Inhibition of inflammatory CCR2 signaling promotes aged muscle regeneration and strength recovery after injury

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Muscle regeneration depends on a robust albeit transient inflammatory response. Persistent inflammation is a feature of age-related regenerative deficits, yet the underlying mechanisms are poorly understood. Here, we find inflammatory-related CC-chemokine-receptor 2 (Ccr2) expression in non-hematopoietic myogenic progenitors (MPs) during regeneration. After injury, the expression of Ccr2 in MPs corresponds to the levels of its ligands, the chemokines Ccl2, 7, and 8. We find stimulation of Ccr2-activity inhibits MP fusion and contribution to myofibers. This occurs in association with increases in MAPKp38/γ signaling, MyoD phosphorylation, and repression of the terminal myogenic commitment factor Myogenin. High levels of Ccr2-chemokines are a feature of regenerating aged muscle. Correspondingly, deletion of Ccr2 in MPs is necessary for proper fusion into regenerating aged muscle. Finally, opportune Ccr2 inhibition after injury enhances aged regeneration and functional recovery. These results demonstrate that inflammatory-induced activation of Ccr2 signaling in myogenic cells contributes to aged muscle regenerative decline.

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During tissue regeneration, the recruitment of inflammatory cells is a critical early response to injury. This recruitment aids in the establishment of a favorable environment for progenitor function and tissue regeneration. Chemokines play an important role in the recruitment of inflammatory cells to sites of injury; however, persistently elevated signaling contributes to chronic inflammation associated with impaired regeneration. Among the large chemokine superfamily members, Ccl2, 7, and 8 bind a shared receptor, Ccr2, and have key roles in the deleterious consequences of chronic chemokine activity. As such, inhibition of Ccr2 is being pursued as a clinical therapy in disease contexts. As a G-protein coupled transmembrane receptor, ligand-mediated activation of Ccr2 mobilizes intracellular G-proteins that help activate several pathways, including Erk and p38Mapk. Although Ccr2 signaling can inactivate several pathways, including Erk and p38Mapk, whether Ccr2 can directly inactivate several pathways, including Erk and p38Mapk, remains to be elucidated.

Ccr2 signaling inhibits terminal myogenic differentiation. Next, we examined if Ccr2 can function as a regulator of active SC and MP fate. To test this, isolated SCs were cultured in growth media for 48 h, and subsequently supplemented for 24 h with vehicle or Ccl2/7/8. Regardless of treatment, all cells were MyoD+ (MP marker); however, Ccl2/7/8 treatment decreased the proportion of Myogenic+ (MyoG+, terminal myogenic commitment/fusion marker) cells without affecting cell density (Supplementary Fig. 2a–d). Importantly, we did not observe a significant effect of Ccr2-ligands on proliferation (EdU+; Supplementary Fig. 2e). These data suggest Ccr2 regulates terminal myogenic commitment. To assess the effect on fusion, we cultured isolated wildtype and Ccr2−/− SCs at a high density for 96 h in growth media, and subsequently switched to low serum media that promotes fusion for 24 h supplemented with combinations of vehicle, Ccl2/7/8, or a Ccr2 small molecule inhibitor BMS CCR2 22 (Ccr2i). Ccr2i is a competitive binding inhibitor with a selective and high affinity for Ccr2’s binding pocket, rendering the receptor inactive albeit stable. Treatment with Ccl2/7/8 reduced the proportion of Ccr2+/− cells expressing MyoG (Fig. 2a, b). This was coupled to a higher proportion of Pax7+ (SC renewal marker) cells (Fig. 2a, c). The capacity for
Ccr2/7/8 to reduce the proportion of MyoG+ in favor of Pax7+ cells was reversed by the addition of Ccr2i and in Ccr2−/− cells (Fig. 2a–c). Next, we quantified the number of nuclei incorporated into skeletal muscle myosin positive myotubes (fusion index). Consistent with the loss of fusion-competent MyoG+ cells (Fig. 2a, b), Ccl2/7/8 treatment impaired myotube formation based on reduced fusion index (Fig. 2d, e). Importantly, Ccl2/7/8 treatment was unable to impair myotube formation in the presence of Ccr2, or when Ccr2 was deficient (Fig. 2d, e). Regardless of treatment or genotype, Ccl2/7/8 treatment did not affect nuclear density (Fig. 2f). Therefore, Ccr2 is dispensable for aspects of active SC and MP function devoted to cell growth. However, in the presence of high ligand levels, Ccr2 can directly inhibit MP fusion capacity and the formation of multinucleated myotubes.

To test whether Ccl2/7/8-mediated reduction in the proportion of MyoG+ cells in differentiating conditions involves transcriptional repression, we examined Myogenin expression. Indeed, treatment of differentiating SC cultures with Ccl2/7/8 resulted in reduced Myogenin expression. Indeed, treatment of differentiating SC cultures with Ccl2/7/8 resulted in reduced Myogenin expression.

