Regulation of skeletal muscle regeneration by CCR2-activating chemokines is directly related to macrophage recruitment

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Departments of 1Surgery, 2Medicine, 3Epidemiology and Biostatistics, 4Pathology, and 5Periodontics, and 6Sam and Ann Barshop Institute for Longevity and Aging Studies, at the University of Texas Health Science Center San Antonio; and 7The South Texas Veterans Health Care System, San Antonio, Texas

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Martinez CO, McHale MJ, Wells JT, Ochoa O, Michalek JE, McManus LM, Shireman PK. Regulation of skeletal muscle regeneration by CCR2-activating chemokines is directly related to macrophage recruitment. Am J Physiol Regul Integr Comp Physiol 299: R832–R842, 2010. First published July 14, 2010; doi:10.1152/ajpregu.00797.2009.—Muscle regeneration requires CCR2-expressing macrophages, which are a prominent CCR2-expressing cell in the regenerative process (34). A better understanding of the mechanisms of muscle regeneration, including how BM-derived cells contribute to these phenomena, could lead to new primary and adjuvant therapies for limb salvage and tissue engineering.

The expression of CC chemokine receptor 2 (CCR2) on BM-derived cells is critical for normal skeletal muscle regeneration (34). This study (34) and related experiments (1, 33, 35), suggest that recruitment of BM-derived cells, especially macrophages, are necessary for skeletal muscle regeneration. In support of these findings, mice lacking macrophage chemotactic protein-1 (MCP-1, also known as CCL2), a chemokine ligand of CCR2, demonstrated impaired muscle regeneration following ischemia injury induced by femoral artery excision (30). Similarly, mice lacking CCR2 demonstrated severe deficits in skeletal muscle regeneration with increased adipocyte accumulation following ischemia (7) in addition to impaired angiogenesis with injury induced by cardiotoxin (CTX) injection (23). In mice, the MCP family members, MCP-1 (5), MCP-3 (CCL7) (15), and MCP-5 (CCL12) (28), bind to CCR2 and influence inflammatory and progenitor cell recruitment.

MCP-1 and CCR2 could conceivably affect angiogenesis and skeletal muscle regeneration in many ways. MCP-1 and MCP-2 are expressed on endothelial cells and in vitro, MCP-1 production is increased after injury (37). After femoral artery excision, MCP-1 expression was increased in ischemic muscle, where angiogenesis and muscle regeneration occurred, but not in the nonischemic thigh muscles, where arteriogenesis, also known as collateral artery formation, occurred (31). While there are many potential sources of MCP-1, in the ischemia model, MCP-1 was localized to macrophages and endothelial cells (31). In the CTX model, MCP-1 was maximally increased in injured muscle 1 day after injection, suggesting that endothelial cells, and not macrophages, were the main source of MCP-1 (23).

The present study used CTX injury, an established model of muscle regeneration (11, 23, 34). CTX contains lytic factors that degrade the muscle plasma membrane (11) resulting in muscle necrosis followed by an inflammatory response with angiogenesis and skeletal muscle regeneration (23). CTX disrupts the endothelial barrier, allowing albumin to extravasate into the injured muscle. IR-820 is a near-infrared contrast agent that binds to albumin. IR-820 signal intensity peaked at 1 h after CTX injection and remained elevated for several days. At 3 days after CTX injection, IR-820 signal intensity decreased, which corresponded to macrophage infiltration, suggesting that macrophages may clear IR-820-bound albumin, in addition to phagocytizing necrotic myofibers (26). Interestingly, the effect of CTX was localized to the injured muscle, as no increase in systemic vascular permeability was detected (26). Given the importance of CCR2-dependent responses in regenerative events, the purpose of this study was to determine

SKELETAL MUSCLE REGENERATION is an important component of limb salvage, defined as saving arms and legs, following traumatic and/or ischemic injury to the extremities, and bone marrow (BM)-derived cells play an important role in this reparative process (34). A better understanding of the mechanisms of muscle regeneration, including how BM-derived cells contribute to these phenomena, could lead to new primary and adjuvant therapies for limb salvage and tissue engineering.

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the role of MCP-1 in these processes. We hypothesized that MCP-1−/− animals will demonstrate similarly significant deficits in macrophage recruitment, adipocyte accumulation, angiogenesis, and skeletal muscle regeneration as CCR2−/− mice following CTX injury.

