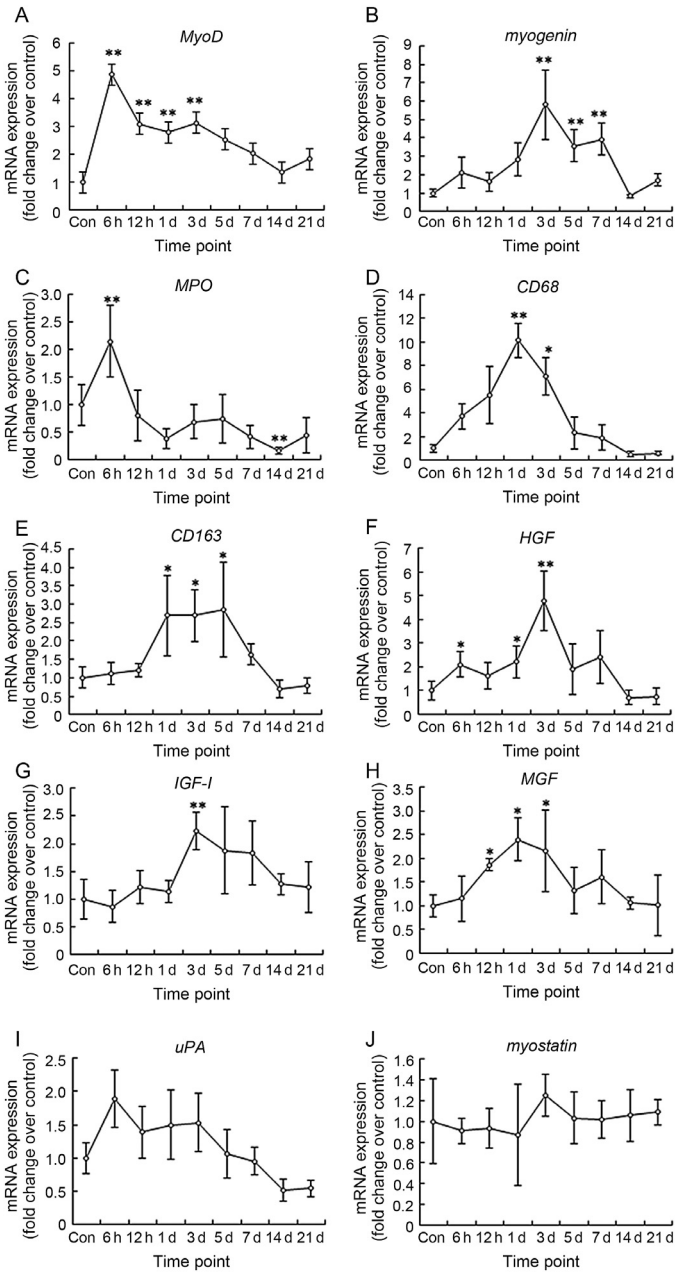

Fig. 2. RNA levels of specific markers of satellite (A–B), immune (C–E) cells. And regeneration regulatory factors (F–J) in mice gastrocnemius muscle post-injury (mean ± SD, $n = 8$). Con = control; d = day. $^{*}p < 0.05$, $^{**}p < 0.01$; compared with Con.

mRNA increased significantly at 3 days (2.23 folds, $p = 0.002$) (Fig. 2G). *MGF*, the splicing isoform of *IGF-1*, increased significantly at 12 h (1.86 folds, $p = 0.041$), Day 1 (2.04 folds, $p = 0.023$), and Day 3 (2.15 folds, $p = 0.038$) (Fig. 2H). However, *uPA* (Fig. 2I) and *myostatin* (Fig. 2J) mRNA did not change significantly from the control group at all time points post-injury ($p > 0.05$).