**Fig. 1 Ccr2 expression in activated SCs and MPs from regenerating muscle.** a, b Representative FACS histograms of Ccr2 reporter fluorescence (eGFP) in a hematopoietic cells (CD45+), and b SCs and MPs (Lin- β1Int + VCAM+) from adult (2 months old) mouse skeletal muscles uninjured (U) and injured muscles, 2- and 5-days post injury (2dpi and 5dpi) (n = 3 mice per condition). c Mean fluorescence intensity of eGFP (Ccr2) in SCs and MPs from adult uninjured and injured limb muscles, 2- and 5-days post injury (2dpi and 5dpi) (n = 3 mice per condition). d Expression of Ccr2 in SCs and MPs from adult uninjured and injured limb muscles, 2- and 5-days post injury (2dpi and 5dpi) (n = 3 mice per condition). e Levels of inflammatory Ccr2 chemokine ligands (Ccl2, Ccl7 and Ccl8) from adult uninjured, 2dpi and 5dpi muscles with multiplex Luminex assay (n = 6 mice per condition). Flow cytometry data are reported as mean ± s.e.m. from at least 500000 events per acquisition. Cells were gated for single cells and live cells (DAPI) prior to specific cell lineage markers (Supplementary Fig. S1) and GFP gating. mRNA levels are reported as fold-change ± s.d. relative to Gapdh and B2m and normalized to experimental control. Luminex data are reported as protein concentration from muscle lysates (mean ± s.e.m., ng/mL). Non-significant (n.s.) P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001, non-parametric one-way ANOVA followed by Tukey’s multiple comparisons test. * is relative to uninjured except if otherwise indicated (bar).
Fig. 2 Ccr2 chemokines prevent SC-derived MP terminal commitment and fusion. a Representative images from immunofluorescence staining of SC cultures derived from adult (2 months old) wildtype (Ccr2+/+) and Ccr2-null mutant (Ccr2−/−) mice. Cells were purified by MACS and, 5000 SCs were plated and cultured for 96 h in growing media prior to low serum condition media (differentiation media; DM) supplemented with vehicle (V), Ccr2-ligands (Ccl2/7/8) or Ccl2/7/8 and Ccr2 small inhibitor (Ccr2i) for 24 hours. Red, Pax7 (SC); Green, MyoG (Differentiating MP); Blue, DAPI (Nuclei). Experiments were performed at least three times. b, c Percentage of b MyoG+ and c Pax7+ cells of total cells (DAPI+) per culture of SCs derived from adult Ccr2+/+ (n = 12) and Ccr2−/− (n = 3) mice. Each dot represents the percentage of positive cells averaged from duplicate cultures from one mouse (n = 3–12 mice). d Representative images from immunofluorescence staining of SC cultures derived from adult wildtype (Ccr2+/+) and Ccr2−/− mice. Cells were purified by MACS and 10000 cultured for differentiation and myotube formation in DM supplemented with vehicle, Ccl2/7/8 or Ccl2/7/8 and Ccr2i for 24 hours. Green, Myosin (Myotubes); Blue, DAPI (Nuclei). Experiments were performed at least three times. e, f MP cultures e fusion index and f cell density (DAPI+/μm²). Fusion index was measured by number of fused myonuclei within myotubes relative to total number of cells (DAPI+) per culture of SCs derived from adult Ccr2+/+ (n = 12) and Ccr2−/− (n = 3) mice. Each dot is the averaged data from duplicate cultures from one mouse. Culture data are percentage mean ± s.e.m. For each experiment, cells were derived from one mouse and cultured into at least two wells per condition and >300 cells were counted. Scale bars, (a) 20 μm, (d) 50 μm. Non-significant (n.s.) P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001, non-parametric two-way ANOVA followed by Tukey’s multiple comparisons test. Stats are relative to Vehicle except if otherwise indicated (bar).
Fig. 3 Ccr2 represses Myogenin expression through p38\(\delta\gamma\) signaling. a Myogenin mRNA expression in SCs derived myotubes from adult (2 months old) wildtype (Ccr2\(^{+/+}\)) (n = 6) and Ccr2-null mutants (Ccr2\(^{-/-}\)) (n = 3) mice treated with vehicle (V), Ccr2-ligands (Ccl2/7/8) or Ccr2 small inhibitor (Ccr2i) for 24 h. b, c Representative immunoblotting from treated SC derived myotubes (b) and c quantification of phospho-p38\(\delta\gamma\) normalized to total p38, and of phospho-MyoD normalized to total protein (n = 3 mice). d Immunofluorescence staining of adult SC derived cultures treated with siRNAs against Mapk12/13 or siGLO (control) after 72 h then directed toward differentiation at 96 h in DM supplemented with vehicle or Ccr2i for 24 h. Pax7 (Red); MyoG (Green); DAPI (Blue). Experiments were performed at least three times. e, f Percentage of e MyoG+ MPs and f Pax7+ SCs normalized to total number of cells (DAPI+). Each dot represents the percentage of positive cells averaged from duplicate cultures from one mouse (n = 3 mice). RTqPCR analysis was performed in triplicate from at least two animal per experiment and condition. mRNA levels are reported as fold-change ± s.d. relative to Gapdh and B2m and normalized to experimental control. Immunoblots were performed from lysates derived from at least two animal per condition from at least three experiments. Data are mean ± s.e.m. For each experiment we plated cells from one mouse into at least two wells per condition and used at least three mice per group and counted >400 cells. siRNA experiments were pre-validated using two different siRNAs for each gene target and efficient siRNAs were controlled for off-target effects toward other p38MAPK gene members (Supplementary Fig. S5e). Scale bars, 37 μm.

