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Role of CC chemokines in skeletal muscle functional restoration after injury

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Warren, Gordon L., Laura O'Farrell, Mukesh Summan, Tracy Hulderman, Dawn Mishra, Michael I. Luster, William A. Kuziel, and Petia P. Simeonova. Role of CC chemokines in skeletal muscle functional restoration after injury. *Am J Physiol Cell Physiol* 286: C1031–C1036, 2004. First published January 14, 2004; 10.1152/ajpcell.00467.2003.—The purpose of this study was to determine whether certain chemokines, which are highly expressed in injured skeletal muscle, are involved in the repair and functional recovery of the muscle after traumatic injury. In wild-type control mice, mRNA transcripts of macrophage inflammatory protein (MIP)-1α, MIP-1β, and monocyte chemoattractant protein (MCP)-1 as well as their major receptors, CCR5 and CCR2, increased after freeze injury and gradually returned to control (uninjured) levels by 14 days. Muscle function and histological characteristics were monitored in injured mice that were genetically deficient for the CCR5 receptor (a major receptor for MIP-1α and MIP-1β) and also rendered MCP-1 deficient with neutralizing antibodies. To dissect the role of these chemokines, additional studies were conducted in CCR5- and CCR2-deficient mice. CCR5−/− mice injected with MCP-1 antiserum for the first 3 days after injury exhibited a twofold greater maximal isometric tetanic torque deficit at 14 days after injury than did controls (i.e., 33% vs. 17%; $P = 0.002$). The impaired functional recovery was accompanied with an increased fat infiltration within the regenerating muscle without a significant difference in the influx of inflammatory cells, including macrophages. Strength recovery was also impaired in mice deficient for the receptor of MCP-1, CCR2, but not in CCR5−/− mice that were not injected with MCP-1 antiserum. The data suggest that MCP-1/CCR2 plays a role in the regeneration and recovery of function after traumatic muscle injury.

inflammation; regeneration; chemokine receptors

CHEMOKINES ARE small-molecular-weight proteins of 70–90 amino acids that possess chemoattractant properties for a number of cell types. These small proteins belong to four different families (C, CC, CXC, CX3C) that are distinguished by the position of one or two cysteine residues near the NH_2-terminal end. Chemokines of the CC family are mainly involved in the migration and activation of monocytes, macrophages, and lymphocytes, whereas those of the CXC family attract polymorphonuclear neutrophils (PMNs) (1). In addition to their chemotactic effects on leukocytes, most chemokines have broader functions including influencing angiogenesis, collagen production, and proliferation of hematopoietic precursor cells (14, 19). Chemokines exert their effects through specific seven transmembrane-spanning, G protein-coupled receptors that are differentially expressed on cell populations mainly of hematopoietic origin (31). For example, macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), and RANTES (regulated upon activation, normal T-expressed, and presumably secreted) (CCL5) bind mainly to the CCR5 receptor, whereas monocyte chemoattractant protein (MCP)-1 (CCL2) and several other members of the MCP family, such as MCP-2 (CCL8) and MCP-3 (CCL7), are ligands for the CCR2 receptor (3, 21).

Many inflammatory diseases with a marked mononuclear cell involvement such as rheumatoid arthritis, asthma, and atherosclerosis have been associated with elevated CC chemokine expression, and the progression of these diseases has been related to the functions of the CC chemokines (4, 7, 23). CC chemokines have also been demonstrated to play a role in traumatic injuries to the central and peripheral nervous systems as well as in skin wound healing, mainly through regulation of monocyte/macrophage recruitment or activation (5, 17, 18, 24). Recently, gene expression profiling demonstrated that enhanced expression of the CC chemokines is also a characteristic of injured skeletal muscle (9, 25). Migration and activation of cells including monocytes/macrophages as well as satellite cells is an obligatory component of the repair process after injury (11, 26). Although the role of chemokines in skeletal muscle injury and repair is not known, it is possible that these mediators may affect cells that express the appropriate receptors and thus influence the repair mechanisms after injury. In this respect, we hypothesized that *1*) MCP-1, MIP-1α, and MIP-1β expression is accompanied by expression of their major receptors including CCR2 and CCR5 in the injured skeletal muscle and *2*) signaling through these receptors may influence the functional recovery of muscle after traumatic injury. Because these three chemokines share redundant physiological activities (1, 19), we first monitored the functional recovery of freeze-injured tibialis anterior (TA) muscles from mice that were genetically deficient for the CCR5 receptor (a major receptor for MIP-1α and MIP-1β) and also rendered MCP-1 deficient with neutralizing antibodies. To dissect the role of these chemokines, additional studies were conducted in CCR5- and CCR2-deficient mice. The results demonstrate that although both CCR2 and CCR5 receptors are expressed in injured muscle, the MCP-1/CCR2 axis, but not signaling through CCR5, plays a role in restoration of skeletal muscle function after traumatic injury.

