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Macrophages and skeletal muscle regeneration: a clodronate-containing liposome depletion study

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Summan, Mukesh, Gordon L. Warren, Robert R. Mercer, Rebecca Chapman, Tracy Hulderman, Nico Van Rooijen, and Petia P. Simeonova. Macrophages and skeletal muscle regeneration: a clodronate-containing liposome depletion study. *Am J Physiol Regul Integr Comp Physiol* 290: R1488–R1495, 2006. First published January 19, 2006; doi:10.1152/ajpregu.00465.2005.—The study evaluates the influence of monocytes/macrophages in the mechanisms of skeletal muscle injury using a mouse model and selective depletion of peripheral monocyte with systemic injections of liposomal clodronate (dichloromethylene bisphosphonate). This pharmacological treatment has been demonstrated to induce specific apoptotic death in monocytes and phagocytic macrophages. In the current studies, the liposomal clodronate injections resulted in a marked attenuation of the peak inflammatory response in the freeze-injured muscle in the first three days after injury. The effect was accompanied by a transient reduction (at *day 1* or *3* postinjury) of the expression of several genes coding for inflammatory, as well as growth-related mediators, including TNF, monocyte chemoattractant protein (MCP)-1, thioredoxin, high-mobility group AT-hook 1, insulin-like growth factor-binding protein (IGFBP), and IGF-1. In contrast, the expression of major myogenic factors (i.e., MyoD and myogenin) directly involved in the activation/proliferation and differentiation of muscle precursor cells was not altered by the clodronate liposome treatment. The repair process in the injured muscle of clodronate liposome-treated mice was characterized by prolonged clearance of necrotic myofibers and a tendency for increased muscle fat accumulation at *day 9* and *14* postinjury, respectively. In conclusion, a significant reduction of the initial monocyte/macrophage influx into the injured muscle is associated with not improved, but moderately impaired, repair processes after skeletal muscle injury.

skeletal muscle injury; inflammation; myogenesis; gene expression

INFLAMMATION IS AN OBLIGATORY event in skeletal muscle injury resulting from a variety of mechanisms, including crush, freezing, overuse, chemical, and biological insults (32). Recovery of skeletal muscle injuries requires severely injured myofibers to be degraded, phagocytized, and replaced via the migration and maturation of satellite cells. It is well established that after a brief initial neutrophil influx into the muscle, macrophages become the dominant inflammatory cell type by ~1 day and with peak levels at *day 3* postinjury (17, 34, 37). Several studies have reported that macrophages can promote both degeneration and regeneration of muscle cells, although the likelihood of this dual role after in vivo muscle injury remains controversial (33). Macrophages are involved in phagocytosis, and removal of cellular debris derived from necrotic muscle tissue (16, 17). It has also been shown that macrophages isolated from dystrophic muscles of mdx mice are highly cytotoxic for muscle cells (40). In contrast, increasing evidence has demonstrated that, in vitro, macrophages can enhance the replication and differentiation of muscle precursor cells (3, 10, 13, 25). It is possible that all phases of muscle injury, including degeneration, regeneration, and fibrosis, are influenced by mediators released from activated macrophages, including inflammatory cytokines, chemokines, and growth factors (8, 33). Consistent with a role of macrophages in regenerative mechanisms are the in vivo observations that recovery of muscle function postinjury begins at approximately the time of peak macrophage infiltration (12, 38, 39). Furthermore, increasing data suggest that resident macrophages and macrophages originating from the systemic circulation, as well as phagocytic and nonphagocytic macrophages, may play different roles during muscle injury (11, 14, 29, 33).

Modulating, either by reducing or enhancing, the influx of macrophages into injured muscle tissue is an attractive target for therapeutic interventions. However, the role of this influx in controlling the outcome of muscle injury is not well understood. Systemic injections of liposome-encapsulated clodronate (dichloromethylene bisphosphonate) in mice have been demonstrated to deplete rapidly (within 24 h) 90% of the peripheral monocytes, as well as tissue macrophages, which are achieved with the liposomes (31, 36). The treatment has been shown to induce selective apoptotic cell death in monocytes and macrophages without affecting lymphocytes and neutrophils (23, 36). Furthermore, unlike other methods of macrophage depletion, the clodronate-mediated anti-monocyte/macrophage approach does not result in secretion of proinflammatory cytokines (35). Although, liposomal clodronate has been applied to study the role of monocytes and macrophages in a variety of disease and injury models, its effects in skeletal muscle disorders have not been studied. The objective of our study was to gain better insight into the role of inflammatory cell influx in muscle injury and repair using the liposomal clodronate pharmacological method for peripheral

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monocyte and macrophage depletion. We hypothesized that minimizing the monocyte/macrophage response to trauma would modulate the muscle's ability to recover from traumatic injury. To test the hypothesis, liposomes loaded with clodronate or PBS (as a control) were injected into mice both before and after induction of a freeze injury to the tibialis anterior (TA) muscle. The injury/repair process was evaluated by histopathological assessment, as well as by quantifying the expression of genes involved in the initial events after injury.

METHODS

Animals. C57BL/6 female wild-type mice (Jackson ImmunoResearch Laboratory, Bar Harbor, ME) were provided food and water ad libitum and maintained on a 12:12-h light-dark cycle. In preparation for muscle injury induction, mice were anesthetized with 0.33 mg/kg fentanyl, 16.7 mg/kg droperidol, and 5.0 mg/kg diazepam administered intraperitoneally. Animal care and use procedures, including death by CO_2 asphyxiation, were conducted in accordance with criteria outlined in the "Public Health Service 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); these procedures were approved by the National Institute for Occupational Safety and Health institutional animal care and use committee.