Myogenin expression (Fig. 3a). The Ccl2/7/8-mediated repression of Myogenin expression was prevented with Ccr2i treatment or Ccr2 deficiency (Ccr2\(^{-/-}\)) (Fig. 3a). Given that Ccr2 activity inhibits Myogenin expression, we investigated downstream signals potentially mediating this effect. Individual chemokines induced rapid phosphorylation of the critical intracellular signaling mediators Erk and p38MAPK, which are known regulators of the myogenic differentiation program (Supplementary Fig. 5a, b). Among these, we found Ccl2/7/8 treatment induced prolonged and enhanced activation of p38MAPK signaling (Supplementary Fig. 5b). The p38MAPK family is composed of four distinct isoforms encoded by four genes often associated by pair due to their similarity and overlapping function across various tissues. In this regard, previous reports show that p38α/β promote, while p38\(\delta\gamma\) inhibit terminal myogenic commitment. Inhibition of myogenic differentiation by activated p38\(\delta\gamma\) involves phosphorylation of MyoD on serine 200 and repression of Myogenin expression. Consistent with the ability to repress Myogenin expression, Ccl2/7/8 increased phosphorylated levels of p38\(\delta\gamma\) and MyoD on serine 200 in a Ccr2-dependent manner (Fig. 3b, c). Furthermore, both Ccl2/7/8-mediated repression of Myogenin expression and p38\(\delta\gamma\) activation were dosage dependent (Supplementary Fig. 5c, d). Subsequently, we sought to determine if p38\(\delta\gamma\) siRNAs (siMapk12/13) could circumvent Ccr2-mediated loss of MyoG+ cells (Supplementary Fig. 5e). Consistent with p38\(\delta\gamma\) genes knockdown as a means to promote terminal myogenic commitment, treatment of cultured SCs with siMapk12/13 increased and decreased the proportion of MyoG+ and Pax7+ cells, respectively (Fig. 3d–f). In response to Ccl2/7/8 supplementation, siMapk12/13 treatment attenuates the loss of MyoG+ cells (Fig. 3d–f). Thus, p38\(\delta\gamma\) signaling contributes to Ccr2-mediated inhibition of MP progression toward fusion competency necessary for myofiber formation.

Five days after degenerative injury to adult muscle, we found Ccr2 chemokines expression had returned to near uninjured baseline levels (Fig. 1e, and Supplementary Fig. 2). At this time, adult regenerating muscle contains a high number of fusion-competent MyoG+ progenitors (Fig. 4b, green). Since Ccr2 chemokines can inhibit Myogenin expression, we tested if Ccr2 chemokines treatment to regenerating adult muscle can lead to a reduction in MyoG+ progenitors. To this end, we locally delivered vehicle or Ccl2/7/8 at 4dpi and subsequently examined muscles at 5dpi for MyoG+ progenitor number (Fig. 4a, b). We found adult regenerating muscle exposed to Ccr2-ligands had reduced MyoG+ cells compared to vehicle (Fig. 4b, c). Next, we sought to determine if the loss of MyoG+ cells reflects in part reduced Myogenin expression in active SCs and MPs. Examination of SCs and progenitors derived from 5dpi muscles revealed that Ccl2/7/8 treatment in vivo reduced Myogenin expression by over 50% (Fig. 4d).

