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IL-10 Triggers Changes in Macrophage Phenotype That Promote Muscle Growth and Regeneration

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We examined the function of IL-10 in regulating changes in macrophage phenotype during muscle growth and regeneration following injury. Our findings showed that the Th1 cytokine response in inflamed muscle is characterized by high levels of expression of CD68, CCL-2, TNF-α, and IL-6 at 1 d postinjury. During transition to the Th2 cytokine response, expression of those transcripts declined, whereas CD163, IL-10, IL-10R1, and arginase-1 increased. Ablation of IL-10 amplified the Th1 response at 1 d postinjury, causing increases in IL-6 and CCL2, while preventing a subsequent increase in CD163 and arginase-1. Reductions in muscle fiber damage that normally occurred between 1 and 4 d postinjury did not occur in IL-10 mutants. In addition, muscle regeneration and growth were greatly slowed by loss of IL-10. Furthermore, myogenin expression increased in IL-10 mutant muscle at 1 d postinjury, suggesting that the mutation Amplified the transition from the proliferative to the early differentiation stages of myogenesis. In vitro assays showed that stimulation of muscle cells with IL-10 had no effect on cell proliferation or expression of MyOD or myogenin. However, coculturing muscle cells with macrophages activated with IL-10 to the M2 phenotype increased myoblast proliferation without affecting MyOD or myogenin expression, showing that M2 macrophages promote the early, proliferative stage of myogenesis. Collectively, these data show that IL-10 plays a central role in regulating the switch of macrophages from a M1 to M2 phenotype in injured muscle in vivo, and this transition is necessary for normal growth and regeneration of muscle. The Journal of Immunology, 2012, 189: 3669–3680.

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hanges in muscle use or acute damage produce a rapid and highly structured inflammatory response that is dominated by successive waves of invasion of myeloid cells that can strongly influence both the magnitude of muscle damage and the rate at which repair occurs (1). Regardless of the perturbation that leads to inflammation, the muscle experiences an initial, rapid invasion by neutrophils and CD68[high] macrophages. In an apparently nonadaptive response, these inflammatory cells amplify damage to the muscle, producing lesions to muscle cell membranes that can lead to necrosis of muscle fibers. These lesions are primarily attributable to the actions of free radicals generated by myeloperoxidase and inducible NO synthase (iNOS) (2–6). However, within days of increased muscle use or acute injury, the numbers of CD68[high] macrophages and neutrophils decline, whereas the numbers of macrophages that express CD163 and CD206 increase and remain elevated as muscle repair, regeneration, and growth proceed (7–10).

In addition to their capacity to exacerbate muscle damage, macrophages have been implicated in promoting muscle regeneration following acute muscle injuries or during increased muscle loading. For example, genetic ablation of CCR2 or its ligand (CCL2) greatly reduced the numbers of macrophages in muscles that were injured by freezing (11, 12), ischemia (13, 14), or cardiotoxin injection (15), and those reductions in macrophages were associated with slower muscle regeneration and growth of the injured tissue. Furthermore, transplantation of wild-type bone marrow into irradiated, CCR2-mutant mice prior to muscle injury was sufficient to restore macrophage invasion and normalize muscle growth to wild-type rates (16). Although other myeloid cells can express CCR2, ablation of CCR2-mediated signaling does not affect chemotraction of neutrophils or lymphoid cells to injured muscle (17), providing strong support that the reported treatment effects of disrupted CCR2 signaling reflect a disruption of macrophage functions in promoting muscle regeneration. Similarly, selective depletion of circulating CD11b-expressing cells prior to muscle injury by toxin injection reduced muscle regeneration (18), lending support to the view that CD11b+ leukocytes, most likely macrophages, promote muscle regeneration.