METHODS

Experimental animals. CCR2−/− (16) and MCP-1−/− (18) mice on a C57Bl/6J background were derived as previously described and backcrossed to C57Bl/6J mice from Jackson Laboratory (Bar Harbor, ME) for six generations. Original CCR2−/− and MCP-1−/− breeders were a kind gift from William A. Kuziel (Daiichi Sankyo Research Institute, Edison, NJ) and Barrett J. Rollins (Dana-Farber Cancer Institute, Harvard University, Boston, MA), respectively. CCR2−/− and MCP-1−/− mice were bred at the Audie Murphy Veterans Hospital (San Antonio, TX), and C57Bl/6J WT control mice were purchased from Jackson Laboratory. Background strain testing of 107 markers performed by Charles River Laboratories (Wilmington, MA) demonstrated a 100% match of both strains with C57Bl/6J WT mice (data not shown). Male mice, 4–6 mo old, were used in this study. All procedures complied with the National Institutes of Health Animal Care and Use Guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and at the South Texas Veterans Health Care Systems, San Antonio, TX.

Mouse CTX model. Myonecrosis was induced by the intramuscular injection of CTX (Calbiochem, San Diego, CA) as previously described (23, 34). In brief, two 50–μl CTX (2.5 μM in normal saline (NS)) injections were delivered uniformly into the muscles of the right hind limb anterior compartment. Similarly, the right hind limb posterior compartment received four 50–μl CTX injections. The left hind limb was injected with identical volumes of NS. Baseline mice that did not receive any injections were used as controls. To increase cell yields for flow cytometry experiments, mice received CTX injections in both hind limbs. Baseline samples were obtained from mice that did not receive any injections.

Histology and histomorphometry. Routine, indirect immunolocalization procedures were used to identify monocytes/macrophages in the tibioischial (TA) muscle of mice following CTX injury. Deparaffinized sections of 10% neutral buffered formalin-fixed muscle were utilized for the indirect immunohistochemical localization of Mac3 following antigen retrieval in 10 mM citrate buffer, pH 6.0, 30 min at 100°C. Sections were incubated (30 min, room temperature) with monoclonal rat anti-mouse Mac3 (clone M3/84; BD Biosciences, San Jose, CA) or the corresponding isotype control (IgG1, κ), each diluted to a final concentration of 0.156 μg/ml in PBS containing 1% BSA (MP Biomedicals, Solon, OH). After incubation (30 min, room temperature) with biotinylated secondary antibody (donkey anti-rat IgG) obtained from Jackson ImmunoResearch Laboratories, West Grove, PA (diluted 1:200 in 1% BSA), sections were treated with streptavidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA) for 30 min (room temperature) followed by incubation in diaminobenzidine/hydrogen peroxide with subsequent counterstaining in hematoxylin.

Myofiber cross-sectional area, %fat, and capillary density in TA muscle were analyzed as previously described (23). Mice were killed at baseline and following CTX and NS injections. Hind limb muscles were collected en bloc from the anterior and posterior compartments, weighed, and fixed in 10% neutral buffered formalin prior to routine paraffin embedding.

The averaged cross-sectional area (μm²) of myofibers in specimen was determined after outlining individual myofibers in representative, digitized images of a given TA muscle. Only regenerated fibers with centrally located nuclei (3) were measured in the post-CTX specimen, whereas, mature myofibers with peripherally located nuclei were measured in baseline specimen. Fat area (%) was calculated after manual outline of intermuscular adipocyte area and division by the total area of the image.

In addition to determination of the averaged cross-sectional area of myofibers in a given TA muscle, the percentage of myofibers in incremental units of 500 μm² was calculated for each specimen. Averaged results were derived from similar animals at comparable time points following CTX administration.

Capillary counts were assessed after treatment of deparaffinized cross sections of TA muscle with a biotinylated lectin, Griffonia (Banisteria) simplicifolia lectin I (Vector Laboratories), at 1:50 dilution followed by streptavidin-horseradish peroxidase and incubation in diaminobenzidine/hydrogen peroxide to identify endothelial cells as previously described (23). The number of capillaries and myofibers in each image were counted. Only capillaries associated with myofibers were included and expressed as capillaries per fiber (C/F). In addition, after subtraction of areas of fat, fibrosis, and residual necrosis from the total area, capillary density was expressed as capillaries per millimeter to the second power. Animal numbers for WT and CCR2−/− mice were increased herein and were similar to previously published data for fiber cross-sectional area, fat area, and capillary density (23).

Necrotic myofibers were readily distinguished on the basis of microscopic myofiber appearance: swollen/enlarged fibers with fragmented, pale, and eosinophilic cytoplasm, compared with both non-injured and regenerated cells, and the absence of centrally-located nuclei that were prevalent in regenerated myofibers.