Deletion of Ccr2 promotes adult MP fusion in aged muscle. Skeletal muscle regenerative capacity declines with age. Early stages of aged muscle regeneration are characterized by delayed activation of SCs; however, similar SC and MP content is observed between adult (2-months-old mice) and aged (24-months-old mice) regenerating muscle at 5dpi (Supplementary Fig. 6a). Furthermore, assessment of SCs and MPs from aged 5dpi muscle demonstrated reduced Myogenin and increased Ccr2 expression compared to adult (Supplementary Fig. 6b). To determine whether higher Ccr2 chemokines levels are a feature of aged muscle regeneration, we compared Ccl2, Ccl7, and Ccl8 mRNA and...
protein expression between adult and aged 5dpi muscles. We found Ccr2-ligands were elevated in aged relative to adult 5dpi regenerating muscle (Fig. 5a and Supplementary Fig. 6c). We next asked whether healthy adult SC-derived progenitors could fuse into aged regenerating myofibers in an environment of high Ccr2 chemokines. Initially, we obtained freshly isolated SCs indelibly labeled with nuclear GFP (nGFP) from adult WT Pax7 CreER/+; Rosa26fltnG/+; Ccr2+/- (SCCcr2+/−; P7nTnG) or Pax7 CreER/+; Rosa26fltnG/+; Ccr2−/- (SCCcr2−/−; P7nTnG) mice, and subsequently transplanted 3,000 of them into regenerating adult and aged muscle hosts (Fig. 5b). Freshly isolated adult SCCcr2+/−; P7nTnG and SCCcr2−/−; P7nTnG displayed similar engraftment efficiency into adult regenerating hosts (Fig. 5c, d). Consistent with previous reports, freshly isolated adult SCCcr2+/−; P7nTnG displayed poor engraftment into an aged regenerating host (Fig. 5c, d)91. In contrast, freshly isolated SCCcr2−/−; P7nTnG engraftment into aged or adult regenerating hosts was similar (Fig. 5c, d). Similar results were obtained when total number of nGFP donor contribution in each treatment group was normalized to muscle volume (Supplementary Fig. 7a). In addition, we found that nGFP+ regenerated myofibers from SCCcr2−/−; P7nTnG recipients were significantly larger in size (Supplementary Fig. 7b). Thus, Ccr2 deficiency in freshly isolated adult SCs was sufficient to improve their engraftment and contribution into aged regenerating hosts.

Next, we wanted to determine if the improved engraftment of adult Ccr2−/−; P7nTnG SC into aged regenerating hosts reflects enhanced fusion competency. To do so, we developed an in vivo MP fusion assay. To obtain fusion-ready MPs, we cultured freshly isolated adult SCCcr2+/−; P7nTnG or SCCcr2−/−; P7nTnG cells in growth media on recombinant extracellular matrix gel for 4 days. Under these conditions SCCcr2+/−; P7nTnG and SCCcr2−/−; P7nTnG display similar growth in culture (Fig. 2f and Supplementary Fig. 8a). Subsequently, 15,000 SCCcr2+/−; P7nTnG and SCCcr2−/−; P7nTnG derived MPs were intramuscularly injected into adult or aged regenerating 4dpi muscle hosts (Fig. 5d). Remaining cells were replated at the same density and displayed similar survival in culture (Supplementary Fig. 8b). Consistent with the propensity for MPs to rapidly proceed toward terminal commitment, SCCcr2+/−; P7nTnG and SCCcr2−/−; P7nTnG derived progenitor fusion was local to the site of intramuscular delivery and along the needle track, which was defined as the area of engraftment (Supplementary Fig. 8c). To quantify SCCcr2+/−; P7nTnG and SCCcr2−/−; P7nTnG derived MP fusion, we assessed the presence of central nGFP-positive myofibers. In adult regenerating myofiber hosts, both SCCcr2+/−; P7nTnG and SCCcr2−/−; P7nTnG derived MPs displayed similar fusion capacity (Fig. 5e, f and Supplementary Fig. 8d). However, SCCcr2+/−; P7nTnG derived progenitors displayed limited, if any, fusion into aged regenerating myofibers (Fig. 5e, f and Supplementary Fig. 8d). In contrast, fusion of SCCcr2−/−; P7nTnG derived MPs was similar regardless of regenerating host myofiber age (Fig. 5e, f and Supplementary Fig. 8d). We also found that nGFP+ aged regenerating myofibers in SCCcr2−/−; P7nTnG recipients displayed larger fiber size (Supplementary Fig. 8e). Thus, Ccr2 deficiency,
specifically in SC derived MPs, is sufficient to overcome the inhibitory effects of the aged regenerating environment enriched in Ccr2 chemokines; thereby, promoting fusion of MPs and their contribution to aged muscle regeneration.

**Timely inhibition of Ccr2 promotes aged muscle recovery.** Next, we sought to determine the consequences of the timely direct delivery of Ccr2i on adult and aged muscle regeneration.

To this end, we intramuscularly injected vehicle or Ccr2i to adult or aged regenerating muscle at 4dpi (Fig. 6a–f). Subsequently, we examined fusion competent MyoG+ MP number at 5dpi (Fig. 6a, b), and regenerated myofiber size at 10dpi (Fig. 6d, e). Consistent with low levels of Ccr2 chemokines, we found no effect of Ccr2i direct delivery on adult muscle regeneration in terms of MyoG+ cell number at 5dpi or regenerated myofiber size at 10dpi (Fig. 6a–f). In contrast, direct intramuscular delivery...
of Ccr2 to regenerating aged muscle at 4dpi resulted in increased MyoG+ cell number at 5dpi, and increased regenerated myofiber size at 10dpi (Fig. 6a–f). To determine if Ccr2i treatment can directly influence MP fate in an in vivo context, fusion-ready SC\textsuperscript{Ccr2i/+;P7nTnG} MPs were pre-treated with Ccr2i, washed, and transplanted into an aged regenerating host (Supplementary Fig. 9a). Pre-treatment of SC\textsuperscript{Ccr2+/+;P7nTnG} MPs with Ccr2i led to their improved fusion into aged regenerating myofibers and larger nGFP+ myofibers (Supplementary Fig. 9b–e). Although adult SCs are sensitive to Ccr2 chemokines, whether aged SCs retain this property is unknown. Similar to adult, aged SC and derived MPs exposed to Ccl2/7/8 in culture displayed no change
in MyoD+ proportions (Supplementary Fig. 10a, b) and had reduced myotube formation, in a Ccr2-dependent fashion (Supplementary Fig. 10c, d).