In vitro observations indicate that macrophages may positively affect muscle growth and regeneration by modulating both the proliferation and differentiation of a population of muscle stem cells, called satellite cells, that normally reside in muscle in a quiescent state. Upon increased muscle loading or injury, satellite cells are activated to proliferate, after which some of the activated cells withdraw from the cell cycle to enter a stage of early differentiation, followed by terminal differentiation when they fuse to form muscle fibers (19). Each of these stages of myogenesis is characterized by shifts in the expression of developmentally regulated genes. For example, during the proliferative stage of myogenesis, muscle cells show elevated expression of the transcription factor MyoD (20, 21). During early differentiation, muscle cells
withdraw from the cell cycle, and the expression of myogenin, also a transcription factor, increases (20, 21). This transition in the myogenic program may be influenced by macrophages. For example, when satellite cells are placed in cocultures with macrophages, satellite cell proliferation increases (22–25), whereas the proportion of muscle cells that express myogenin is reduced (23).

Although some in vitro observations of macrophage/satellite cell cocultures show that macrophages can promote proliferation and inhibit differentiation of satellite cells, other findings indicate that some macrophage populations can promote muscle differentiation. For example, if muscle cells are cocultured with CD163+ macrophages, muscle cell fusion to form myotubes is increased (26), which is a morphological indicator of increased differentiation. CD163, a hemoglobin/haptoglobin receptor, is selectively expressed on macrophages that are activated to an anti-inflammatory, M2 phenotype (27–30). In injured tissues, CD163+ macrophages are in a state of alternative activation in which they secrete anti-inflammatory cytokines that can deactivate neutrophils and CD68+ macrophages and, thereby, reduce tissue damage. Thus, in vitro findings suggest that M2 macrophages could improve repair and regeneration of muscle by deactivation of cytototoxic neutrophils and CD68+ macrophages, as well as by directly influencing muscle cell differentiation. Similarly, in vivo observations support a role for CD163+ macrophages in promoting muscle growth following increased muscle loading or injury. Depletion of macrophages from injured muscles at the stage when CD68+ macrophages decline and CD163+ macrophages increase resulted in reductions in muscle repair and greatly slowed muscle growth and regeneration (31).

Collectively, these observations indicate that the transition of macrophage populations in injured muscle from a CD68+CD163- population to a CD68high/CD163+ population reflects a shift of macrophages to the M2 phenotype that influence muscle growth and regeneration following injury. If that were true, then modulation of the expression of signaling molecules that promote the CD163+ M2 phenotype would affect the course of muscle differentiation, regeneration, and growth. Although several cytokines can drive macrophages to the M2 phenotype, IL-10 provides an especially good candidate molecule for regulating changes in macrophage phenotype that influence muscle growth and regeneration. IL-10 is a strong activator of CD163 expression in vitro (29), and ablation of IL-10 expression in dystrophic muscle reduces the numbers of CD163+ macrophages in the muscle, indicating that IL-10 is also capable of modulating muscle macrophage phenotype in vivo (32). In addition, IL-10 promotes phagocytosis by macrophages (32), and induction of phagocytosis can produce a phenotype switch from proinflammatory M1 macrophages to the anti-inflammatory M2 phenotype, at least in vitro (18).

In the present investigation, we tested whether IL-10 expression affects muscle injury, regeneration, or growth that occurs following muscle damage caused by increased muscle loading. We anticipate that null mutation of IL-10 in mice experiencing muscle reloading after disuse atrophy will attenuate the shift of macrophages to the M2 phenotype that is caused by increased muscle loading and help us to identify the functional importance of that shift in the context of muscle growth and regeneration. Finally, we test whether IL-10 has direct effects on muscle cell growth or differentiation in vitro, to assess whether IL-10–mediated influences on muscle can reflect direct actions on muscle cells in addition to effects mediated through the myeloid compartment.

Materials and Methods

Animals

All experimental protocols involving the use of animals were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of California, Los Angeles Institutional Animal Care and Use Committee. Adult C57BL/6J mice and IL-10-null mice (B10.129P2[BJ6]-Il10tm1Cgn/J) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mutant mice were back-crossed onto the C57BL/6J background for at least eight generations by the supplier.