The area of muscle injury and residual necrosis in the TA muscle of individual animals obtained at day 7 post-CTX was measured on hematoxylin and eosin-stained cross-sections after digital scanning of microscope slides using a model CS ScanScope system (Aperio Technologies, Vista, CA). The digitally captured slide was analyzed via ImageScope software (version v10.3.6.1805; Aperio Technologies) to measure the total area of the TA, area of TA injury, and area of residual TA necrosis. The entire area of injury for a given TA muscle in cross section was defined as the area of regenerated muscle cells with centrally-located nuclei in combination with the area of residual necrotic myofibers. Percent necrosis was calculated as the area of necrosis relative to the entire area of injury. Percent injury was calculated as the entire area of injury relative to the entire cross-sectional area of the TA.

Determination of percent total body fat. Determination of percent total body fat in baseline mice was performed using a PIXimus Mouse Densitometer (General Electric, Waukesha, WI) after anesthesia following the intraperitoneal injection of pentobarbital (60 mg/kg; Abbott Laboratories, Chicago, IL). Data for WT mice was previously published (23).

Tissue levels of MCP-5. MCP-5 tissue levels were measured by ELISA in muscle tissue lysates as previously described (23, 30). MCP-5 was chosen because it is closely related to human MCP-1 in structure (28), and blocking antibodies to murine MCP-5 are available (22). Anterior compartment muscles of WT and MCP-1−/− mice were harvested after CTX injection into the right hind limb and NS injection into the left hind limb and utilized to prepare tissue lysates as previously described (23). MCP-5 levels in tissue lysates were assessed by ELISA (R&D Systems, Minneapols, MN), according to the manufacturer’s protocol with slight modifications. Standards and unknowns were diluted in lysate buffer and results were expressed as picograms per milligrams protein. Samples from WT and MCP-1−/− mice were processed concurrently.

Protein in the tissue lysates was determined by the BCA protein assay (Pierce Biotechnology, Rockford, IL) using a microtiter plate format; BSA (ICN Biomedical, Costa Mesa, CA) in tissue lysate buffer (23) was used as the standard. Absorption in microtiter plate assays was monitored using a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA), and results were analyzed with SOFTmax PRO software (Molecular Devices).
MCP-5 blocking-antibody. MCP-5 blocking antibody was a kind gift from Anuk Das (Centocor Research and Development, Radnor, PA). MCP-1−/− mice received intraperitoneal injections of MCP-5 blocking antibody (0.5 mg) or rat IgG control antibody (Jackson Immunoresearch Laboratories, West Grove, PA) 4 h prior to CTX injections and weekly injections thereafter until the mice were euthanized. An additional control group consisted of MCP-1−/− mice that only received CTX injection.

Tissue immune cell, macrophage, neutrophil, and CD45+/Sca-1+ cell quantification. Tissue CD45+ immune cells, macrophages, neutrophils, and CD45+/Sca-1+ cells were measured using flow cytometry. All flow cytometry analyses were performed on a FACSAria equipped with FACSDiva software (BD Biosciences).

TA muscle and posterior compartments of bilateral hind limbs were harvested at baseline or after CTX injection and used to create two separate cell suspensions for each mouse for analysis. Tissues underwent enzymatic dissociation (HBSS (Invitrogen, Carlsbad, CA) supplemented with 2,500 units/ml collagenase II (Invitrogen), 4.0 µg/ml Dispase (Invitrogen), and 2.5 mM CaCl2 (Sigma-Aldrich)) at 37°C for 90 min with a 40-µm strainer (BD Biosciences). The filtrate was centrifuged at 700 g to harvest dissociated cells that were further quantified. Trypan Blue (Invitrogen) was added to an aliquot of the filtrate to harvest cells from each population was calculated by using the percent data divided by the weight of the tissue. Percent of cells from each population was quantified. Trypan Blue (Invitrogen) was added to an aliquot of the filtrate to harvest cells that was counted on a hemocytometer to derive a total cell count and divided by the weight of the tissue. Percent of cells from each population was determined by flow cytometry (described below) and absolute number of each cell population was calculated by using the percent data multiplied by the total number of cells per gram of tissue.

Single cell suspensions were treated with mAb 2.4G2 for 20 min on ice to block Fcγ II/III receptors followed by incubation with conjugated antibodies at 4°C for 30 min. Cell populations were defined as follows: neutrophils (CD45+/CD11b+/Ly-6G+), macrophages (CD45+/CD11b+/Ly-6G−) (8, 9, 17), immune cells (CD45+), and BM progenitor cells (CD45+/Sca-1+) (25). The fluorescent-conjugated rat anti-mouse monoclonal antibodies (all antibodies were from BD Biosciences) were as follows: FITC CD45, PE Sca-1, APC Ly-6G, and PE CD11b as well as the corresponding isotype control antibodies. Isotype controls were used to titrate each antibody to minimize background staining, propidium iodide (1 µg/ml; Sigma-Aldrich) was used for dead cell exclusion, and fluorescence-minus-one controls were used to generate gates (27).