Finally, as a fundamental test of functional recovery, we assessed if direct Ccr2 delivery improved muscle strength of aged muscle post injury. To this end, we examined intrinsic absolute and specific force recovery of regenerating EDL muscles at 10dpi relative to contralateral (CL) uninjured muscles, after intramuscular injection of Ccr2i or vehicle at 4dpi (Fig. 6d and Supplementary Fig. 11a). As expected, injured muscles produced less force than their uninjured counterparts regardless of age (Fig. 6g and Supplementary Fig. 11b, c). However, in comparison to adult the extent of aged 10dpi muscle force recovery relative to uninjured contralateral controls was reduced (20% vs 60%) (Fig. 6g-i and Supplementary Fig. 11b, c). Furthermore, half of vehicle treated aged regenerating muscles at 10dpi were composed primarily of connective tissue (in left picture) and unable to elicit recognizable force values in response to direct ex vivo muscle stimulation (Fig. 6g-i and Supplementary Fig. 11a–c). Strikingly, all Ccr2i treated aged regenerating muscles at 10dpi were able to generate force. In addition, Ccr2i treatment promoted force recovery in injured aged muscle (Fig. 6h, i and Supplementary Fig. 11b, c). Collectively, our findings demonstrate that timely pharmacological inhibition of Ccr2 is a viable strategy to promote both the regeneration and functional recovery of aged muscles after a traumatic injury.

Discussion
In response to injury, the coordinated activation, proliferation and fusion of SC derived MPs is required for skeletal muscle regeneration. The complex aged inflammatory environment disrupts this synchronization33,40. The CC chemokine receptor Ccr2 is a regulator of immune cell recruitment and function during muscle regeneration; however, a role of Ccr2 in non-hematopoietic cells in the context of tissue regeneration and aging is unclear. In this study, we demonstrate that active SCs and MPs in regenerating muscle express Ccr2. Although dispensable for MP expansion and growth, elevated Ccr2 chemokine activity inhibits MP fusion and contribution to multinucleated myofiber formation. After an acute injury, aged muscle is characterized by high Ccr2 chemokines levels. We demonstrated that Ccr2 deficiency or timely Ccr2 inhibition promotes MP fusion and aged skeletal muscle regeneration, respectively.

Although one study employed antibody labeling to detect Ccr2 in MPs of regenerating muscle23, the use of Ccr2 reporter mice has been the gold standard method to evaluate Ccr2 expression, due to the elevated turnover of G protein-coupled receptors26,36. We found SCs from uninjured muscle express negligible levels of Ccr2 reporter. In contrast, we demonstrate, using multiple methods, that active SCs and MPs from regenerating skeletal muscle express relatively higher levels of Ccr2. Accordingly, RNA-seq analysis of SCs and MPs from uninjured and regenerating muscle demonstrate similar patterns of Ccr2 expression as observed here55. Furthermore, we observe that Ccr2 expression in active SCs and MPs is positively associated with Ccr2 chemokines levels, suggesting Ccr2 expression can be enhanced by its own ligands.

We find that ectopic treatment with Ccr2 chemokines inhibited the capacity of adult SCs and derived MPs to contribute to multinucleated myofiber formation. A previous report demonstrated alterations in proliferation are not observed in adult Ccr2+/− MP cells; however, neither Ccr2+/- nor Ccr2−/− MPs were treated with Ccr2 chemokines39. Although we find Ccr2 deficiency did not alter SC growth in standard culture conditions, we did find Ccr2−/− SC-derived MPs were resistant to Ccr2 chemokines-mediated inhibition of terminal differentiation and fusion. Previous groups have demonstrated roles for p38MAPK signaling in regulating SC fate and MP function42–44. The p38MAPK family is composed of four genes Mapk14, Mapk11, Mapk12, and Mapk13, encoding for the kinases p38α, p38β, p38γ and p38δ, respectively. Activation of p38α/β, often referred to as p38MAPK, induces myogenic differentiation42, whereas activation of p38δ/γ inhibits myogenic differentiation43,44. Consistent with the latter, we found that Ccr2-mediated inhibition of MP fusion and myotube formation is associated with activation of p38δ/γ43,44. Considering the recent discovery of proteins with critical roles in MP fusion46–58; it will be of interest to determine if Ccr2 and p38δ/γ activity can modulate these fusion regulators.