3.5. Inflammatory cytokine levels

We studied the expression of pro-inflammatory cytokines (i.e., *TNF-α*, *IL-1β*, and *IL-6*). The data showed that *TNF-α*

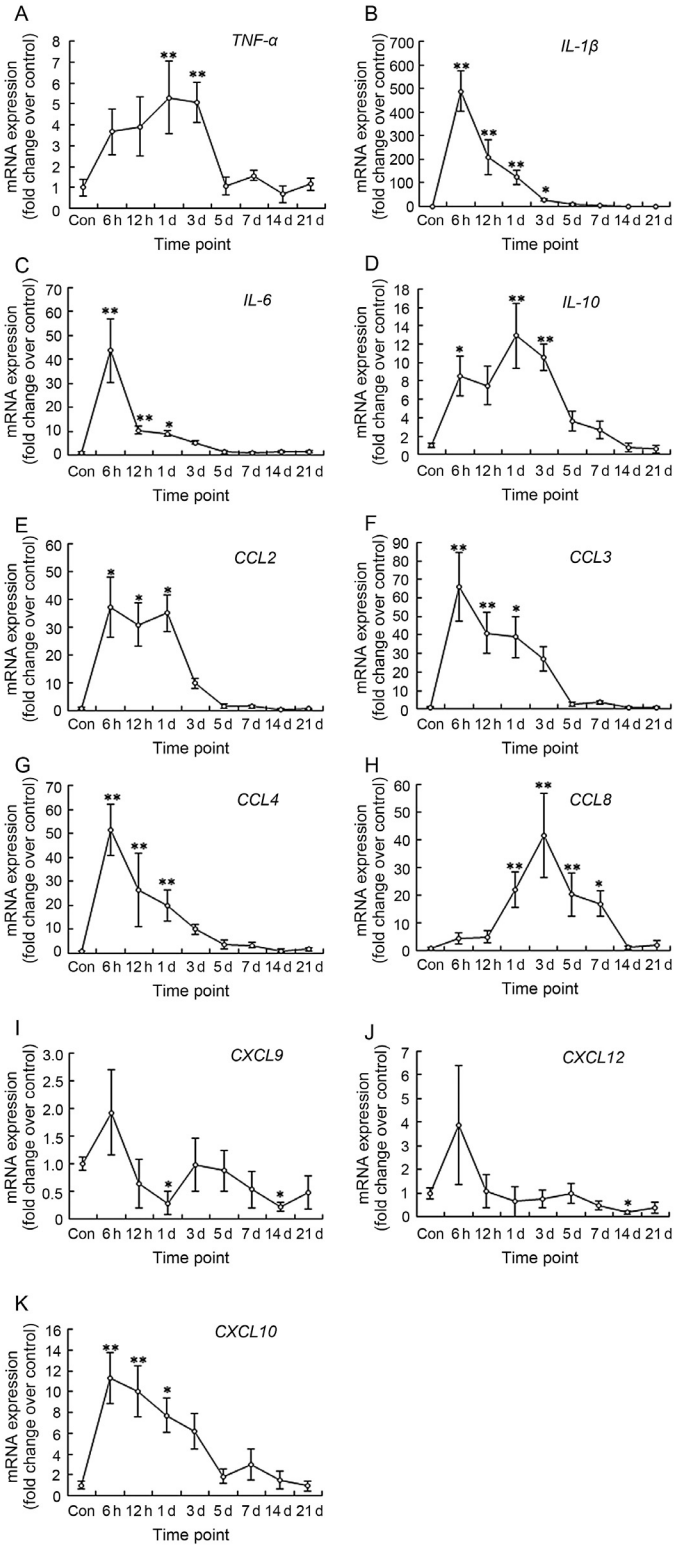

Fig. 3. mRNA levels of inflammatory cytokines (A–D), CC chemokines (E–H), and CXC chemokines (I–K) in mice gastrocnemius muscle post-injury (mean ± SD, $n = 8$). Con = control; d = day. $^{*}p < 0.05$, $^{**}p < 0.01$; compared with Con.

mRNA in muscle increased at 6 h and 12 h, and a high expression at 1 day (5.29 folds, $p = 0.006$) and 3 days (5.09 folds, $p = 0.009$) post-injury (Fig. 3A). *IL-1β* mRNA increased dramatically at 6 h (489.52 folds, $p = 0.000$), 12 h ($p = 0.000$),

Day 1 ($p = 0.002$), and Day 3 ($p = 0.043$) (Fig. 3B). The expression of *IL-6* was similar to *IL-1β*, but the extent of change was much smaller (Fig. 3C). On the other hand, a test on the mRNA level of *IL-10* (anti-inflammatory cytokine) showed that *IL-10* mRNA increased significantly at 6 h (8.56 folds, $p = 0.041$), Day 1 (12.95 folds, $p = 0.001$), and Day 3 (10.58 folds, $p = 0.004$) (Fig. 3D).