METHODS

Animals

CCR2−/− mice on a mixed C57BL/6 × strain 129 genetic background were generated as described previously (15, 16). The

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CCR2−/− were then backcrossed to C57BL/G mice for 10 generations. CCR5−/− mice (B6 and C57BL/6J;129P2-Ccr5^{tm1Kuz}) as well as wild-type controls (B6;129PF2/J) were obtained from the Jackson Laboratory (Bar Harbor, ME). Mean (±SD) weights for the CCR5−/− mice ($n = 37$) and their controls ($n = 47$) were 21.5 ± 2.1 and 21.3 ± 2.4 g, respectively; ages for all mice were between 13 and 16 wk. Mean weights for the CCR2−/− mice ($n = 9$) and their controls ($n = 8$) were 24.4 ± 2.1 and 21.5 ± 0.6 g, respectively; ages for these mice were between 18 and 24 wk. The mice were provided food and water ad libitum and were maintained on a 12:12-h light-dark cycle. Animal care and use 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 pub. no. 86–23, 1996). These procedures were approved by the Georgia State University and National Institute for Occupational Safety and Health institutional animal care and use committees.

Polyclonal antiserum to murine MCP-1 was prepared (Biosource International, Camarillo, CA) by injecting New Zealand White rabbits with recombinant murine MCP-1 (PeproTech, Rocky Hill, NJ) as described previously (13). The antiserum completely neutralizes the activity of 500 pg/ml of mouse MCP-1 and does not react with mouse MIP-1α or MIP-1β, as measured by a competitive ELISA (data not shown). CCR5−/− mice received intraperitoneal injections of MCP-1 antiserum (10 μl/g body wt) 2 h before injury and every 12 h for the first 3 days after injury. Some CCR5−/− mice received MCP-1 antiserum injections for 14 days (injected as described above for the first 3 days plus once daily from 4 to 14 days after injury).

Induction of Freeze-Induced Muscle Injury

The procedure used was identical to that previously described (27). 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. The skin incision was then closed with silk suture. In preparation for muscle injury induction and isometric strength testing, mice were anesthetized with 0.33 mg/kg fentanyl, 16.7 mg/kg droperidol, and 5.0 mg/kg diazepam administered intraperitoneally. We previously determined (12) that this anesthetic regimen is optimal for in vivo muscle strength testing in mice.

Measurement of In Vivo Isometric Strength

Maximal isometric tetanic torque of the left anterior crural muscles was measured before and after freeze injury with a miniature dynamometer as described previously (27, 28). In brief, the lateral surface of the lower left hindlimb was shaved and aseptically prepared. Two 36-gauge needle electrodes were inserted percutaneously to positions adjacent to the common peroneal nerve. These electrodes were connected to a stimulator, and the muscle was stimulated with 200-ms trains of 0.1-ms biphasic pulses at 300 Hz. Voltage and position of the electrodes were adjusted to yield the maximal isometric tetanic torque of the anterior crural muscle group. The TA muscle was then injured as described in *Induction of Freeze-Induced Muscle Injury*. Two minutes after injury, the maximal isometric tetanic torque measurement was repeated. In all mice, maximal isometric tetanic torque was measured at one additional time point after injury induction (i.e., 7, 14, or 28 days after injury). These time points were selected on the basis of previous work with this injury model in which we found minimal recovery of strength in the first days after injury, rapid recovery during the second week, and near-complete recovery after 4 wk (Ref. 27; unpublished observations).