Data analysis. Fiber cross-sectional area, capillary density, and MCP-5 tissue levels were analyzed with Dunnett’s multiple-comparison procedure using a two-way ANOVA of least-square means to determine whether significant differences existed at different time points post-CTX injection compared with baseline values. ANOVA with Bonferroni-corrected P values was used to determine significant differences between WT, MCP-1−/−, and CCR2−/− mice at individual time points. For lysate samples with MCP-5 below the level of detection (≤15.625 pg/ml), a value of 15.625/2 was assigned to these samples (12), and this value was corrected for the protein in each sample. Fiber distribution was analyzed by subtracting the smallest (0–500) from all larger percent fiber ranges (501–1,000 through 5001+), and differences from baseline and between mouse strains were determined using mean contrast based on a mixed linear model in terms of size range and day with Bonferroni correction. Percent intermuscular fat, percent injury, and percent necrosis were analyzed by the Exact Wilcoxon Test using a Bonferroni correction, and percent total body fat was analyzed by an unpaired Student’s t-test.

Flow cytometry data was analyzed by a Dunnett’s-corrected multiple comparisons procedure utilizing a two-way ANOVA of least square means to determine whether significant differences existed at different time points (1, 3, 7, and 14 days after injection) compared with baseline values. Bonferroni-corrected P values were used to determine significant differences between WT, MCP-1−/−, and CCR2−/− mice at individual time points. Total cells isolated from muscle data was first log transformed and analyzed the same as the flow cytometry data.
baseline, mature myofiber size from days 7–21, with most fibers being smaller than 1,500 μm² at day 7. The regenerated myofiber distribution was similar to baseline by day 28. In contrast, CCR2−/− mouse regenerated myofiber size distribution was even more decreased with most myofibers being smaller than 500 μm² at day 7. The regenerated myofibers gradually increased in size, but still had a smaller (P < 0.001) myofiber size distribution at all postinjury time points compared with baseline. Similar to the cross-sectional area, the MCP-1−/− mice exhibited an intermediate phenotype in myofiber size distribution with most fibers being smaller than 1,000 μm² at day 7 but still demonstrating a smaller (P ≤ 0.004) myofiber size distribution at all postinjury time points compared with baseline. Furthermore, all three strains were significantly different (P ≤ 0.004) from one another at all postinjury time points.

Intersmuscular fat in regenerated muscle was also estimated by histomorphometry. At baseline, no intersmuscular fat was identified in any of the three mouse strains (Fig. 2B). Following injury, the percent fat in WT, MCP-1−/−, and CCR2−/− mice at 7–28 days following CTX injection was significantly increased (P ≤ 0.001) compared with baseline. Furthermore, MCP-1−/− mice demonstrated increased (P = 0.008) percent fat at 7 days compared with WT mice. CCR2−/− mice had increased percent fat compared with WT mice at days 7–28 (P ≤ 0.008) and compared with MCP-1−/− animals at days 7–21 (P ≤ 0.02). MCP-1−/− mice demonstrated increased intersmuscular fat after injury compared with WT, but less intersmuscular fat compared with CCR2−/− animals, providing further evidence of the intermediate muscle regeneration phenotype present in MCP-1−/− mice.

Percent total body fat of baseline WT or MCP−/− mice was comparable (15.7% ± 0.6 and 15.2% ± 0.5, respectively, n = 10 mice/strain) and similar to percent total body fat previously published (23) for CCR2−/− mice. This suggests that the increased percent intersmuscular fat observed after injury in MCP−/− and CCR2−/− mice was not attributable to differences present in the three mouse strains at baseline.
following CTX injection, C/F was significantly increased (P ≤ 0.007) in WT mice at 21 and 28 days compared with baseline (Fig. 4A). MCP-1−/− mice did not exhibit a significant difference in C/F compared with baseline, but did demonstrate a significant difference (P = 0.05) to WT mice at 21 days. In contrast, as previously published (23), CCR2−/− mice demonstrated a significant decrease in C/F (P < 0.001) at 14–28 days compared with baseline. Additionally, CCR2−/− mice demonstrated significantly decreased C/F at 14–28 days following injury compared with WT (P < 0.001) and MCP-1−/− (P < 0.001) mice.