Injection of liposomes. Clodronate- and PBS-loaded liposomes were prepared as described earlier (36). Clodronate was a gift of Roche Diagnostics (Mannheim, Germany). Mice were injected with clodronate liposomes or PBS liposomes 2 days (0.2 ml iv of liposomes through the tail vein) and 2 h (0.1 ml ip of liposomes) before muscle injury. Additionally, in the 9- and 14-day postinjury studies, liposomes (clodronate or PBS, respectively) (0.1 ml ip) were administered every third day after the injury. This protocol was based on previous publications (21), as well as discussions through the Web site www.ClodronateLiposomes.org. To evaluate the effects of clodronate liposome treatment on peripheral leukocytes, blood smears were prepared from the foot vein for all of the experimental animals; the smears were stained with the Wright stain; 100 cells per slide and two slides per animal were counted. There was no difference, measurable by this method, in the leukocyte counts between the two treatments at any time point (the average count was 87 ± 5% lymphocytes, 12 ± 5% neutrophils, and <2% monocytes.

Induction of muscle injury. The muscle injury induction procedure was identical to that described previously (37). In brief, a 1.5-cm-long incision was made through aseptically-prepared skin overlying the left TA 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. This injury results in damage of ~60% of the TA; influx of inflammatory cells, mainly monocytes and macrophages; and activation of the regeneration in the first 3 days after injury, as well as recovery of the histopathology by 14 days after injury (37).

Histopathology. All histopathological and immunohistological evaluations were conducted on at least four mice per treatment group. For cross-section histology and immunohistology, muscles were embedded in Tissue Tek OCT (Miles Scientific), frozen in melting isopentane, and stored at −80°C. With the use of a microtome cryostat at −20°C, 10 cross sections (10 μm thick) were cut at each of six levels equally spaced along the length of the TA muscle. Sections at each level were used for routine hematoxylin and eosin (H&E) staining or for immunohistology. Myofiber cross-sectional areas (CSAs) in regenerating muscles at 9 days postinjury were determined using H&E-stained sections, as described before (38). Briefly, the average uninjured and regenerating myofiber areas were determined for each muscle. Sections chosen for analysis were the ones: *1)* with the highest fraction of the muscle area showing injury and regeneration and *2)* a well-demarcated border between the regions of regenerating and uninjured myofibers; only fibers in the vicinity of the border were analyzed. Regenerating myofibers chosen for analysis were centronucleated fibers, not bordering a fiber that was not centronucleated. Likewise, uninjured myofibers chosen for analysis were noncentronucleated fibers that did not border a centronucleated fiber. Between 190 and 255 myofibers were analyzed per muscle. For each muscle, the average uninjured and regenerating myofiber areas were determined using at least 5 mice per group. Measurements were performed using a Leica Leitz DMRB microscope, ×20 objective, and MediaCybernetics Optimas 6.5 software (Media Cybernetics, Silver Spring, MD). Muscles processed for longitudinal-section histology were fixed by immersion in 10% neutral-buffered formalin. Masson's trichrome stain was applied to visualize collagen, as described originally (27). Fixed tissues were embedded in paraffin, cut into 6-μm longitudinal sections, and stained with H&E for blinded histopathological assessment. The histopathology was graded independently by two researchers using a 1-to-5 scale, which represents a relative fold increase compared to the uninjured muscle (1 = minimal; 2 = slight/mild; 3 = moderate; 4 = moderately severe; 5 = severe/high), and sections from seven mice per treatment group. Standard morphometric methods of point counting (quantitative stereology) were used to determine the muscle response to injury in tissue sections of the muscle. Categories used to measure the response included the proportion of normal and necrotic muscle fibers, as well as, normal interstitial spaces, and interstitial spaces containing inflammatory cells or fat cells. These categories were measured using point counting of a grid, which covered a ±0.5-mm region over the central area of injury in H&E-stained sections from each muscle specimen. A stage micrometer was used to identify the midpoint of each muscle specimen. For counting, a Leica Lablux 5 equipped with an 11 × 11 graticule eyepiece (×15) and ×40 objective was used. The proportion of the tissue volume occupied by each category was obtained for each muscle specimen by dividing the sum of all points over the category by the total points of the counting grid. Results were expressed as means (SD) with six animals per group.

Rat monoclonal antibodies specific for Mac-1, Mac-3, and Gr-1, markers of inflammatory cells, (BD PharMingen, San Diego, CA) were applied in an immunostaining protocol conducted on acetone-fixed cross-cryosections as described before (38). The positive staining was evaluated visually by two independent researchers and quantitatively by Optimas image analysis software, version 6.51. Images of stained sections (two regions of the injured portion of each muscle section, 2 sections per animal, 4 animals per group, ×20 magnification) obtained with Leica DMRB microscope and captured by a Sony DXC9000 color video camera were used for the image analysis. After we set red, green, and blue thresholds to estimate the stained areas, the Optimas software measured the total area, stained area, and percent stained area. The same thresholds and lighting conditions were used for all sections for a given stain.

Immunostainings for MyoD, a marker of muscle precursor cell activation/proliferation, was performed on formalin-fixed cross-cryosections using polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), as described previously (37). Control experiments omitting the primary antibodies were used for demonstrating specificity of the immunostaining.