Real Time RT-PCR

TA muscles were collected in RNAlater (Qiagen, Valencia, CA) and homogenized, and total RNA was extracted with a commercial kit (RNeasy, Qiagen) following the manufacturer's protocol. cDNA was synthesized from 1 μg of RNA with Superscript II (Life Technologies, Gaithersburg, MD). Real-time PCR for CCR2, CCR5, MIP-1α, MIP-1β, MCP-1, and 18S/rRNA was performed with predeveloped primers and probes (TaqMan assay reagents, PE Applied Biosystems, Foster City, CA) on an ABI Prism 7700 Sequence detector (PE Applied Biosystems). The comparative threshold cycle (C_T) method was used to calculate the relative concentrations as described previously (25). This method involved obtaining C_T values for the transcript of interest, normalizing to the housekeeping gene 18S/rRNA (TaqMan assays), and comparing relative increases between controls and experimental samples.

Histopathology

Muscles processed for paraffin-embedded histology were removed and fixed by immersion in 10% neutral-buffered formalin. The tissues were then embedded in paraffin, cut into 6-μm-thick longitudinal sections, and stained with hematoxylin and eosin for blinded histopathological assessment. Histopathological findings were graded from 1 to 5 depending on severity (1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe, 5 = severe/high) in six mice per group. Masson's trichrome stain to visualize collagen was applied as described originally (20).

Representative muscle tissue was embedded in Tissue Tek OCT (Miles Scientific), frozen in melting isopentane, and stored at −80°C. Ten cross sections (10 μm thick) were cut at each of six levels equally spaced along the length of the TA muscle in a microtome cryostat at −20°C. Immunostaining for Mac-3 was conducted as described previously (27). Immunostaining for MIP-1β, MCP-1, CCR2, and CCR5 was conducted on formalin-fixed cryosections with specific polyclonal antibodies prepared in goats (Santa Cruz Biotechnology, Santa Cruz, CA). The antibodies were diluted 1:300 in phosphate-buffered saline (PBS) and applied to the sections for 18 h at 4°C. The slides were visualized with the ImmunoCruz staining system (Santa Cruz Biotechnology) and Tyramide Signal amplification kit (Molecular Probes, Eugene, OR) according to the manufacturers' instructions.

Statistics

The strength differences among groups of mice were analyzed with a two-way (group × time) repeated-measures ANOVA. When significant interactions were found, single degree of freedom contrasts were applied as post hoc tests. For comparison of mRNA transcript levels between control and injured muscles, unpaired Student *t*-tests were used. An α-level of 0.05 was used for all analyses. All statistical testing was conducted with SPSS (version 10.0). Values presented are means ± SE.

RESULTS

Expression of MIP-1α, MIP-1β, MCP-1, CCR2, and CCR5 in Injured TA Muscles

RNA, isolated from an injured or a contralateral uninjured (control) muscle, was examined for transcripts of MIP-1α, MIP-1β, MCP-1, CCR5, and CCR2 by TaqMan real-time PCR after muscle freeze injury. The largest increases in MIP-1α, MIP-1β, and MCP-1 expression occurred within 24 h of injury. The rapid increases in the chemokine mRNA transcript levels were followed by gradual reductions over *days 3–7* and a return to control levels by *day 14*. CCR2 and CCR5 mRNA expression in the injured TA muscle were increased only in the first 24 h after injury (Fig. 1*B*). The contralateral uninjured muscles expressed low constitutive levels of MIP-1α, MIP-1β, MCP-1, CCR2, and CCR5 mRNA that did not vary over time after injury. Immuno-