Hindlimb muscle unloading and reloading

In the present investigation, we use the rodent hindlimb unloading followed by reloading model to cause muscle damage, regeneration, and growth that occur over a well-delineated time course (33). In this manipulation, rodent hindlimbs are elevated for a period of time so that they are no longer weight-bearing, which causes a rapid loss of mass in some muscles as they adapt to the unloaded condition. The soleus muscle is most rapidly affected and can lose 30 to 40% of its mass in 10 d through mechanisms that resemble those that occur in the muscles of patients subjected to prolonged bedrest (34). Reloading the hindlimbs by returning the animals to normal weight-bearing and ambulation provides a reproducible model for studying muscle adaptation to increased loading. During reloading, muscle experiences growth and regeneration that are characterized by the appearance of central-nucleated muscle fibers that show renewed expression of developmental genes, as well as a rapid increase in fiber cross-sectional area, so that the fibers return to their original, presuspension size within 4–7 d of reloading. This response of soleus muscle to reloading provides a unique in vivo analysis of the contributions of specific leukocyte populations to injury, regeneration, and growth that result from increased muscle use.

Four-month-old female mice were subjected to 10 d of hindlimb unloading using a previously described device (33), followed by no reloading or by 1 or 4 d of reloading by normal weight-bearing. At the end of the treatment period, mice were euthanized with inhalation of isoflurane, and soleus muscles were rapidly dissected and collected. Ambulatory, control mice were subjected to normal cage activity until euthanized for tissue collection.

Immunohistochemistry

One soleus muscle from each mouse was dissected and then rapidly frozen in isopentane cooled in liquid nitrogen. Frozen cross-sections were cut from the midbelly of each muscle at a thickness of 10 μm. The frozen sections were then air-dried and fixed in cold acetone for 10 min, and endogenous peroxidase activity was quenched with 0.03% H2O2. Sections were blocked in 3% BSA and 2% gelatin in 50 mM Tris buffer (pH 7.2) for 1 h and then immunolabeled with rat anti-CD68 (Serotec) or rat anti-CD163 (Serotec) for 3 h or with mouse anti-MyoD (BD Bioscience) or mouse anti-myogenin (BD Bioscience) overnight. Negative control sections were prepared for each primary Ab using isotype control IgG in place of the specific primary Ab. Sections were washed with 50 mM sodium phosphate (pH 7.2) containing 150 mM sodium chloride (PBS) and then incubated with biotin-conjugated secondary Ab (Vector) for 1 h and Avidin D-conjugated HRP (Vector) for 30 min. Immunoreactive cells were visualized using the enzyme substrate, 3-amin0-9-ethylcarbazole, which yields red reaction products in the presence of peroxidase (AEC kit; Vector). The number of immunolabeled cells/volume of muscle tissue was then determined by first measuring the total volume of each section using a stereological, point-counting technique to determine section area (31) and then multiplying that value by the section thickness (10 μm). The number of immunolabeled cells/unit volume of each section was calculated as the number of cells/unit volume of each section.

Assay for muscle fiber injury

Muscle fibers experiencing membrane lesions were identified by IgG staining, as previously described (35). Presence of IgG in the cytosol indicates the presence of muscle membrane lesions large enough to allow the unregulated transit of large molecules through lesions in the cell membrane. Frozen sections were air-dried and blocked in 1% gelatin diluted in PBS and then labeled with FITC-conjugated mouse anti-IgG (Vector) for 1 h. After washing with PBS, the staining was visualized by epifluorescence microscopy, and the number of injured fibers showing cytosolic fluorescence and total number of fibers were counted. The extent of muscle injury was expressed as the number of injured fibers relative to the total number of fibers in the muscle cross-section.
Assay for muscle fiber regeneration

Centrally located nuclei are morphological markers of muscle fibers undergoing regeneration. The number of fibers containing central nuclei in complete cross-sections of soleus muscles was counted in unfixed sections that were air-dried and stained with hematoxylin. Central-nucleated, regenerative fibers are expressed as the percentage of the total number of fibers in each section.