Real-time RT-PCR. Muscles were collected in RNAlater (Qiagen, Valencia, CA), homogenized, and total RNA was extracted using a commercial kit (RNeasy, Qiagen) following the manufacturer's protocol. cDNA (200 μl) was synthesized from 1 μg of RNA using Superscript II (Life Technologies, Gaithersburg, MD) and random hexamers. Amplification reactions were performed with 1× SYBR Green PCR mastermix (PE Applied Biosystems, Foster City, CA), 1 μM primers, and 4 μl of cDNA in a 50-μl final volume. Amplification reactions were performed in an ABI Prism 7700 spectrofluorometric thermal cycler (PE Applied Biosystems), according to the manufacturer's instructions [10 min at 95°C, 15 s at 95°C, and 1 min at 60°C (40 cycles)] with the following designed primers (Invitrogen Life Technologies, Carlsbad, CA): thioredoxin (53 bp): sense 5′-GTT-

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CTGCTGAGACGCGTGTG-3′ and antisense: 5′-TTCACCATTTT-GGCTGTTGC-3′; high-mobility group AT-hook1 (HMGA) (54 bp): sense: 5′-GAGTCGGGCTCAAAGTCCAG-3′ and antisense: 5′-ATCCTTTTCCTGTTTGGAGGC -3′; Mac-1 (210 bp): sense: 5′-GATGAGACAAAGAACAACACAC-3′ and antisense: 5′-TGAAGAACCTCTGAGCATCC-3′; MyoD (104 bp): sense: 5′-ACCCAGGAACTGGGATATGGA-3′ and antisense: 5′-AAGTCGTCTGCTGTCTCAAA -3′; myogenin (114 bp): sense: 5′-GCACTGGAGTTCGGTCCCAA-3′ and antisense: 5′-TATC-CTCCACCGTGATGCTG-3′; insulin-like growth factor binding protein (IGFBP) (53 bp): sense: 5′-GGGAGTGTGGAAAGCC-AGGT-3′ and antisense: 5′-GGAATCGGTCACTCGGTGTG-3′.

Real-time RT-PCR for monocyte chemoattractant protein 1 (MCP-1), TNF-α, and 18S/rRNA was performed using predeveloped primers and probes (TaqMan assay reagents; PE Applied Biosystems). Similarly, real-time RT-PCR for colony-stimulating factor (CSF)-1, insulin-like growth factor 1 (IGF-1), and transforming growth factor beta 1 (TGF-β1) was performed with predeveloped primers and probes (Assays-On-Demand Gene Expression Products, PE Applied Biosystems). PCR products were amplified on the ABI Prism 7700 spectrofluorometric thermal cycler. The differences in mRNA expression between control and treatment groups were determined by the relative quantification method (18), using the threshold cycle (C_T) method and real-time PCR efficiencies of the target gene normalized to the housekeeping gene 18S/rRNA. The housekeeping gene expression was not influenced by the injury or by the pharmacological treatment.

Statistics. The expression of each gene was obtained from five animals per group and triplicate measures. Differences in mRNA transcript levels between the two treatments were analyzed using Student's unpaired *t*-tests or Mann-Whitney *U* tests when assumptions of normality or equal variance were violated; Bonferroni adjustments were applied to control for type I error inflation due to the multiple comparisons.

For comparison of myofiber CSAs between the two groups of mice, first an average CSA for each myofiber type (regenerating or uninjured) in each muscle was calculated. These averaged CSA values were then analyzed using the two-way (group × myofiber type) repeated-measures ANOVA. For immunostaining comparison, the percent stained area was averaged for each muscle and analyzed by a one-way ANOVA.

RESULTS

Monocyte and macrophage depletion. To evaluate the depleting effects of clodronate liposome treatment in injured muscle, the muscle was assessed by mRNA expression for Mac-1 (integrin β2 subunit, CD18β subunit), a marker of leukocytes, including monocytes and macrophages and neutrophils (Fig. 1). Control (uninjured) muscle expressed low constitutive levels of Mac-1 mRNA transcripts irrespective of clodronate or PBS liposome treatment. Mac-1 expression was significantly increased in the injured muscle of PBS liposome-treated mice at 1 and 3 days postinjury, consistent with the influx of inflammatory cells. However, compared to PBS liposome-treated mice, the Mac-1 mRNA transcript levels in injured muscles from mice treated with clodronate liposomes were reduced by ~80% and ~50% at 1 and 3 days postinjury, respectively. Furthermore, the inflammatory cell influx was evaluated by immunostaining for Mac-1 or more specifically by Mac-3, a marker of monocytes/macrophages (24) and Gr-1, a marker of neutrophils (5). Consistent with the gene expression data, immunohistological evaluation demonstrated low levels of inflammatory cells in the uninjured muscle, which were not altered by the clodronate liposome treatment. These cells stained positively for Mac-3 (representative images Fig. 2*A*) and Mac-1 but not for Gr-1. At 3 days after injury, massive infiltration of Mac-1- or Mac-3-positive cells occurred in the damaged muscle area from mice treated with PBS-liposomes (representative images Fig. 2, *B* and *C*). In contrast, recruitment of Mac-1 or Mac-3 positive cells into the injured muscle was reduced by more than 50% upon treatment of mice with clodronate liposomes (Fig. 2, *B*, *C*, and *F*). As we have reported before (38), there was minimal immunostaining for Gr-1, a marker of neutrophils, in the muscle 3 days after injury (6.5 ± 2.8% stained area, $n = 4$), and this immunostaining was not modified by the clodronate liposome treatment (7.2 ± 3.5% stained area, $n = 4$) (representative images Fig. 2*D*).

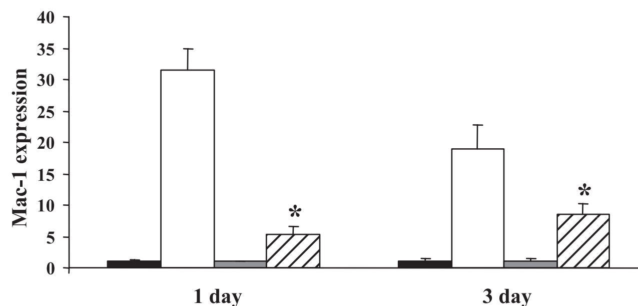

Fig. 1. Effects of clodronate liposome treatment on Mac-1 gene expression in freeze-injured tibialis anterior (TA) muscle. Injured and uninjured (control) TA muscles were obtained from mice at the time points indicated and analyzed for mRNA transcripts using real-time PCR. Expression was normalized to 18S/rRNA from the same samples and presented as a fold-increase above control. Uninjured (solid) and injured (open) TA from PBS liposome-treated mice; Uninjured (gray) and injured (cross-hatched pattern) TA from clodronate liposome-treated mice. Values represent mean ± SE ($n = 5$). *Significantly different expression in clodronate liposome-treated mice compared with PBS liposome-treated mice ($P < 0.05$).