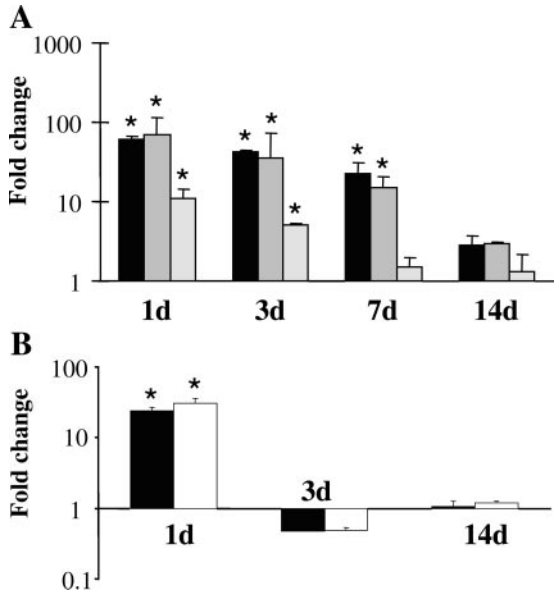

Fig. 1. Time course of monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, CCR2, and CCR5 expression in muscle tissue after freeze injury. Injured and uninjured (control) tibialis anterior (TA) muscles were obtained from each mouse at the times indicated (in days) and analyzed for mRNA transcripts with real-time PCR. The expression was normalized to 18S RNA from the same samples and presented as a fold increase above control. *Significantly different from controls ($n = 3$, $P < 0.05$). *A*: MCP-1 (black bars), MIP-1α (gray bars), and MIP-1β (light gray bars) fold increase. *B*: CCR2 (filled bars) and CCR5 (open bars) fold increase.

staining for CCR5, MIP-1β, CCR2, and MCP-1 revealed that mRNA changes were accompanied by increases in the corresponding proteins within the freeze-injured TA muscle (Fig. 2). Minimal immunostaining was observed in the first 24 h of injury (data not shown), but considerable staining was observed by postinjury *day 3*. The immunoreactive proteins were not detected in uninjured muscles but were readily observed in the damaged muscle region adjacent to the deep, uninjured region.

Evaluation of Effects of CCR5/MCP-1 Deficiency on TA Muscle Injury and Recovery

Muscle strength in vivo. Wild-type controls and chemokine-deficient mice (CCR5−/− mice injected with MCP-1 antiserum for the first 3 days after injury) experienced nearly identical losses of maximal isometric tetanic torque immediately after injury (i.e., 75 vs. 73%; Fig. 3). The torque deficits for the two groups after 7 days were also very similar (i.e., ~55%). However, the chemokine-deficient group recovered more slowly during the second week, and at 14 days after injury the torque deficit for these mice was double that of the control mice (i.e., 33 vs. 17%; $P = 0.002$). By 28 days after injury, neither group showed significant deficits in maximal isometric tetanic torque. The difference in torque between the two groups at 14 days after injury was not exacerbated by a more prolonged administration of the MCP-1 antiserum. Compared with control mice, CCR5−/− mice injected with antiserum for 14 days exhibited a twofold greater torque deficit at 14 days after injury (i.e., 31.0 ± 5.1% vs. 16.1 ± 2.9%; $P = 0.018$). These findings were