Measurement of muscle fiber cross-sectional area

Muscle fiber atrophy or growth was analyzed by measuring the muscle fiber cross-sectional area using a digital imaging system (BIOQUANT). In each soleus muscle cross-section, 250 muscle fibers were randomly chosen and measured for each of six mice in each treatment group. The average of the group of 250 fibers was calculated; that value was used as a single datum that was used to calculate the mean and SEM for fiber cross-sectional area for each treatment group.

RNA isolation and quantitative real-time PCR

During tissue collection, one soleus muscle from each animal was rapidly frozen in liquid nitrogen and used for RNA isolation. Total RNA was isolated with TriZol reagent, according to the manufacturer’s protocol (Invitrogen). First-strand cDNA was synthesized using random primers (Roche Diagnostics) and Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (QPCR) reactions with cDNA were performed using SYBR Green Master Mix (Bio-Rad). The real-time amplification of genes was measured with an iCycler thermocycler system and IQ5 optical system software (Bio-Rad). Data were normalized to β-actin transcript levels. The PCR primers used are listed in Table I.

Macrophage isolation

Peritoneal macrophages were isolated from 3–6-mo-old C57BL/6J mice. Mice received an i.p. injection of 12% sodium casein in 0.9% sodium chloride and were euthanized with isoflurane 3 d later. Immediately after euthanization, the peritoneal cavity of the mouse was opened and rinsed with PBS. The peritoneal exudates were then collected and filtered through a 70-μm cell strainer (BD Biosciences). Filtered peritoneal cells were centrifuged and resuspended in proliferation medium (DMEM [Sigma-Aldrich] supplemented with 10% FBS and 1% penicillin and streptomycin [Life Technologies]). Cell suspensions were overlaid on an equal volume of Histopaque-1077 (Sigma-Aldrich) and centrifuged at 400 × g for 30 min. Macrophages were collected from the interface of Histopaque and DMEM, centrifuged, and resuspended in DMEM media for later use. Some isolated cells were adhered to microscope slides by centrifugation at 14,000 × g for 3 min using 2% Cytochalasin (Shandon) and stained with rat anti-F4/80 (2.0 μg/ml) to confirm that they were macrophages. Rat anti-F4/80 was prepared previously by ammonium sulfate precipitation of IgG from F4/80 (0.2 μg/ml) to confirm that they were macrophages. Rat anti-F4/80 was prepared previously by ammonium sulfate precipitation of IgG from F4/80 (0.2 μg/ml) to confirm that they were macrophages. Rat anti-F4/80 was prepared previously by ammonium sulfate precipitation of IgG from F4/80 (0.2 μg/ml) to confirm that they were macrophages.

Assay for muscle cell proliferation or differentiation

Peritoneal macrophages were seeded at 4.0 × 10⁵ cells/well in six-well plates and stimulated for 24 h with IL-10 (10 ng/ml) to induce the M2 phenotype or IFN-γ and TNF-α (10 ng/ml of each) to induce the M1 phenotype. After stimulation, cytokines were washed away with DMEM. C2C12 cells that were grown previously in proliferation medium under subconfluent conditions were plated on top of stimulated macrophages at 4.0 × 10⁵ cells/well in differentiation media in which 10% FBS was replaced by 2% horse serum. Fresh differentiation media were added to the cultures every 24 h. Myoblast proliferation was assayed by harvesting cells from 2-d-old cocultures in 0.05% trypsin-EDTA, after which the cells were pelleted, resuspended in PBS, and counted using a hemocytometer. Macrophages remained adherent to the culture dishes during trypsinization. Myoblast differentiation was assayed by collecting cell lysates from 4-d cocultures in reducing sample buffer (80 mM Tris [pH 6.8], 0.1 M DTT, and 70 mM SDS) and protease inhibitor mixture (1:100, Sigma-Aldrich) for Western blot analysis.