Nine days after injury, Mac-1 and Mac-3 immunostaining was significantly increased in the injured muscles of clodronate-treated mice compared to the control treatment (representative images Fig. 2*E*), but this difference had disappeared by 14 days postinjury (Fig. 2*F*). At these time points, positive immunostaining for Gr-1 was not detectable in the injured muscle of PBS liposome- or clodronate liposome-treated mice (data not shown).

Histological evaluation of the effects of monocyte and macrophage depletion on muscle injury and repair. The time course for the histological appearance of the freeze-injured muscle was compared between clodronate liposome- and PBS liposome-treated mice. Uninjured muscles from clodronate liposome- and PBS liposome-treated animals showed no histopathology and no differences (data not shown). Consistent with the Mac-1 and Mac-3 immunostaining data, hematoxylin and eosin staining indicated considerable inflammatory cell infiltration, mainly mononuclear cells, in the 3-day postinjury muscle of mice treated with PBS-liposomes (a representative image is in Fig. 3*A*). The necrotic myofibers were covered by mononuclear inflammatory cells. In contrast, the influx of inflammatory cells into injured muscle of clodronate liposome-treated mice was significantly reduced (a representative image is in Fig. 3*A*). At 9 days postinjury, the damaged muscle area in the PBS liposome-treated mice had been replaced mostly by centronucleated myofibers (indicating regenerating myofibers), whereas numerous necrotic myofibers and inflammatory cells dominated the injured muscle regions of clodronate liposome-

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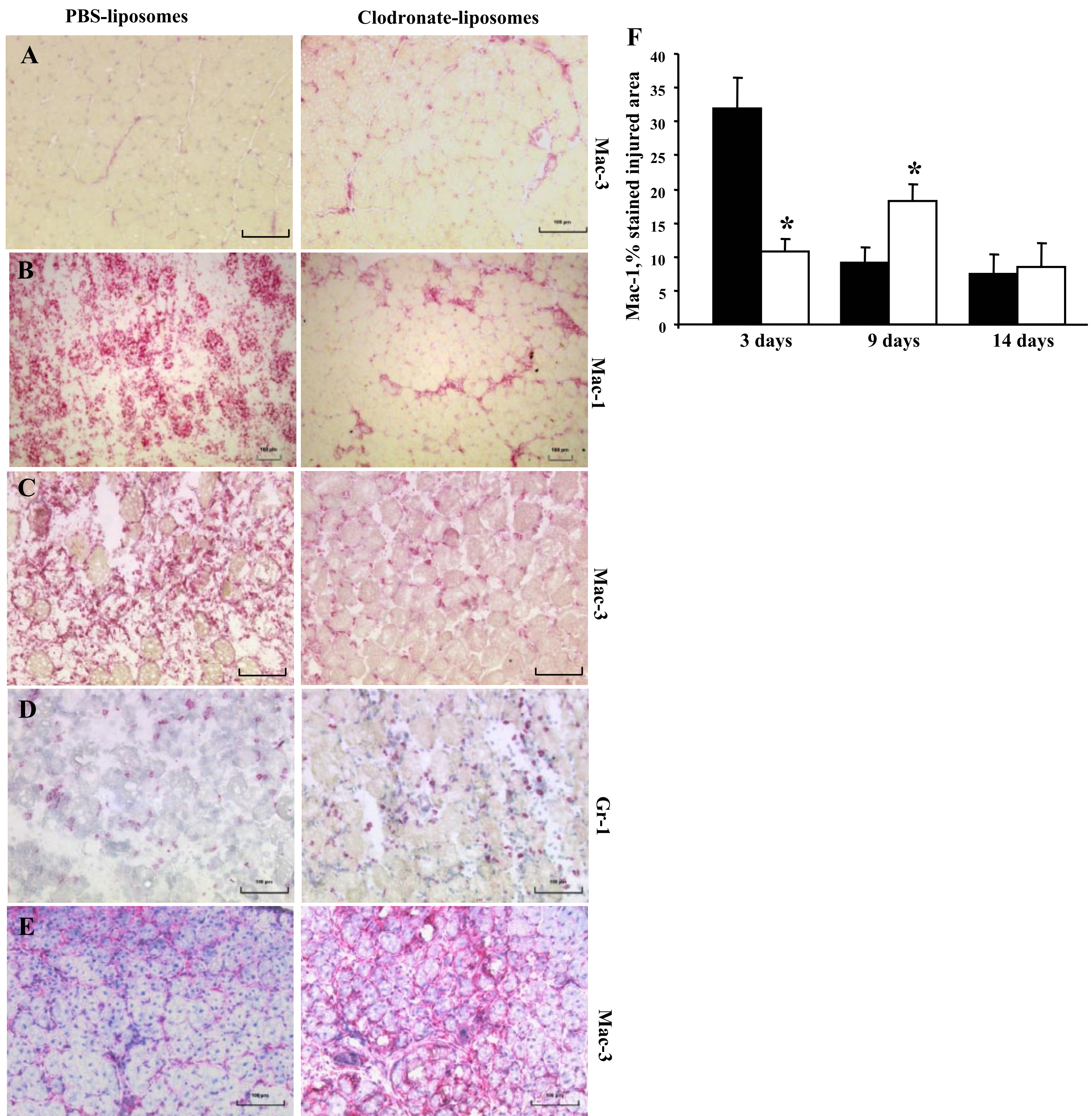