3.6. Chemokine levels

We tested the CC chemokines which were involved in the chemotaxis of immune cells. The data showed that the expression patterns of *CCL2*, *CCL3*, and *CCL4* were very similar. All of them peaked at 6 h (37.19 folds, $p = 0.010$; 65.86 folds, $p = 0.000$; 51.64 folds, $p = 0.000$; respectively) and then declined, but the mRNA levels at 12 h and 1 day were still much higher than the control group (Fig. 3E-G). However, *CCL8* had different gene expression patterns. *CCL8* mRNA increased significantly at Day 1 ($p = 0.003$), Day 3 ($p = 0.000$), Day 5 ($p = 0.009$), and Day 7 ($p = 0.032$) (Fig. 3H). Furthermore, we investigated the CXC chemokines. The data showed that *CXCL9* and *CXCL12* mRNA increased slightly only at 6 h ($p > 0.05$). At other time points, there was the tendency for the mRNA levels to decline, especially at 1 day (*CXCL9*, $p < 0.05$) and 14 days post-injury (*CXCL9*, *CXCL12*, $p < 0.05$) (Fig. 3I and J). However, different from *CXCL9* and *CXCL12*, the expression of *CXCL10* was similar to some CC chemokines (i.e., *CCL3* and *CCL4*) (Fig. 3K).

4. Discussion

4.1. The sequence of immune cells invade after muscle contusion

Acute muscle injuries initiate a series of responses by specific myeloid cell populations. Studies have shown that type I macrophages are associated with muscle necrosis, whereas type II macrophages are associated with regenerative myofibers.[3] In this study, we tested the specific markers of neutrophils (*MPO*),[11] M1 macrophages (*CD68*), and M2 macrophages (*CD163*).[3,12] The data showed that neutrophils (*MPO*) were the first to respond and peak in concentration at 6 h post-injury before declining rapidly. Following the onset of neutrophil invasion, M1 macrophages also began to invade until it reached significant elevated concentrations at about 24 h post-injury and then declined sharply. Their invasion preceded the elevation of a population of M2 macrophages that remained significantly elevated for many days (from 1 day to 5 days post-injury). The fact that the invasion of M1 macrophages preceded that of M2 macrophages conformed to their functions. Indeed while type I macrophages enhance the proliferation of local myogenic precursor cells, type II macrophages stimulate their fusion and differentiation.[31] Though our study was only based on genetic levels, we acknowledge that the results would have been more convincing had protein levels been tested too.

The sequence of immune cells invasion after muscle contusion, as described above, was similar to other injury models.[3] Meanwhile, it coincided with the expression of the inflammatory cytokines. The high-energy blunt injury used in this study induced intense inflammatory response. Pro-inflammatory cytokines (i.e., *TNF-α*, *IL-1β*, and *IL-6*) were highly expressive in the first 3 days. Moreover, we studied the anti-inflammatory cytokine (*IL-10*) which plays a central role in regulating the switch of muscle macrophages from M1 to M2 phenotype in injured muscle.[32] The study showed that ablation of *IL-10* prevented a subsequent increase in *CD163* post-injury. Furthermore, muscle regeneration and growth were greatly slowed by the loss of *IL-10*. In addition, *in vitro* assays showed that coculturing muscle cells with macrophages activated with *IL-10* to the M2 phenotype increased myoblast proliferation.[32] In this study, we found that the expression of *IL-10* was faster than the specific marker of M2 macrophages (*CD163*). We speculate that *IL-10* may be involved in the transition of macrophage phenotype after muscle contusion; however, this needs further study.