Western blot analysis

Cell lysates collected in reducing sample buffer with protease inhibitor mixture were electrophoresed in polyacrylamide gels and then transferred electrophoretically to nitrocellulose membranes. Thirty micrograms of sample was used in each lane for all gels and blots analyzed. Following transfer, the membranes were stained with Porcine S solution (Sigma-Aldrich) to confirm the transfer of loading of samples. Nitrocellulose membranes were blocked with 3% nonfat milk and then incubated with mouse anti-MyoD (BD Biosciences), mouse anti-myogenin (BD Biosciences), rabbit anti-CD163 (Santa Cruz Biotechnology), or rabbit anti-mouse iNOS (Upstate Biotechnology) for 3 h at room temperature. The Abs were diluted in 50 mM Tris (pH 7.6) containing 150 mM NaCl, 0.1% NaN₃, 0.05% Tween 20, and 3% BSA. After washing with PBS buffer containing 0.1% Tween 20, the blots were incubated with a specific secondary Ab conjugated with HRP (Amersham) for 1 h at room temperature. After washing, the signal was detected by ECL (Amersham) and a fluorochem imaging system (Alpha Innotech).

Statistics

Data are presented as mean ± SEM. One-way ANOVA (GraphPad InStat version 2.03) was used to test whether differences between groups were significant at P < 0.05. Comparisons of two groups of values were analyzed using the unpaired, two-tailed t test. Differences were considered significant at P < 0.05.

Results

Increased muscle mass following periods of disuse induces an initial Th1 cytokine response followed by a Th2 cytokine response

Previous investigations demonstrated that CD68high macrophages appeared at elevated numbers in the initial inflammatory infiltrate in muscle experiencing increased loading, but their numbers then declined, whereas a CD163+ macrophage population subsequently increased (7–9). Our immunohistochemical observations confirm the early invasion of CD68high macrophages into muscle that has experienced 1 d of reloading and show that these macrophages can invade the cytoplasm of injured muscle fibers (Fig. 1). We also confirmed that their decline between days 1 and 4 of reloading was accompanied by an increase in CD163− macrophages that do not invade damaged muscle fibers (Figs. 2, 3). CD68+ and CD163+ macrophages were found codistributed in the same inflammatory lesions at each stage of reloading that was assessed (Supplemental Fig. 1).

We assayed for changes in expression levels of CD68 and Th1 cytokines that are associated with classical activation of macrophages to the M1 phenotype and found that there is a large, significant increase in CD68 expression at 1 d of reloading in wild-type muscle that is largely attenuated by 4 d of reloading (Figs. 3, 4), consistent with changes in the numbers of CD68high macrophages that occur during this period of reloading (31). This change in expression of CD68 coincided with changes in the expression of TNF-α, IL-6, and CCL2 (Fig. 4), all of which are cytokines that typify a Th1 cytokine response (27). Other transcripts associated with a Th1 cytokine response (CCR2, IRF-5, and IL-12) were not elevated concurrently (Fig. 4). In contrast, CD163 expression was not significantly elevated at 1 d of reloading, but it was increased at 4 d in wild-type muscle. This increase in CD163 coincided with a tremendous increase in arginase-1 (Arg1) expression, a marker for M2 activation of macrophages (36), along with substantial increases in the expression of IL-10 and its receptor, IL-10R1 (Fig. 5). However, expression of heme oxygenase-1 (HMOX-1), which can be activated by CD163 ligation (37), was not increased at 4 d (Fig. 5). Collectively, these data indicate that the CD68high/CD163− early invading macrophages are characteristic of a Th1 cytokine response, and they are subsequently replaced by a population of CD68low/CD163+ M2 macrophages.

Null mutation of IL-10 attenuates the shift of muscle macrophages to the M2 phenotype