Fig. 2. Microscopic evaluation for Mac-1-, Mac-3-, and Gr-1-positive cells in injured TA muscle. The images are representative of those obtained from muscles of 4 mice per group. Scale bars = 100 μm. *A*: Mac-3 immunostaining on frozen transverse sections of uninjured TA muscle. *B*: Mac-1 immunostaining on frozen transverse sections of TA muscle 3 days after injury. *C*: Mac-3 immunostaining on frozen transverse sections of TA muscle 3 days after injury. *D*: Gr-1 immunostaining on frozen transverse sections of TA muscle 3 days after injury. *E*: Mac-3 immunostaining on frozen transverse sections of TA muscle 9 days after injury. *F*: average percent stained area in the injured muscle at the time points indicated (see METHODS for the positive staining evaluation technique). PBS liposome-treated mice (solid bars) and clodronate liposome-treated mice (open bars). Values represent means ± SE (n = 4). *Significantly different positive staining in clodronate liposome-treated mice compared with PBS liposome-treated mice ($P < 0.05$).

treated mice (Fig. 3*B*). However, the mean regenerating myofiber CSA was not different between PBS liposome- and clodronate liposome-treated mice at 9 days postinjury (0.53 ± 0.09 and 0.527 ± 0.053 ratio of regenerating to uninjured fiber CSAs, respectively). Two weeks after injury, no sign of damage was detectable in the PBS liposome-treated muscles. The histopathology at this time point was comparable for the mice treated with clodronate liposomes, except increased fat infiltration (severity grade of 3 for 5 and 2 for 2 mice) into the interstitium of the injured TA muscle (Fig. 3*D*). Collagen staining was minimal in the regenerating muscles from both treatment groups (representative images are in Fig. 3*D*). Fur-

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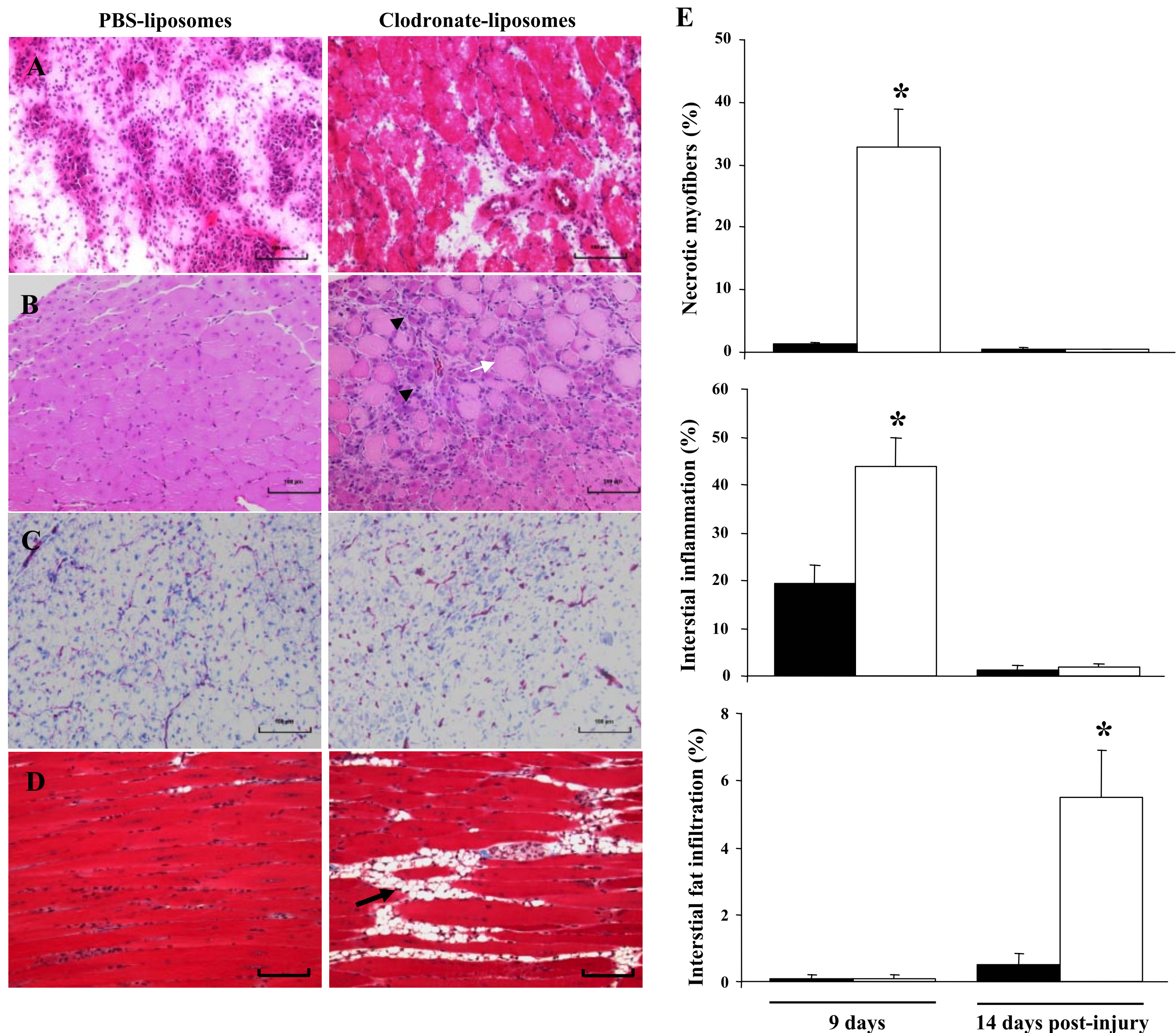