Capillary density, expressed as capillaries per millimeter to the second power (Fig. 4B) or capillaries per millimeter to the second power (Fig. 4A) or capillaries per millimeter to the second power (Fig. 4B) in the TA of WT, MCP-1−/−, and CCR2−/− mice. However, there were no significant differences between WT and MCP-1−/− mice at corresponding time points.

Capillary density in injured skeletal muscle. At baseline, there were no significant differences in C/F (Fig. 4A) or capillaries per millimeter to the second power (Fig. 4B) in the TA of WT, MCP-1−/−, and CCR2−/− mice. However, the percent injury and residual necrosis within the TA muscle at 7 days following CTX injection was evaluated with histomorphometry in all three mouse strains. The extent of skeletal muscle injury within the TA muscle at baseline (no injury) and after CTX-induced injury, n = 8–15 mice/strain/time point. A: cross-sectional area (μm²) of muscle fibers in WT, MCP-1−/−, and CCR2−/− mice of mature myofibers at baseline and after injury (regenerated myofibers). B: fat area (%) at baseline was not detectable (ND) in all 3 mouse strains but increased after injury. Data are means ± SE. *Significant difference (P < 0.001) compared with baseline for each mouse strain; †significant difference (P ≤ 0.008) between WT and MCP-1−/− mice at corresponding time points; #significant difference (P ≤ 0.008) between WT and CCR2−/− mice at corresponding time points; $significant difference (P ≤ 0.02) between MCP-1−/− and CCR2−/− mice at corresponding time points.

Fig. 2. Impaired muscle regeneration, myofiber cross-sectional area, and fat area in MCP-1−/− and CCR2−/− mice. Measurements were performed in the TA muscle at baseline (no injury) and after CTX-induced injury, n = 8–15 mice/strain/time point. A: cross-sectional area (μm²) of muscle fibers in WT, MCP-1−/−, and CCR2−/− mice of mature myofibers at baseline and after injury (regenerated myofibers). B: fat area (%) at baseline was not detectable (ND) in all 3 mouse strains but increased after injury. Data are means ± SE. *Significant difference (P < 0.001) compared with baseline for each mouse strain; †significant difference (P ≤ 0.008) between WT and MCP-1−/− mice at corresponding time points; #significant difference (P ≤ 0.008) between WT and CCR2−/− mice at corresponding time points; $significant difference (P ≤ 0.02) between MCP-1−/− and CCR2−/− mice at corresponding time points.

The percent injury and residual necrosis within the TA muscle at 7 days following CTX injection was evaluated with histomorphometry in all three mouse strains. The extent of skeletal muscle injury within the TA muscle following CTX injection was similar in WT, MCP-1−/−, and CCR2−/− mice (Table 1). However, residual necrosis was highest in CCR2−/−, intermediate in MCP-1−/−, and lowest in WT animals (Table 1). Thus, while all three mouse strains exhibited similar injury, the removal of necrotic tissue was differentially impaired in MCP-1−/− compared with CCR2−/− mice.

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total cells at day 3 compared with both WT (P < 0.001) and MCP-1−/− mice (P = 0.01). Recruitment of CD45+ immune cells into injured skeletal muscle followed a similar pattern as total cell recruitment (Fig. 6B). Compared with baseline, there was an immediate and sustained increase in both WT and MCP-1−/− animals. In contrast, CCR2−/− mice exhibited an increase (P ≤ 0.007) in CD45+ cells compared with baseline at all postinjury time points except day 3. In addition, CD45+ cell recruitment was significantly different (P ≤ 0.002) in all three strains at day 3, with WT exhibiting the largest increase, MCP-1−/− mice were intermediate, and CCR2−/− mice had the fewest CD45+ cells.

The recruitment of polymorphonuclear neutrophilic leukocytes (neutrophils or PMN) (CD45+/CD11b+/Ly-6G+ cells) into injured skeletal muscle peaked at day 1 after injury in all three mouse strains and remained elevated compared with baseline through day 7 in WT (P ≤ 0.04) and MCP-1−/− mice (P < 0.001) and through day 14 in CCR2−/− (P ≤ 0.02) mice (Fig. 6C). PMN were similar between the mouse strains at all time points except for day 14; CCR2−/− mice exhibited small but significant elevations compared with both WT (P = 0.002) and MCP-1−/− animals (P = 0.02). Thus, baseline numbers of PMN and recruitment into injured muscle were similar in all three mouse strains except for a sustained elevation at day 14 in CCR2−/− mice.