During adult muscle regeneration, high levels of Ccr2-ligands coincide with the recruitment of inflammatory cells and SC expansion. Later stages of adult muscle regeneration are associated with the resolution of inflammation, progression toward terminal myogenic differentiation and decreased levels of Ccr2-ligands in the environment59. Following an acute injury, we uncovered the prolonged elevated levels of Ccr2 chemokines in aged regenerating muscles. This was consistent with a decrease in the numbers of fusion competent myogenic cells in 5dpi regenerating aged in comparison to adult muscles in vivo. Indeed, Ccr2 deficiency promoted SC-derived MP fusion to aged regenerating myofibers. Furthermore, timely Ccr2 inhibition stimulated aged muscle regeneration and functional recovery after an acute degenerative injury. Ccr2-signaling acts at the nexus of events regulating muscle regeneration in what is likely a complex interplay between immune, and myogenic cells1. Therefore, timely Ccr2 treatment could alter the levels of inflammatory derived factors that influence MP fate60. A subset of aged SCs retain extensive regenerative potential, thus, timely Ccr2 treatment may primarily alter the response of relatively healthy aged SCs and derived MPs to high Ccr2 chemokines in the aged environment31,32. Alternatively, opportune Ccr2i treatment could restore the function of some SCs prone to senescence22,50. Indeed, Ccr2 signaling is part of the senescence associated secretory pathway, and can reinforce senescence programs3,61,62. Therefore, it is conceivable that transient Ccr2i treatment could attenuate senescence-like phenotypes induced by age-related dysregulation in protein and mitochondrial quality control in some SCs and derived MPs22,63.

Although aged human skeletal muscle can regenerate40, delays in this process cause persistent physical discomfort that compromises recovery, mobility, and independence in the growing elderly population64–66. Here, we identify a previously characterized role for Ccr2 signaling as an inhibitor of aged muscle regeneration. Furthermore, we demonstrate targeting Ccr2 can promote MP function in an aged environment, and thereby restore aged muscle function after a degenerative injury. In light of our observations, the identification of similar modes of stem cell and progenitor fate regulation in other systems could prove useful to improve aged tissue regeneration. Specifically, timely inhibition of Ccr2 could be used to rejuvenate aged tissue regeneration or improve cell-based therapies in a high inflammatory environment. Finally, the recent clinical trials using Ccr2 inhibitors in diverse human disorders suggest targeting Ccr2 is a viable treatment16–19,67. These studies could give us further insight into the systemic effects of Ccr2 inhibition and help to develop future therapeutic strategies for aged tissue regeneration in a clinically relevant context.

Methods
Animal studies. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures and protocols involving animals were approved and authorized by the Institutional Animal Care and Use Committee (IACUC) at the University of Rochester called the University Committee on
Animal Resources (UCAR). C57BL6 adult (2–4 months; Jackson Labs) and aged (24–25 months; National Institute on Aging) mice were used for indicated experiments. C57-KO (Ccr2−/−) (004999) and C57-ZE/ZE (Ccr2+/-EGFP) (027619) mice were obtained from Jackson Labs. For transplant experiments, we used Pax5+Ccr2+/-/ Rosa262Rosa26EGFP mice (10). All mice were housed in the Vivarium animal housing areas at URMC and were cared for by the facility staff following the programs for animal care set by the University of Rochester. The Animal Resource Programs for animal care set by the University of Rochester.

Skeletal muscle injury. Mice were anesthetized by 3% isoflurane inhalation. Buprenorphine (0.1 mg/kg) was administered prior to the procedure and approximately every 12 h as needed. The skin overlaying the tibias anterior (TA) muscle was shaved and the TA and extensor digitorum longus (EDL) were directly injected with a 1.2% solution of BaCl2 in normal saline.

Transplant and in vivo fusion assay. For freshly isolated SC transplant, cells were derived from uninjured limb from adult Pax5+Ccr2+/-/ Rosa262Rosa26EGFP mice wild type for Ccr2 (SC5a2x2+/-) or mutant (SC5a2x2+/–). Cells were counted and 3000 SCs were transplanted into a pre-injured TA/EDL of an adult or aged mouse host (4dpi). 35 days post injury, transplanted muscles were collected, frozen, sectioned and stained for GFP, Laminin and DAPI. Engraftment and contribution to regeneration was assessed by quantifying number of centrally nucleated fiber with GFP+ nuclei within the area of engraftment.

For in vivo fusion assay, the experiment was similar, except we collected muscles for 96 h post transplant. Cell growth was controlled throughout by quantifying number of live nGFP cells in culture (Supplementary Fig. 8a), while survival post-trypsinization was verified by re-plating extra cells (Supplementary Fig. 8b). For in vivo fusion assays, 15000 cells were injected into an injured limb (4dpi). Area of engraftment was determined within the field of view containing centrally nucleated nGFP+ fibers, as depicted in Supplementary Fig. 10c.