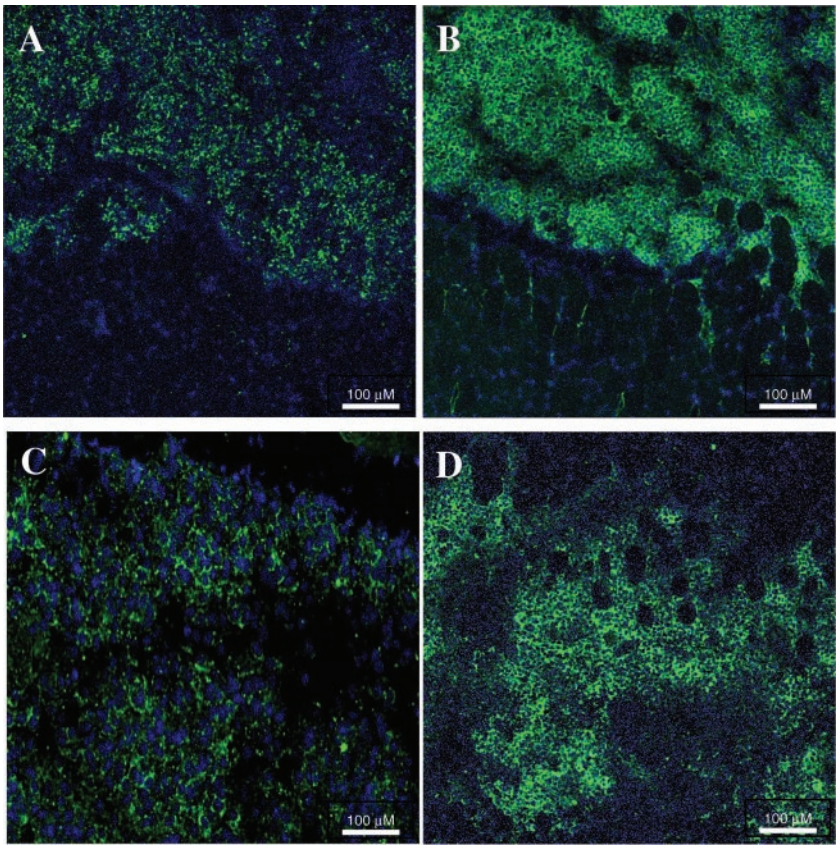

Fig. 2. Immunostaining for MCP-1, MIP-1β, CCR2, and CCR5 in TA muscle 3 days after freeze injury. The proteins of interest were stained green, and all nuclei were stained blue (DAPI). *A*: CCR2. *B*: MCP-1. *C*: CCR5. *D*: MIP-1β. Original magnification, ×100.

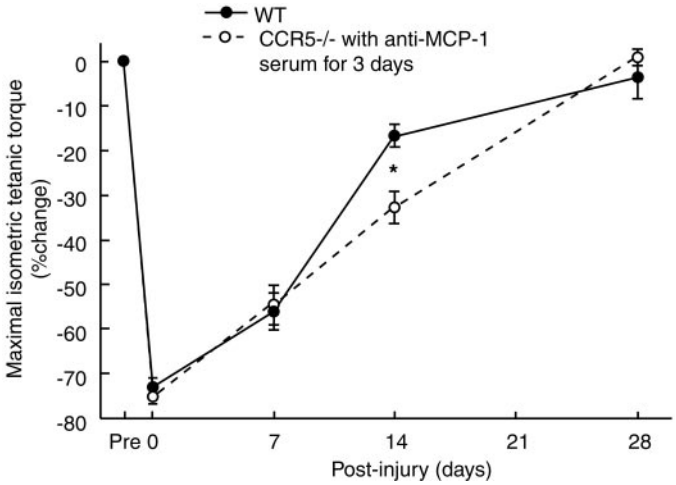

Fig. 3. Effect of chemokine deficiency on anterior crural muscle maximal isometric tetanic torque over the 28 days after freeze injury. Chemokine-deficient mice were CCR5−/− mice that were injected with MCP-1 antiserum for the first 3 days after injury, whereas wild-type (WT) controls were B6;129PF2/J mice. *Significant difference between the 2 groups of mice ($P < 0.05$; $n = 8$–9 mice/group at this time point).

virtually identical to those obtained for the CCR5−/− mice receiving antiserum for only 3 days (Fig. 3).