The finding that the expression of IL-10, its receptor, and two major indicators of macrophage activation to the M2 phenotype (CD163 and Arg1) increased in muscle experiencing 4 d of reloading following a period of atrophy led us to test whether the shift to the M2 phenotype in reloading muscle was regulated by IL-10. Because IL-4 expression levels remained very low throughout the unloading and reloading process and did not differ significantly between any two time points assayed (data not shown), we inferred that IL-4...
mediated signaling was not an important modulator of macrophage phenotype switching in this model. Expression levels of markers of the M1 phenotype were assessed by QPCR (Table I) in both wild-type ($n = 6$) and IL-10$^{-/-}$ muscle ($n = 6$) after 1 d of reloading, showing that IL-10 ablation produced increased expression of two Th1-associated transcripts, CCL2 and IL-6 (Fig. 4). However, the expression levels of CD68 and TNF-$\alpha$ were not significantly affected by the IL-10 mutation. Similarly, expression levels of markers of the M2 phenotype were measured in muscle after 4 d of reloading, showing large, significant reductions in the expression levels of CD163 and Arg1, although the expression level of IL-10R1 was not significantly affected (Fig. 5). Together, these data show that IL-10 plays a significant role in regulating the induction of the M2 macrophage phenotype switch in reloaded muscle, although IL-10-mediated signaling does not influence the expression of all Th1 or Th2 cytokines in injured muscles in vivo.

Null mutation of IL-10 slows muscle fiber repair and regeneration in vivo

We assayed whether the attenuated shift to a M2 macrophage phenotype in IL-10$^{-/-}$ mutants influenced muscle fiber injury and regeneration that occur during the first 4 d of muscle reloading. Sections of soleus muscles were immunolabeled with FITC-conjugated anti-mouse IgG to assay for the presence of extracellular protein (Ig) in the muscle fiber cytoplasm, indicating the presence of muscle membrane lesions that permitted unregulated transit of large molecules across the cell membrane. The number of muscle fibers containing IgG did not differ between wild-type and IL-10$^{-/-}$ mice in 1-d reloaded muscles (Fig. 6). In wild-type mice, there was an 80% reduction in the percentage of IgG+ muscle fibers from days 1 to 4 of reloading; this indicates that the majority of muscle membrane repair occurred between 1 and 4 d reloading, which is consistent with previous findings (31). However, the number of injured muscle fibers in IL-10$^{-/-}$ mice was not reduced at 4 d of reloading compared with 1 d of reloading (Fig. 6C),...
indicating that null mutation of IL-10 either impairs muscle membrane repair during reloading or that recurring membrane damage persists for longer periods in the absence of IL-10.

Null mutation of IL-10 also impairs muscle regeneration during muscle reloading. No significant difference in muscle fiber central nucleation was observed between IL-10−/− and wild-type mice at 1 d of reloading (Fig. 6D). However, there was a 4-fold increase in central nucleation between 1 and 4 d of reloading in wild-type mice, suggesting that muscle experiences regeneration during this period of reloading. However, the increase in central nucleation at 4 d of reloading was less in IL-10−/− muscles (Fig. 6D), indicating that IL-10 mediates processes that promote muscle regeneration.

Loss of IL-10-mediated signaling perturbs muscle differentiation and reduces growth during regeneration

Our qualitative observations of muscle fiber histology (Figs. 1, 2) suggested that muscle fibers in IL-10−/− mice regained their normal size during reloading more slowly than did wild-type muscle fibers, suggesting possible defects in growth and differentiation. We assayed changes in muscle growth in IL-10-null mutant mice by