Tissue monocyte/macrophages (CD45+/CD11b+/Ly-6G-cells) exhibited the most striking differences between the three animal strains (Fig. 6D). Compared with baseline, WT mice exhibited significant increases (P < 0.001) in macrophage recruitment after injury through day 7 with maximal numbers of macrophages present at day 3 (21 ± 3 × 10⁶ macrophages/g tissue). MCP-1−/− mice demonstrated a much slower, but increased (P ≤ 0.005) macrophages, compared with baseline at days 1–7, with maximal macrophages present at day 7 (8 ± 2 × 10⁶ macrophages/g tissue). CCR2−/− animals exhibited significant increases (P ≤ 0.008) in macrophages compared with baseline from days 3–14 after injury with maximal numbers occurring at day 7 (3 ± 1 × 10⁶ macrophages/g tissue). Furthermore, significant differences (P ≤ 0.03) existed be-

**Table 1. Extent of injury and residual necrosis in tibialis anterior (TA) muscle at 7 days following the administration of cardiotoxin (CTX) in wild-type (WT), monocyte chemotactic protein-1−/− (MCP-1−/−), and CC chemokine receptor-2−/− (CCR2−/−) mice**

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<tr>
<td>Injury</td>
<td>85.2 ± 3.3</td>
<td>86.8 ± 2.9</td>
<td>92.0 ± 2.3</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0.5 ± 0.2</td>
<td>7.1 ± 3.0†</td>
<td>38.7 ± 1.7†</td>
</tr>
</tbody>
</table>

Values are means ± SE in percent. Histomorphometric measurements of the TA muscle in cross section were used to determine the extent of injury and residual necrosis at day 7 post-CTX injection; n = 12–15 mice/strain. *Significant difference (P < 0.001) between WT and MCP-1−/− mice; †significant difference (P < 0.001) between WT and CCR2−/− mice; ‡significant difference (P < 0.001) between MCP-1−/− and CCR2−/− mice.
cells/g tissue) were macrophages \((21 \pm 3 \times 10^6 \text{ macrophages/g tissue})\). Thus, the \(5 \pm 1 \times 10^6 \text{ CD45+/Sca-1+ cells/g tissue at least partially represent a subset of macrophages in WT mice.}\]

**Monocyte/macrophage tissue distribution following injury and during regeneration of mouse skeletal muscle.** Consistent with results obtained by flow cytometry, immunohistochemistry confirmed that the majority of infiltrating cells in the TA of WT mice at day 3 post-CTX carried the monocyte/macrophage marker, Mac3 (Fig. 7A). Interestingly, at day 7 post-CTX in WT mice, interstitial mononuclear cells present at the perimeter of all regenerated myofibers were also Mac3+ (Fig. 7D). Subsequently, Mac3+ cells were progressively reduced in regenerated muscle through day 14 and virtually absent by day 21 (Fig. 7, G and J). In MCP-1−/− mice, macrophage accumulation in injured TA muscle was significantly attenuated at day 3 (Fig. 7B), increased by day 7 in association with necrotic myofibers that persisted among newly-regenerated cells (Fig. 7E), and then decreased and redistributed to an interstitial pattern within 14–21 days post-CTX (Fig. 7, H and K). Thus, the accumulation of Mac3+ cells in MCP-1−/− mice was delayed and reduced compared with that of WT animals; however, the tissue distribution of these cells was ultimately comparable although displaced in time following injury; the pattern of Mac3+ cells surrounding injured myofibers at 7 days postinjury in a MCP-1−/− animal most closely resembled that of muscle obtained at 3 days from a WT mouse. A similar, but even further diminished and delayed pattern of macrophage accumulation was observed in CTX-injured TA muscle in CCR2−/− mice (Fig. 7, C, F, I, and L); note that at later time points postinjury (14 and 21 days) in these animals, aggregates of Mac3+ cells were associated with foci of residual necrotic myofibers (Fig. 7, I and L).

**DISCUSSION**

The present study examined the role of MCP-1 and CCR2 in immune/inflammatory cell recruitment to regenerating skeletal muscle. Despite MCP-1 being a major ligand for CCR2, MCP-1−/− mice demonstrated an intermediate phenotype to WT and CCR2−/− animals with respect to macrophage recruitment, regenerating fiber cross-sectional area, and intermuscular adipocyte accumulation, but surprisingly, similar capillary density compared with WT mice.