Histopathology. Hematoxylin and eosin staining indicated that ~60% of the TA muscle was affected by the freeze injury. Approximately three-quarters of the muscle cross section were damaged directly beneath where the steel probe had been applied, whereas the deepest one-quarter of the muscle cross section appeared histologically normal. By postinjury *day 3*, the levels of inflammatory cell infiltration had peaked in the injured muscles of both wild-type controls and chemokine-deficient mice including CCR5−/− mice injected with anti-MCP-1 for 3 days. Furthermore, there were no marked differences in the degree of tissue damage or inflammatory cell response between control mice and chemokine-deficient mice at this time point (Fig. 4*A*). The majority of the inflammatory cells in the injured TA muscles from both control and experimental groups were positive for Mac-3, a marker of activated monocytes/macrophages (Fig. 4*B*). However, by postinjury *day 14*, no sign of damage was detectable in the mice from the control group, indicating a complete regeneration, whereas the chemokine-deficient mice exhibited increased fat infiltration (severity grade of 2 for 4 mice or 3 for 2 mice) into the interstitium of the injured TA muscles (Fig. 4*C*). We next investigated the presence of collagen in the injured muscle with the Masson's trichrome procedure, which stains collagen blue. As shown in Fig. 4*D*, small amounts of collagen were seen in the interstitium of injured muscles from the chemokine-deficient mice (severity grade of 1) but not in muscles from control animals.

Muscle strength recovery in CCR5−/− and CCR2−/− mice. To evaluate whether the delay in muscle function recovery was dependent more on the CCR5 signaling chemokines or on MCP-1, which signals through CCR2, maximal isometric tetanic torque at 14 days after injury was measured in CCR2−/− mice and CCR5−/− mice not injected with MCP-1 antiserum. The CCR5−/− mice not injected with MCP-1 antiserum exhibited a torque deficit at 14 days after injury similar to that of the wild-type controls (i.e., 20.3 vs. 19.6%, respectively; $P = 0.90$; Fig. 5*A*). In contrast, injured CCR2−/− mice retested at 14 days after injury exhibited a greater torque deficit compared with the wild-type controls (i.e., 38 vs. 25%, respectively; $P = 0.03$; Fig. 5*B*). The effect of the CCR2 deficiency on the strength