4.2. CC and CXC chemokines may be involved in the chemotaxis of immune cells after muscle contusion

Chemokines are small cytokines with chemoattractant properties, belonging to one of the four families: C, CC, CXC, and CX3C.[33] These families are differentiated by the number and order of the amino-terminal cysteine residues and vary in their specific actions by binding to specific seven transmembrane-domain G protein-coupled receptors of the same family.[34,35] The C and CX3C families are chemotactic for lymphocytes,[36] while CC chemokines and their receptors are primarily involved in the migration and activation of monocytes, macrophages and lymphocytes.[34] The CXC chemokines that contain three residue motifs (ELR) have neutrophil specificity.[36] Our study showed a rapid and coordinated elevation in *CCL2*, *CCL3*, and *CCL4* in muscle post-injury, which is similar to the study of Warren et al.[37,38] CC chemokines increased dramatically post-injury. This has very important physiological significance. There were enough evidence to indicate that CC chemokines play a significant role in attracting immune cells to the injury site.[3,37,38] Disruption of *CCL2/CCR2* signaling in injured muscle may have multiple impacts on tissue response to injury that can lead to slower regeneration. Apart from attracting immune cells, CC chemokines were involved in the proliferation of myoblast. The study of Yahiaoui et al.[39] showed that myoblasts constitutively expressed receptors for *CCL2* (*CCR2*), *CCL3* (*CCR1* and *CCR5*), and *CCL4* (*CCR5*); and that stimulation with either *CCL2* or *CCL4* was sufficient to promote myoblast proliferation. However, unlike *CCL2*, *CCL3* and *CCL4*, *CCL8* had different expression pattern. *CCL8* did not increase significantly at 6 h and 12 h after injury; it did after 1 day. Our results are very similar to the study of Nicholas et al.,[40] who found that *CCL2* and *CCL3* mRNA peaked at 8 h after muscle contusion, while *CCL8* mRNA peaked at 48 h after injury. Immune cells invasion preceded the elevation of *CCL8*, meaning that *CCL2*, *CCL3*, and *CCL4*, but not *CCL8*, may be involved in the chemotaxis of immune cells after muscle contusion.

In addition, according to the literature,[41] we studied three *CXC* chemokines which may be involved in the chemotaxis of immune cells after muscle injury. The data showed that *CXCL9*

and *CXCL12* did not change significantly in the whole process of muscle recovery. However, *CXCL10* increased rapidly after muscle injury, similar to *CCL2*, *CCL3*, and *CCL4*. In the study of Koh and Pizza,[41] *CXCL10* produced skeletal muscle cells or immune cells that can influence the chemotaxis of inflammatory cells. Therefore, *CXCL10*, but not *CXCL9* and *CXCL12*, may be involved in the chemotaxis of immune cells after muscle contusion.

4.3. HGF may be the primary factor to activate muscle satellite cells after muscle contusion

Muscle regeneration consists of activation, proliferation, and differentiation of satellite cells into myotubes, which ultimately fuse with other existing myofibers or fuse together to form new myofibers.[42,43] The activation and differentiation of satellite cells are respectively indicated by the up-regulation of myogenic regulatory factor (*MRF*) *MyoD* and *myogenin*.[12–14] In our study, we found that *MyoD* is expressed early in proliferating satellite cells, and *myogenin* appeared after the decline in *MyoD* expression (Fig. 2A and B). These phenomena also existed in other injury models in some previous studies.[44,45] *MyoD* acts as an early *MRF* that is mainly involved in satellite cell activation and proliferation, whereas myogenin is a late-acting *MRF* expressed during differentiation.[13]

Satellite cells can be activated by some growth factors after muscle injury. However, there still exists a controversy as to which specific factor is involved after muscle contusion. Most studies have chosen the 1 day post-injury as the first time point. They pointed out that *IGF-1*, *MGF*, *uPA*, or *HGF* may be involved in the activation of satellite cells.[15,16,18] Researchers of early studies opined that *IGF-1* was the activate factor of satellite cells;[46] as a result, *MGF*, the isoform of *IGF-1*, was deemed to be involved.[15] On the other hand, some studies found that *uPA* may play an important role in satellite cells activation.[47,48]