Physiologic muscle force generation assay. Muscle force generation capacity was analyzed in EDL muscles using an ASI muscle contraction system (Aurora Scientific). Mice were maintained under isoflurane throughout the procedure. TA was removed, and EDL were dissected, adjusted to optimal length (OL), and force of recovery was calculated based on the injured normalized to contralateral uninjured force values at 150 Hz stimulation.

Cell isolation and culture. For magnetic activated cell sorting (MACS), skeletal muscles were dissociated in F10+ media (F10 supplemented with 10% horse serum, 2% B2M) containing 0.2% Collagenase II and 0.4% Dispase using the Gentle MACS tissue dissociator for 30–60 min at 37 °C. Single cell suspension was spun down at 500 g for 20 min at 4 °C, washed with F10+ media and filtered using Smart Filters (Miltenyi Biotech). Cells were resuspended in ice-cold PBS 0.5% BSA prior to filtration through a FACS tube cap. SCs were then freshly isolated using first the Satellite Cell Isolation Kit (Miltenyi Biotech) for negative selection, followed by positive selection using α7Integrin beads following the manufacturers’ protocols. SC cultures and related experiments were performed as previously described (34, 39).

Briefly, 5000 SCs (72 h cultures) or 10000 SCs (differentiation assay) were plated on 0.5% ECM in growing media (GM; DMEM, 10%horse serum, FGF2, 1% pen/strep, 2% B2M). GM was changed every 48 h and differentiation media (GM, DMEM, 10%horse serum, FGF2, 1% pen/strep, 1% HEPES) was added at 96 h for 18–24 h, monitoring the formation of long and healthy myotubes. All cells were incubated at 37 °C in 5% CO2 and humid air conditions.

FACS and flow cytometry analysis. For flow cytometry analysis, skeletal muscles were dissociated using the Gentle MACS (see above), and SCs were isolated as previously described (34). Briefly, SCs were characterized as C4d−, Sca1−, CD31− (Lineage negative; Lin−) (BD Biosciences; Biolegend), and VCAM + and Fl1Integrin + (Biolegend). Muscle-derived monocyes (Cd11b ultrapure beads; Milteny) were used as controls for the Ccr2-GFP mouse experiments as they express high levels of Ccr2 in response to injures and then to the damaged tissues and were detected as C4d5− (BioLegend). Gating strategy is described in Supplementary Supplemental Fig. 1a and depict cell populations from injured muscles while representative FMO and single stain controls for the antibodies used in flow experiments can be found in Supplementary Fig. 12. Flow cytometry experiments were always performed with single stain, FMO and appropriate experimental controls. For cell sorting purposes, we used BD FACSDiva software v8, while flow analysis was performed on FCS Express v6.

Transfection and siRNA treatments. Cells were transfected with siRNAs (Drmaacon) using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer’s instructions. ssGLO was used as control. We used siRNA targeting other p38 genes as a control of specificity. None of the other p38 genes was regulated when using ssRNA targeting a specific p38 gene member (Supplementary Fig. 5e).

Recombinant chemokines and Ccr2 inhibitor treatments. In culture, recombinant chemokines were delivered in PBS at a final concentration of 0.5–50 ng/mL as indicated, while BMS CCR2 22 inhibitor (Ccr2i) was used at 1 nM as previously described (35, 36). For in vivo experiments, Ccr2i was intramuscularly delivered in 90% Saline-10%DHEO at 0.5 ng/ul as previously described (37, 38). Recombinant chemokines were delivered in PBS at a final concentration of 5 ng/ml body weight.

Tissue section and immunostaining. For injury or transplant studies, TA muscles were harvested and frozen in isopentane cooled in liquid nitrogen and stored at −80 °C prior to sectioning or prepared for magnetic activated cell sorting (MACS). Frozen muscles were sectioned at 10 μm. For Hematoxilin and Eosin staining, sections were treated as previously described (39). Muscle sections were fixed for 3 min in 4% paraformaldehyde (PFA), permeabilized with PBS-T (0.2% TN, pH-X100) for 10 min and blocked in 10% Normal Goat Serum (NGS; Jackson Immuno Research, West Grove, PA) in PBS-T for 30 min at room temperature. When mouse primary antibodies were used, sections were additionally blocked in 3% AffiniPure Fab fragment goat anti-mouse IgG1(±1) (Jackson Immuno Research) with 2% NGS in PBS at room temperature for 1 h. Primary antibody incubation in 10% NGS/PBS-T was carried out at 4 °C overnight or 2 hr at RT and sections were incubated with secondary antibodies in 10% NGS/PBS-T for 1 h at RT. DAPI staining was used to label nuclei. All slides were mounted with Fluoromount-G (SouthernBiotec, Birmingham, AL). At least three sections from three slides were analyzed per sample. Sections and cells were imaged on a Zeiss Axios Observer D1 microscope (Germany) or Echo Revolve and processed and analyzed in ImageJ. Sample analysis was conducted in a double-blind manner. For transplant analysis, each transplanted muscle was sectioned at 10 μm through–2500 μm. Area of transplant was determined by staining >20 muscle sections throughout the muscle for each sample and was then contained in a 20X field of view for analysis of nGFP+ centrally nucleated fibers. Analysis was performed using at least 60 fields of view.