Fig. 3. Microscopic appearance of TA muscle of PBS liposome- and clodronate liposome-treated mice at 3, 9, and 14 days after freeze injury. The images are representative of those obtained on muscles from at least 4 mice from each group. Scale bars = 100 μm. *A*: hematoxylin and eosin (H&E) staining of transverse sections of TA muscle at 3 days after injury. *B*: H&E staining of transverse sections of TA muscle at 9 days after injury. The white arrow indicates necrotic myofibers, and black arrows indicate inflammatory cells. *C*: CD31 immunostaining on frozen transverse sections of TA muscle at 9 days after injury. *D*: Masson's trichrome staining of longitudinal sections of TA muscle 14 days postinjury (collagen stains blue). The arrow indicates fat accumulation. *E*: morphometric analysis of the proportion of the tissue volume occupied by necrotic myofibers, inflammation, or fat infiltration on longitudinal sections of TA muscle 9 or 14 days postinjury. PBS-liposome-treated mice (solid bars) and clodronate liposome-treated mice (open bars). Values represent means (SD; $n = 6$). *Significantly different expression in clodronate liposome-treated mice compared with PBS-liposome-treated mice ($P < 0.05$).

thermore, immunohistochemistry for a vascular endothelial cell marker, CD31/PECAM-1 (19), was conducted to assess whether angiogenesis was altered in regenerating muscle of clodronate-treated mice. We observed no differences in the number of CD31-positive cells between the PBS-and clodronate liposome-treated groups (representative images are in Fig. 3*C*). However, we cannot rule out the possibility that small differences in the number or diameter of microvessels may exist between the treatment groups. Morphometric analysis using quantitative stereology confirmed that at 9 days after injury, the percentage of the necrotic myofibers, as well as the interstitial inflammation, was significantly higher in the clodronate-treated mice compared to the PBS control-treated group (Fig. 3*E*). Furthermore, this analysis confirmed that at 14 days, the interstitial fat accumulation was significantly higher in the clodronate-treated group compared to the PBS control-treated group (Fig. 3*E*).

Effect of monocyte and macrophage depletion on expression of genes coding for mediators related to muscle injury/repair. Real-time RT-PCR was applied to evaluate whether the macrophage influx into the injured muscle affects the expression of genes associated with initial stages of the muscle injury/repair process. As we have demonstrated before, using mainly DNA microarray gene expression analysis, the expression of genes

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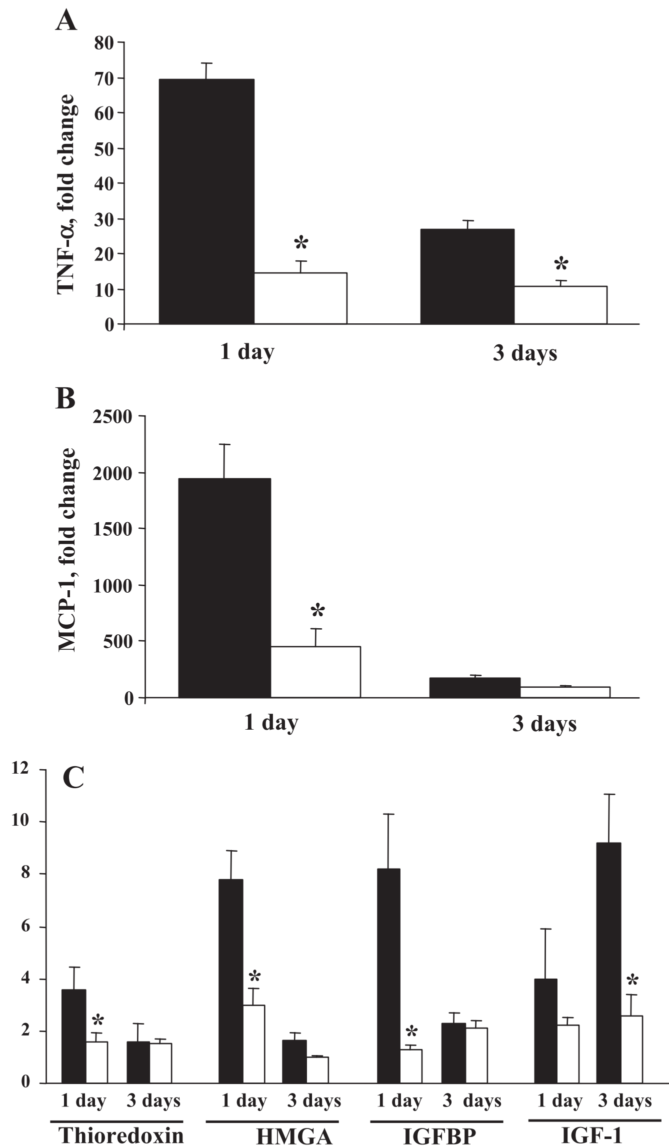

Fig. 4. Injury-induced expression of genes, coding early mediators of injury/repair process in TA muscle of PBS-and clodronate liposome-treated mice. Injured and uninjured (control) TA muscles were obtained from mice at the times indicated and analyzed for mRNA transcripts using real-time PCR. Expression was normalized to 18S/rRNA from the same samples and presented as the fold-increase above control (uninjured TA muscle). The mRNA transcripts of all of the tested genes were not significantly different between the control TA muscles from mice treated with PBS or clodronate liposomes. PBS liposome-treated mice (solid bars) and clodronate liposome-treated mice (open bars). Values represent means ± SE (n = 5). *Significantly different expression in clodronate liposome-treated mice compared with PBS-liposome treated mice ($P < 0.05$). *A*: TNF-α mRNA expression. *B*: MCP-1 mRNA expression. *C*: thioredoxin, high-mobility group AT-hook 1 (HMGA), insulin-like growth factor-binding protein (IGRBP), and IGF-I mRNA expression.