With the development of the intermediate phenotype in the MCP-1−/− mice, the role of MCP-5 (28), a CCR2-activating chemokine, was investigated. CCR2 is the only known receptor for MCP-5 (28). In the present study, MCP-5 exhibited a sustained and significant elevation in the injured muscle of MCP-1−/− mice compared with WT mice. Thus, elevated MCP-5 may play a role in the partial rescue of muscle regeneration in MCP-1−/− mice. Interestingly, by day 3, when maximal macrophage recruitment had occurred in WT, MCP-5 levels were significantly greater in the MCP-1−/− mice, potentially signifying the continuing attempt to recruit macrophages in the MCP-1−/− animals. If elevated MCP-5 levels could partially rescue muscle regeneration in MCP-1−/− mice, then blocking MCP-5 in MCP-1−/− mice may result in a similarly impaired muscle regeneration phenotype as observed in CCR2−/− animals. However, administration of a MCP-5 neutralizing antibody did not alter the myofiber cross-sectional area or fat accumulation in MCP-1−/− mice.

Between all three mouse strains at days 1 and 3 after injury; for these time points, WT had the highest numbers of macrophages, MCP-1−/− mice were intermediate, and CCR2−/− animals had the fewest macrophages. Thus, the kinetics of macrophage recruitment and absolute number of macrophages were severely and moderately impaired in CCR2−/− and MCP-1−/− mice, respectively.

CD45+/Sca-1+ cells (Fig. 6E) demonstrated a similar recruitment pattern in the three mouse strains as macrophages. While there were no significant differences between WT and MCP-1−/− mice at any time point, CD45+/Sca-1+ cells were decreased in CCR2−/− mice compared with both WT \((P < 0.001)\) and MCP-1−/− mice \((P < 0.001)\) at day 3. Furthermore, in WT mice at day 3, almost all of the CD45+ cells \((22 \pm 3 \times 10^6\) cells/g tissue) were macrophages \((21 \pm 3 \times 10^6 \text{ macrophages/g tissue})\). Thus, the \(5 \pm 1 \times 10^6 \text{ CD45+/Sca-1+ cells/g tissue at least partially represent a subset of macrophages in WT mice.}\]
Interestingly, the same MCP-5 blocking antibody used herein was able to prevent CCR2-dependent fibrocyte recruitment in a lung injury model (22). Possible explanations may be that other chemokines besides MCP-5, such as MCP-3, are important in activating CCR2 within MCP-1−/− mice or the blocking antibody did not sufficiently suppress MCP-5. Recent studies have demonstrated that MCP-3 and MCP-1 are important for maintenance of blood monocytes (36). However, additional studies suggest that functional MCP-1 is required for MCP-3 activity and that MCP-1−/− mice express very low levels of MCP-3 (6, 13). If the MCP-1−/− mice herein did not have functional levels of MCP-3, then additional, and possibly undefined, CCR2-activating chemokines may be important in monocyte/macrophage recruitment.

Previous studies in chimeric mice demonstrated that expression of CCR2 on BM-derived cells regulated the myofiber cross-sectional area and macrophage recruitment (34). We quantified BM-derived cells in injured muscle to test whether the intermediate phenotype observed in MCP-1−/− mice could be due to differential recruitment of BM-derived cells and macrophages. CD45+ immune cells at day 3 post-CTX was the only time point that demonstrated a significant difference in cells between the three mouse strains, potentially indicating that immune cell recruitment during day 3 may be crucial for the normal reparative process of skeletal muscle regeneration. While neutrophil recruitment was relatively similar between the three mouse strains, striking differences in macrophage recruitment that paralleled CD45+ cell accumulation also occurred at the day 3 time point. Recruitment of macrophages to sites of injury has been demonstrated to be crucial for skeletal muscle regeneration (1, 33, 35). While MCP-1 is thought to be the main chemokine involved in this process (4), the differential recruitment of macrophages in MCP-1−/− and CCR2−/− mice suggest that other CCR2-activating chemokines are also critical for macrophage recruitment. The difference in day 3 post-CTX macrophage recruitment appeared to account for the difference in CD45+ cells between WT and MCP-1−/− mice for the same time point. Unlike the CCR2−/− mice that demonstrated severe impairments in macrophage recruitment following injury, MCP-1−/− animals were able to recruit some macrophages, albeit at a significantly reduced number compared with WT. These macrophages may have been able to partially regenerate injured skeletal muscle producing the intermediate phenotype of MCP-1−/− animals between WT and CCR2−/− mice. While injury was similar between all three strains, the amount of residual necrosis in the injured tissue inversely paralleled the macrophage recruitment with the most necrosis present in CCR2−/−, intermediate in MCP-1−/−, and least necrosis in WT mice, suggesting that macrophage levels were important in the resolution of necrosis.