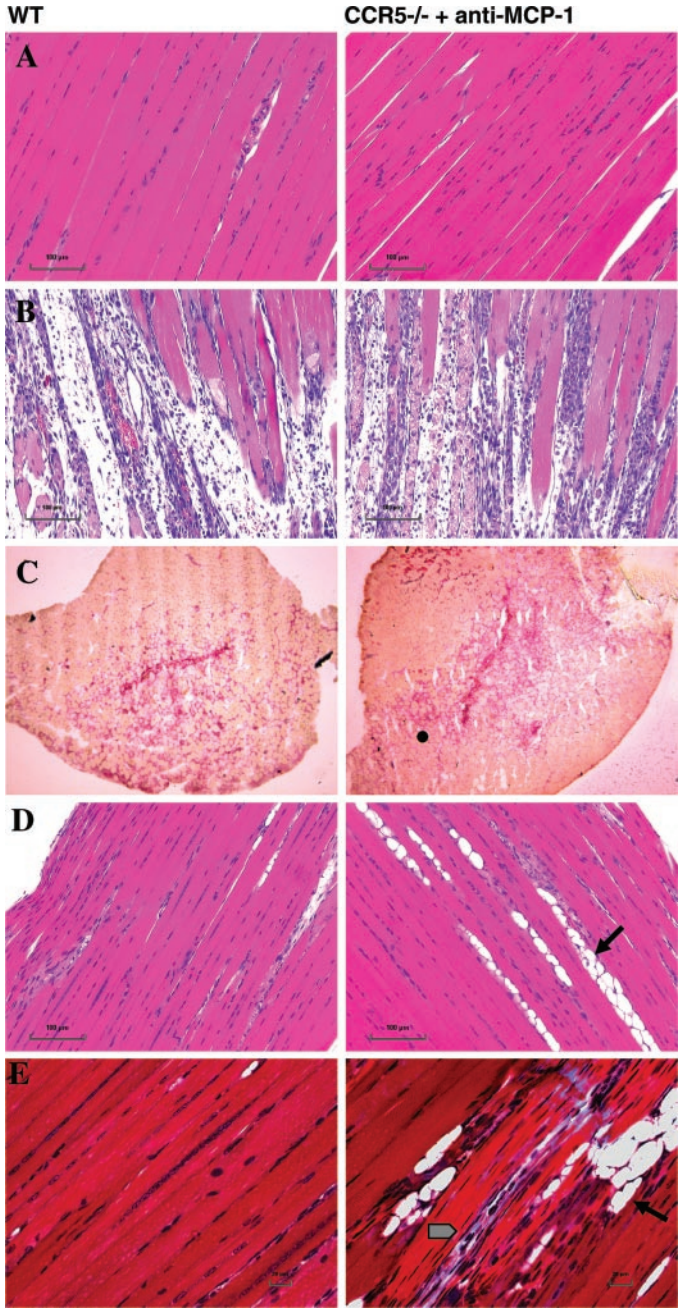

Fig. 4. Microscopic comparison of TA muscle of wild-type and CCR5−/− mice injected with anti-MCP-1. *A*: hematoxylin and eosin staining of longitudinal sections of uninjured TA muscle. *B*: hematoxylin and eosin staining of longitudinal sections of TA muscle 3 days after injury. *C*: Mac-3 immunostaining on frozen transverse sections of TA muscle 3 days after injury. *D*: hematoxylin and eosin staining of longitudinal sections of TA muscle 14 days after injury (original magnification ×200). *E*: trichrome staining of longitudinal sections of TA muscle 14 days after injury (original magnification ×400). Collagen is stained blue. The black arrows show fat accumulation; the gray arrow shows collagen.

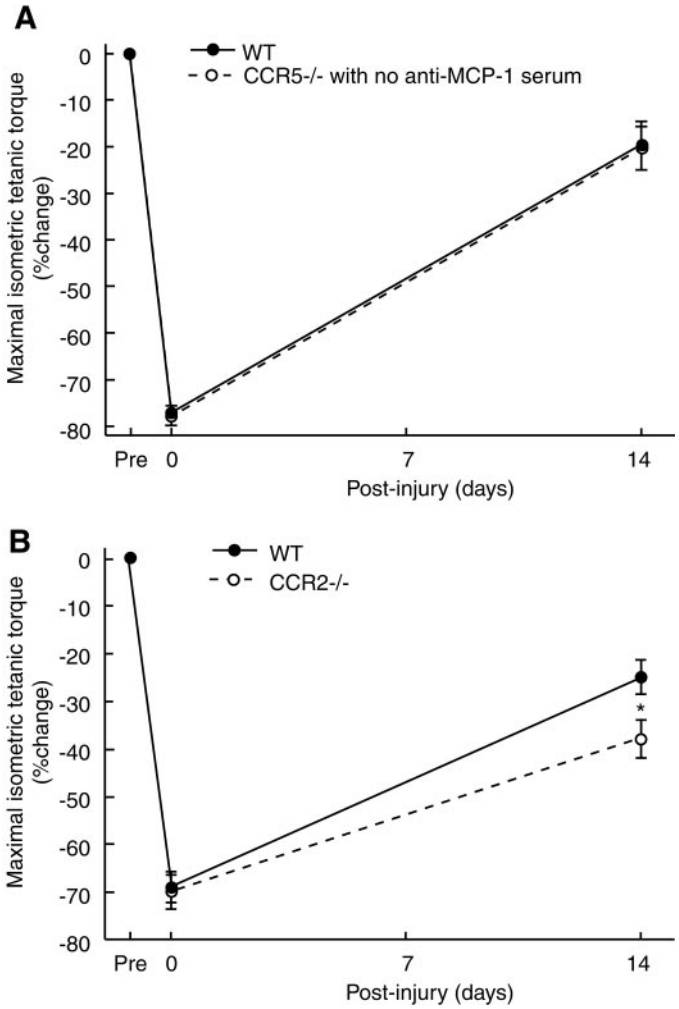

Fig. 5. Effect of chemokine receptor deficiency on anterior crural muscle maximal isometric tetanic torque immediately and 14 days after freeze injury. *A*: maximal isometric tetanic torque of the left anterior crural muscles in CCR5-deficient mice and wild-type controls (B6;129PF2/J) after injury. *B*: maximal isometric tetanic torque of the left anterior crural muscles in CCR2-deficient mice and wild-type controls (C57BL/6J) after injury. *Significant difference between the two groups of mice ($P < 0.05$; $n = 8$–9 mice/group).

deficit was comparable in magnitude to that of MCP-1 neutralization.