coding for mediators related to inflammation, oxidative stress, or cell growth, including TNF-α, MCP-1, thioredoxin, HMGA, IGFBP, IGF-1, CSF-1, and TGF-β, is up-regulated in TA muscle in the first days of postfreeze injury (30). The clodronate liposome treatment did not modify the mRNA transcript levels in the control (uninjured) muscles for all of the genes of interest. In contrast, this treatment significantly reduced injury-induced mRNA transcripts of TNF-α, MCP-1, thioredoxin, HMGA, and IGFBP at 1 day postinjury (Fig. 4, *A–C*). Of these five genes, only TNF-α mRNA expression continued to show a statistically significant decrease at 3 days postinjury (Fig. 4*A*). Furthermore, at *day 3* postinjury, injured muscles from clodronate liposome-treated mice also expressed less IGF-1 mRNA compared to injured muscles from PBS liposome-treated mice (Fig. 4*C*). In contrast, CSF-1, as well as TGF-β gene expression, was not affected by the monocyte and macrophage depletion at either 1 or 3 days postinjury (data not shown).

To evaluate the effect of clodronate liposome treatment on the maturation of muscle precursor cells, real-time RT-PCR was applied to determine the expression of MyoD and myogenin, markers of activated/proliferating and differentiating muscle precursor cells, respectively (26). When compared with uninjured control muscles, increases in MyoD and myogenin expression occurred in the injured muscles of both the PBS liposome- and clodronate liposome-treated mice at 3 days postinjury ($P < 0.05$) (Fig. 5). However, the transcript levels of these myogenic factors in the injured muscle did not differ between the two treatment groups. Consistent with the MyoD RT-PCR data, MyoD-immunostained nuclei, which belonged to activated and proliferating myoblasts, were found by *day 3* postinjury in the injured muscles of both PBS liposome- and clodronate liposome-treated groups (Fig. 6).

DISCUSSION

The clodronate liposome treatment, which targets mainly peripheral monocytes/macrophages, results in a marked attenuation of the peak inflammatory response in the injured muscle in the first 3 days after injury. This effect is accompanied with a transient reduction of the expression of genes coding for mediators that may affect a variety of cells involved in the injury/repair process of the muscle. However, the expression of major myogenic factors (i.e., MyoD and myogenin), directly involved in the activation and proliferation and differentiation of muscle precursor cells, is not altered by the clodronate liposome treatment. Under these circumstances, the injured muscle regenerates, but the repair process is impaired by prolonged clearance of necrotic myofibers and a tendency for increased muscle fat accumulation.

Growing evidence demonstrates that monocytes and macrophages (hematogenous or resident) are involved in wound-healing

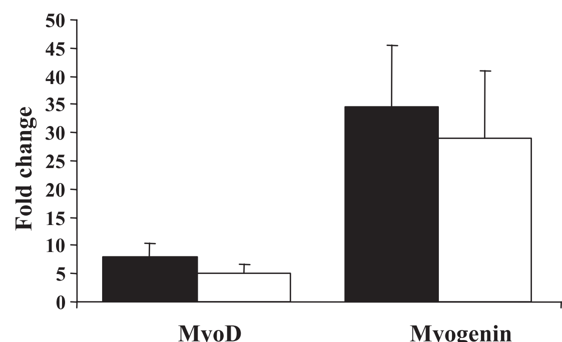

Fig. 5. Injury-induced MyoD and myogenin gene expression in TA muscle (at *day 3* postinjury) of PBS liposome- and clodronate liposome-treated mice. Injured and uninjured (control) TA muscles were obtained from mice on *day 3* and analyzed for mRNA transcripts using real-time PCR. Expression was normalized to 18S/rRNA from the same samples and presented as the fold-increase above control. PBS liposome-treated mice (solid bars) and clodronate liposome-treated mice (open bars). Values represent means ± SE (n = 5).

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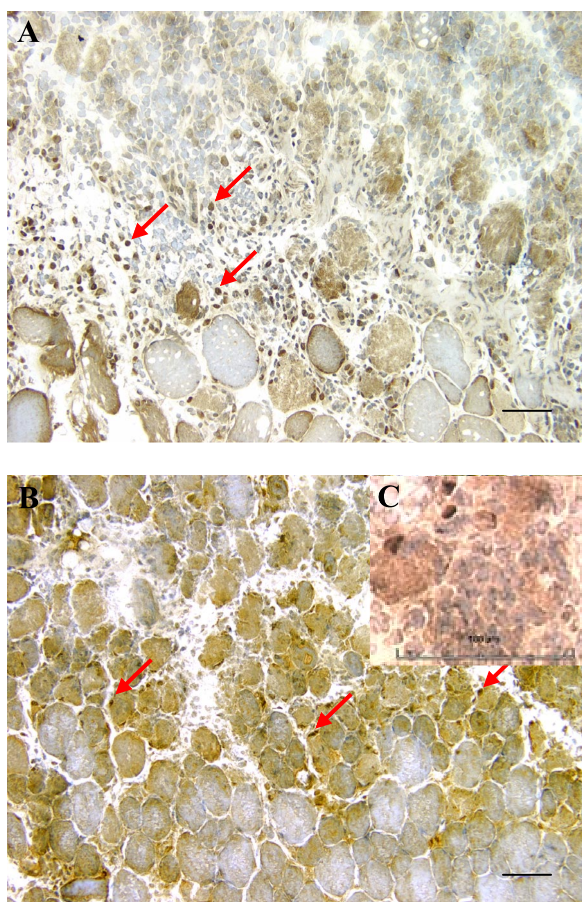