In this study, we added two time points, 6 h and 12 h, before the 1 day post-injury. We speculated that the negative regulatory factor of muscle regeneration, *myostatin*, would increase after muscle contusion, as in other injury models.[49] However, interestingly, *myostatin* did not change in the whole process of recovery. On the other hand, when we tested some positive regulatory factors such as *HGF, IGF-1, MGF,* and *uPA*, the data showed that the expression of *IGF-1* and *MGF* increased significantly after muscle injury, though they trailed the activation of satellite cell (*MyoD*). In addition, *uPA*, an activate factor, as described in some studies, did not change after muscle contusion. Nonetheless, due to the unavailability of protein data, we cannot certainly conclude that these factors (*IGF-1*, *MGF*, and *uPA*) are not involved in the activation of satellite cells. However, *HGF* mRNA increased significantly at 6 h post-injury, which is synchronous with the activation of satellite cell. We speculate that *HGF* may be the primary factor to activate muscle satellite cells after muscle contusion. *HGF* is a mesenchyme-derived heparin-binding glycoprotein that regulates cell proliferation, cell survival, cell motility, and morphogenesis.[50] The evidence from early experiments showed that *HGF* can activate quiescent satellite cells *in vivo* or *in vitro*. The activation of satellite cells by *HGF* can undergo both paracrine and autocrine signaling.[51] *HGF* is essential in inducing the migration of myogenic precursor cells in embryonic myogenesis.[51] Furthermore, *HGF* is the only growth factor that has been established to have the ability to stimulate quiescent satellite cells to enter the cell cycle early in a culture assay and *in vivo*.[18,52]

4.4. Two weeks is needed to recover when acute muscle contusion happens

In order to investigate the process of muscle recovery, we did the morphological analysis. The high-energy blunt injury induced a large hematoma and edema, widened the interstitial spaces between the muscles fibers, infiltrated the mononuclear cell into the interstitial spaces, and disorganized the muscle architecture on the first day of post-injury[53] (Fig. 1). On Day 3 of post-injury, central nucleation started and became more obviously on Days 7 and 14. However, on Day 21 post-injury, central nucleation almost disappeared. Since central nucleated myofibers are a sign of regeneration in injured muscle,[47] it means that muscle regeneration was substantially completed on Day 21 post-injury.

Furthermore, we studied many genes expression at different times post-injury. The data showed that many genes, e.g., specific markers of immune cells (*MPO* and *CD68*), regeneration regulatory factors (*HGF, IGF-1, MGF*), inflammatory cytokines (*TNF-α, IL-1β, IL-6, IL-10*), and chemokines (*CCL2, CCL3, CCL4, CXCL10*), increased in the first phase of the recovery, especially in the first 3 days. In the second phase of regeneration, specific markers of satellite cells differentiation (*myogenin*) and M2 macrophages (*CD163*) increased significantly. In addition, we found that intense inflammatory response and muscle regeneration existed simultaneously (6 h after injury), which suggested that there were no strict boundaries between the first and the second stages of regeneration. However, almost all the genes returned to normal at 14 days post-injury. Some genes such as *MPO*, *CXCL9*, and *CXCL12* even decreased significantly at 14 days. Though we are currently not sure about the reasons why these inflammatory genes inhibited in the later stage of recovery, we speculate that it may be related to the severe inflammatory reaction of the early stage of recovery. Accordingly, these data suggest that skeletal muscle needs no less than 2 weeks to recover after acute contusion, as used in our study. However, it is unclear whether or not 2 weeks are sufficient for the recovery of muscle function since our study only focused on morphology and genes. This needs further study.

5. Conclusion

From the genetic perspective, we can conclude that the sequence of immune cells invaded after muscle contusion was neutrophils, M1 macrophages and M2 macrophages. Some CC (*CCL2*, *CCL3*, and *CCL4*) and *CXC* (*CXCL10*) chemokines may be involved in the chemotaxis of these immune cells. *HGF* may be the primary factor to activate the satellite cells. In addition, intense inflammatory response and regeneration existed simultaneously, which suggested that there were no

strict boundaries between the first and second stages of regeneration. Almost all index returned to normal at 14 days. This means that 2 weeks are needed to recover when the acute contusion, as used in the study, happens.

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Authors' contributions

WX, BL, LZ, and XL carried out the genetic studies. YL and ZZ carried out the morphological analysis. WX performed the statistical analysis and drafted the manuscript; PC conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors have read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

Competing interests

None of the authors declare competing financial interests.

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