DISCUSSION

Several members of the CC family of chemokines, including MCP-1 with signaling through CCR2 as well as MIP-1α and MIP-1β with major signaling through CCR5, are found at high levels in the early phases of skeletal muscle injury (9, 25). The CC chemokines are structurally related molecules that display redundant functional activities, especially regarding regulation of inflammatory cell influx and activation (1, 14, 19). We used the combination of CCR5 genetic deficiency and MCP-1 neutralization to determine the role that chemokines play in degenerative or repair mechanisms after traumatic muscle injury. Freeze injury to muscle is associated with early expression (peak levels at 24 h after injury) of not only the chemokines MCP-1, MIP-1α, and MIP-1β but also their major receptors, CCR2 and CCR5. Our findings demonstrate that the regenerative mechanisms that lead to functional restoration of the freeze-injured TA muscle are influenced by the chemokine response. Consistent with the time course for MCP-1 expression, which peaks 3 days after injury, MCP-1 neutralization in the first 3 days after injury delayed the restoration of muscle strength at postinjury *day 14*. The effect of chemokine deficiency was transient, because muscle function in the chemokine-deficient mice had fully recovered by postinjury *day 28*, demonstrating that MCP-1 is more of a modifier than an essential regulator of muscle regeneration. In contrast to the MCP-1 signaling, although the expression of MIP-1α and MIP-1β as well as CCR5 is increased after injury, signaling through this receptor does not affect the recovery of skeletal muscle function. Because MIP-1α is a ligand for CCR1 and both MIP-1α and MIP-1β are ligands for CCR9 (14), it is possible that these chemokines may modulate muscle repair via receptors other than CCR5 or, alternatively, that MIP-1α and MIP-1β may play a negligible role in muscle repair. In contrast to the normal recovery in CCR5−/− mice, CCR2−/− mice exhibited a significant impairment in strength recovery after injury, confirming that the MCP-1/CCR2 axis plays a role in the recovery from muscle injury. Understanding the specific roles of the various inflammatory mediators in skeletal muscle regeneration will be important, particularly in terms of directing therapeutic interventions.

Strength loss is the most important functional consequence and a reliable indicator of muscle injury (29). Satellite cells, myogenic precursor cells, have been demonstrated to be necessary for the recovery of strength after injury, probably by assisting in the restoration of contractile protein content (22). Resident satellite cells migrate from surrounding uninjured areas to the injured site and fuse together to form myoblasts, which in turn fuse to form myotubes (8). In addition, it was demonstrated recently that bone marrow-derived cells can migrate to areas of induced degeneration to undergo myogenic differentiation and participate in the regeneration of damage fibers (6). Many growth factors, such as hepatocyte growth factor (HGF) and insulin-like growth factor (IGF), have been studied and demonstrated as potential activator and chemotactic factors for the muscle precursor cells (2, 8, 30). Other immune mediators, such as IL-4, have been shown to modulate the fusion process of the satellite cells through induction of calcium-dependent cell signals (10). Many of these mediators, like chemokines, are released by activated macrophages involved in the injury process. Although the role of chemokines in modulating satellite cell migration and activation has not been studied, our findings of a delay in the functional restoration of animals deficient for MCP-1/CCR2 suggest that the chemokines may modulate the satellite cell response after injury. This effect may be by stimulating migratory, proliferative, or fusion activities of the satellite cells or indirectly through macrophage activation. Although neutralization of MCP-1 did not influence the inflammatory cell accumulation after injury, a role of MCP-1 in macrophage activation is not excluded. Future studies should be directed toward identifying the mechanisms through which MCP-1 and/or CCR2 modulate skeletal muscle regeneration and how they affect the recruitment and activation of the satellite cells.

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