Immunoblotting and Luminex. Cell lysates (50 mM HEPES [pH 7.4], 150 mM NaCl, and 1% Triton X-100) and freshly added EDTA-free protease and phospha-tase inhibitor cocktails (Roche) were immunoblotted with primary antibodies overnight at 4 °C. HRP-conjugated secondaries and ECL from BioRad was used for chemiluminescence detection, while Dylight secondary antibodies were used for infrared detection on LI-COR Odyssey and using LI-COR Image Studio software or ImageJ. Light for analysis and band quantification. Fluorescent images were saved in grey scale and therefore displayed as such. Uncropped and unprocessed blot images with molecular weight are available in the Source Data File.

For Luminex assays, samples were prepared using a customized Mouse Magnetic Luminex Assay (R&D Systems) following manufacturer’s protocol and using BioRad BioPlex 200 apparatus and software.

RNA extraction and qPCR. RNA was extracted using TRIzol reagent (Life Technologies) following the manufacturer’s protocol. RNA was quantified, normalized, and used for RT-PCR followed by qPCR using EvaGreen (Bio-Rad) and the 7500 Fast Real-Time PCR system (Applied Biosystems) with StepOnePlus software to collect and analyze data. Transcript levels were normalized to an average of GAPDH and B2M 5′−4′ to the control condition. For qPCR, we used the following primers:

- **MyoG Forward Primer 5′-GTCCTAACACCCACGAGCATC-3′**
- **MyoG Reverse Primer 5′-CCAGATGGAAGCTAAGGGAGG-3′**
- **Ccr2 Forward Primer 5′-AAGGAGCATACTGTGAAATGC-3′**
- **Ccr2 Reverse Primer 5′-TGTTGCTGAATCCAGTGCCT-3′**
- **Mapk14 Forward Primer 5′-TGACCTTATGACGAGCTCGTCTT-3′**
- **Mapk14 Reverse Primer 5′-GTACAGGTCTCCATCACCTCTTAT-3′**
- **Mapk11 Forward Primer 5′-GGGAAAATCTTACCCGGCAGAAG-3′**
- **Mapk11 Reverse Primer 5′-GGGACAGCTGAGGCGTAAAGG-3′**
- **Ccr2 Reverse Primer 5′-TGTTGCTGAATCCAGTGCCT-3′**
- **Mapk12 Forward Primer 5′-GAGGCGCTTTTACCGGCCAGG-3′**
- **Mapk12 Reverse Primer 5′-GGGCACTCCTCTGTAGGCCC-3′**

Antibodies. The following antibodies were used: mouse anti-Pax7 (1:100, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA), mouse anti-Mysd (BD Biosciences #554130), mouse anti-phospho-Mysd (1:1000, Sigma) rabbit anti-Mmp2 (1:250, Abcam), rabbit anti-Mmp9 (1:250, Alomone), rabbit anti-Lat (1:200, Sigma Aldrich), L0663 or L9393), rabbit anti-skeletal muscle myosin (1:500, Sigma-Aldrich HPA1239), rabbit anti-Ccr2 (1:500, AbCam), rabbit or mouse anti-p38 (1:1000, Cell Signaling), rabbit or mouse anti-phospho-p38 (1:1000, Cell Signaling), rabbit anti-phospho-p38delta/gamma (1:1000, Thermo/Fisher), rabbit anti-Exk2 (1:1000, Cell Signaling), mouse anti-Phospho ERK1/2 (1:1000, Cell Signaling). All antibodies were previously confirmed for specificity in the literature and/or by the manufacturers.
Statistical analysis. Statistical significance was assessed using Prism® software via Student’s or Welch’s t-test (unpaired, two-sided, 95% confidence interval [CI]) or ANOVA (one or two way followed by Tukey post hoc test; 95% CI). Error bars are reported as s.e.m. or s.d. and displayed in appropriate graphs. p values < 0.05 were considered statistically significant (*p < 0.05, **p < 0.01, and ***p < 0.001). No power analysis was performed. Sample size was based on previous experiments.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All the supporting findings of this study are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions
R.S.B., J.G.K., J.F.B., A.M., and N.D.P. performed experiments. R.S.B., and J.V.C. analyzed and interpreted the data. R.S.B., and J.V.C. designed the experiments. R.S.B., and J.V.C. wrote the paper.

Competing interests
The authors declare no competing interests.

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