Interestingly, macrophages are also important in angiogenesis (21). Capillary density was similar between WT and MCP-1−/− mice, while CCR2−/− mice demonstrated im-

![Fig. 5](http://ajpregu.physiology.org/). Elevated MCP-5 in injured muscle of MCP-1−/− mice but similar muscle regeneration with MCP-5 blocking antibody. A: measurement of tissue MCP-5 levels in the anterior compartment of WT and MCP-1−/− mice (5–9 mice/strain/time point) at baseline and after CTX-induced injury. *Significant difference compared with baseline (P < 0.02) for each mouse strain; †significant difference (P = 0.03) between WT and MCP-1−/− mice at corresponding time points. Histomorphometric measurements performed in the TA muscle of MCP-1−/− mice after CTX-induced injury and while receiving MCP-5 neutralizing antibody, control antibody, or no antibody. n = 6–21 mice/strain/antibody treatment/time point. B: cross-sectional area (μm²) of regenerating myofibers. C: fat area (%) after injury. Data are means ± SE.
Fig. 6. Flow cytometry analysis of cell populations present in baseline and injured muscles of WT, MCP-1−/−, and CCR2−/− mice. Measurements of absolute cell numbers performed in the TA muscle at baseline (no injury) and after CTX-induced injury, \( n = 4–11 \) mice/strain/time point. Total cells isolated (A), CD45+ immune cells (B), neutrophils (CD45+/CD11b+/Ly6G+ cells) (C), macrophages (CD45+/CD11b+/Ly6G- cells) (D), and CD45+/Sca-1+ cells (E). Data are means ± SE. *Significant difference compared with baseline (\( P \leq 0.05 \)) for each mouse strain, †significant difference (\( P \leq 0.03 \)) between WT and MCP-1−/− mice at corresponding time points, #significant difference (\( P \leq 0.002 \)) between WT and CCR2−/− mice at corresponding time points, §significant difference (\( P \leq 0.04 \)) between MCP-1−/− and CCR2−/− mice at corresponding time points.
paired angiogenesis. The partial recruitment of macrophages in MCP-1−/− mice may have been able to restore normal angiogenesis. Proinflammatory, or M1 macrophages, express CCR2 and are initially recruited to injured tissues (10). Anti-inflammatory, or M2 macrophages, do not express CCR2 and are important in angiogenesis (10, 19). Thus, the absolute numbers and possibly differences in types of macrophages present after injury may be crucial for angiogenesis. Alternatively, angiogenesis may have been impaired in MCP-1−/− mice at earlier time points than were studied herein. Extensive muscle necrosis precludes the accurate determination of capillary density (23); thus, earlier time points could not be studied using the methods in the present study. Current studies are developing new measures of in vivo angiogenesis that can be used at earlier time points as well as determining differences in macrophage types in the three mouse strains to resolve these intriguing possibilities.

Although the main source of cells for skeletal muscle regeneration are satellite cells (24, 29, 38), several interesting populations of progenitor cells reside within muscle (2, 14, 24). One such population is the CD45+/Sca-1+ cells that are present in muscle and may participate in regeneration. Although CD45+/Sca-1+ cells cultured from baseline muscle do not exhibit myogenic properties (20), with muscle injury these cells increase and attain myogenic potential in vitro, demonstrating that nonsatellite cell populations may participate in muscle regeneration (25). Similar numbers of CD45+/Sca-1+ cells were present in WT and MCP-1−/− mice but were significantly decreased in CCR2−/− animals. Thus, MCP-1−/− mice may retain the ability to recruit some populations of BM-derived cells that may assist in muscle regeneration, leading to the intermediate phenotype compared with CCR2−/− animals. Further studies to determine the recruitment of different populations of BM-derived cells in the three mouse strains are ongoing.

**Perspectives and Significance**

In conclusion, while the absence of MCP-1 results in impaired macrophage recruitment and muscle regeneration, MCP-1−/− mice exhibited an intermediate phenotype compared with CCR2−/− mice in regard to macrophage recruitment to the site of injury, resolution of necrosis, and skeletal muscle regeneration. Intermediate macrophage recruitment in MCP-1−/− mice was associated with similar capillary density to WT mice, suggesting that fewer macrophages maybe needed to restore angiogenesis vs. muscle regeneration. Finally, other chemokines, in addition to MCP-1, may activate CCR2-dependent regenerative processes resulting in an intermediate phenotype in MCP-1−/− mice. Additional studies are required to further define the chemokines involved in macrophage recruitment and the necessary cell populations that are needed for the normal reparative process of skeletal muscle regeneration and angiogenesis.
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