Fig. 6. Microscopic evaluation for MyoD-positive nuclei in injured TA muscle (at *day 3* postinjury). Images are representative of those obtained on muscles from 4 mice from each of the 2 groups. Scale bars: 100 μm. A: PBS liposome-treated mice. *B*: clodronate liposome-treated mice. Arrows show positive nuclei. *C*: MyoD-immunopositive nuclei in higher magnification (×400).

mechanisms, including those from muscle injury (for a review, see Ref. 33). Many physiological processes, such as aging, hypoperfusion, and local or systemic infection likely exert some of their negative effects on muscle regeneration secondary to suppression of the innate immune system (e.g., inflammatory cell activation). The strongest evidence that impaired inflammatory responses are detrimental to muscle regeneration has arisen from the numerous studies investigating the use of anti-inflammatory drugs (e.g., corticosteroids and nonsteroidal anti-inflammatory drugs) after muscle injury (for reviews, see Refs. 1 and 22). These drugs may be mildly beneficial in the short term (i.e., 2–3 days postinjury) but have adverse effects in the long term, with an impaired regeneration and functional restoration. Anti-inflammatory drugs of great recent interest, i.e., cyclooxygenase-2 (COX-2) inhibitors, have recently been shown to attenuate macrophage infiltration and to impair regeneration of injured muscle (2, 15). Additionally, if inflammatory responses are modified by targeted genetic manipulation or immune neutralization, muscle regeneration can be attenuated in mice. We have shown that immune neutralization of TNF-α, a major secretory product of activated macrophages, or genetic elimination of its receptors delays muscle regeneration and functional restoration after traumatic injury (37). More recently, we found MCP-1, a highly expressed chemokine in injured muscle, and its receptor CCR2 to play essential roles in the regeneration and functional recovery of injured muscle (38, 39). Suppression of the MCP-1/CCR2 signaling axis leads to impaired regenerative processes with development of compensatory fat infiltration and fibrous scar formation. Modulation of prostaglandin synthesis or suppression of the TNF-α or MCP-1/CCR2 signaling axis may target not only macrophages but all cell types resident in muscle, including muscle precursor cells (2, 38, 39). In contrast, clodronate liposome treatment only affects phagocytic monocytes and macrophages because the treatment is not toxic to nonphagocytic cells (23, 36). Although multiple injections of clodronate liposomes were administered, which should provide long-term depletion of blood-borne macrophages (21), the inflammatory response was upregulated at 9 days postinjury, compared with that in injured muscles from mice treated with control liposomes. Apparently, as a result, the clearance of necrotic myofibers in muscles from clodronate liposome-treated mice was hastened between 9 and 14 days postinjury, and the histopathological differences between the two treatment groups had become minimal by the latter time point. This late macrophage response may have resulted from the activation and proliferation of macrophages, which are present in the damaged muscle of the liposomal clodronate-treated mice or through continuous infiltration of monocytes from the circulation. Consistent with previous results that liposomes are not able to cross intact capillary walls (36), the liposomal clodronate treatment did not result in a reduced number of residential macrophages at least in the uninjured muscle. The greatest reduction (i.e., 90%) in circulating monocytes has been reported to take place at 18–24 h after a single clodronate liposome injection, but repopulation occurs in 3–4 days (31). Thus our clodronate liposome injection protocol, which called for injections every third day, provided an opportunity for circulating monocytes, although in a reduced number, to infiltrate the injured tissue. Additionally, the clodronate liposome treatment did not affect the expression of CSF-1 and only diminished the expression of MCP-1, two major factors involved in recruitment and activation of macrophages (6, 20).

A reduced monocyte and macrophage infiltration into injured muscle was associated with diminished expression not only of inflammatory mediators, such as TNF-α and MCP-1, but also of the growth factors IGF1 and IGFBP. The members of the IGF family are known to play an essential role in the regulation of myogenesis. These growth factors are able to alter both muscle precursor cell proliferation and differentiation in vitro (for a review, see Ref. 42). IGFs exert their effects through binding to two types of receptors: insulin-like growth factor receptor-1 (IGFR-1) and IGFR-2 (7). Additionally, IGF bioavailability is modulated by IGFBPs, which have a higher affinity for IGF than the IGFRs (9). It is possible that the infiltrating macrophages are a source of IGF-1/IGFBPs, or they may provide cytokines such as TNF-α, which may control IGF gene expression by resident muscle cells. However, the reduced IGF-1 gene expression in the injured muscle of clodronate liposome-treated mice was not associated with reduced expression of MyoD and myogenin, which demonstrates that the level of IGF-1 in that muscle was in excess or, alternatively, that there are redundant myogenic factor-stimulating mechanisms in the injured muscle. Furthermore, monocyte/macrophage depletion resulted in reduced gene expression of HMGA and thioredoxin, mediators that have been shown to participate in a variety of inflammatory, cell-death, and growth-related processes (4, 28). Although the cellular sources and role of these mediators in muscle injury and repair are not characterized,

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the upregulation of these genes after muscle injury has been recognized by recent gene expression profiling studies (30, 41).

In conclusion, pharmacological depletion of hematogenous monocytes/macrophages leads to a significant reduction of the inflammatory response in the first days postinjury. This is associated with neither improved nor abrogated, but moderately impaired, repair processes in the injured muscle. Thus redundant cellular sources, including resident macrophages, may provide mediators necessary for complete regeneration of trauma-injured muscle. Further studies should be directed to evaluate the role of immunotherapies targeting the resident macrophages in skeletal muscle regeneration, for example via local Toll-like receptor activation.

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