FIGURE 3. Null mutation of IL-10 amplifies CD68high macrophage numbers, reduces M2 macrophage numbers, and perturbs myogenin expression in injured muscle. Numbers of CD68+ (A), CD163+ (B), MyoD+ (C), and myogenin+ (D) cells in soleus muscles of wild-type and IL-10–null mutants over the time course of muscle unloading and reloading. (A) Numbers of CD68+ macrophages are increased at 1 d of reloading in wild-type and mutant muscles, and CD68+ cell numbers begin to decline after 1 d of muscle reloading of wild-type muscle. However, IL-10–null mutants do not experience a decline in CD68+ cells at 4 d of reloading. (B) Numbers of CD163+ macrophages are increased slightly in wild-type muscles at 1 d of reloading but not in mutant muscles at that time point. Numbers of CD163+ cells are increased in both wild-type and mutant muscles at 4 d of reloading, but the numbers are greatly amplified in wild-type muscles. (C) The numbers of MyoD-expressing satellite cells show similar increases in wild-type and mutant muscles at 1 d of reloading and similar reductions in numbers at 4 d of reloading. (D) The numbers of myogenin-expressing satellite cells show large increases in mutant muscle at 1 d of reloading, although their numbers do not change significantly in wild-type muscles at that time point. QPCR data showing changes in the levels of expression of MyoD (E) and myogenin (F) over the course of muscle unloading and reloading. The changes in gene expression for the two transcripts resemble the changes in the numbers of MyoD+ and myogenin+ cells shown in (C) and (D). Each bar represents the mean and SEM for the muscles collected from five mice in each data set. All data in each set were normalized relative to expression levels in ambulatory, wild-type muscles, which were set at 1.0. (G) Image showing an anti-MyoD–labeled satellite cell (arrow) at the surface of a muscle fiber in 1-d reloaded, IL-10-null muscle. Scale bar, 40 μm. (H) Image showing an anti-myogenin–labeled satellite cell (arrow) at the surface of a muscle fiber in 4-d reloaded, IL-10–null muscle. Scale bar, 40 μm. *p < 0.05 versus ambulatory control muscle of same genotype, †p < 0.05 versus wild-type muscle under the same treatment conditions, ‡p < 0.05 versus 1-d reloaded muscle of same genotype.
measuring changes in muscle fiber size during muscle unloading and reloading. Consistent with previous findings in rodents subjected to hindlimb unloading (31), 10 d of unloading in wild-type mice caused ∼40% atrophy in soleus muscle. No significant increase in the cross-sectional area of muscle fibers was observed at 1 d of reloading compared with the fiber size of mice that were assayed immediately following completion of unloading. However, there was a rapid increase in the fiber cross-sectional area from 1 to 4 d of reloading in wild-type mice, but that increase did not occur in IL-10−/− mice (Fig. 6E). We assayed muscle differentiation by quantifying the expression of key myogenic transcription factors by QPCR and by immunohistochemistry. Neither the expression level of MyoD nor the number of MyoD-expressing cells was significantly affected by IL-10 mutation at any stage of muscle unloading or reloading (Fig. 3). However, IL-10–null mutant mice displayed a greatly increased expression of myogenin

FIGURE 4. Levels of expression of transcripts related to the Th1 cytokine response during muscle unloading and reloading. Expression levels of CD68 (A), IL-6 (B), CCL2 (C), and TNF-α (D) were all increased at 1 d of reloading in wild-type muscles, reflecting a Th1 cytokine response, although IRF-5 (E), CCR2 (F), and IL-12 (G) were not increased at that stage. The transcripts that were increased at 1 d of reloading were all significantly reduced by 4 d of reloading, reflecting resolution of the Th1 cytokine response. Null mutation of IL-10 amplified the Th1 cytokine response at 1 d of reloading, reflected in significant increases in IL-6 and CCL-2. Each bar represents the mean and SEM for the muscles collected from six mice in each data set. All data in each set were normalized relative to expression levels in ambulatory, wild-type muscles, which were set at 1.0. *p < 0.05, versus ambulatory control muscle of same genotype, ǂp < 0.05 versus wild-type muscle under the same treatment conditions, # p < 0.05 versus 1-d reloaded muscle of same genotype.
and significantly more myogenin-expressing cells in 1-d reloaded muscle (Fig. 3), indicating that normal patterns of muscle differentiation are disrupted by null mutation of IL-10 during muscle reloading.

M2 macrophages promote muscle cell proliferation without direct effects on differentiation in vitro

Our in vivo data show that null mutation of IL-10 increased myogenin expression in reloaded muscle, indicating that IL-10 could affect muscle differentiation either by direct actions on myogenic cells or by acting through M2 macrophages. We tested these possibilities by analyzing the effects of IL-10 or IL-10–activated M2 macrophages on muscle cell proliferation and differentiation in vitro. Although we were unable to detect IL-10R expression in myoblasts at the protein level, expression of both IL-10R1 and IL-10R2 was confirmed by RT-PCR in proliferative myoblasts, although IL-10R1 was barely detectable (Fig. 7A).

We assayed whether IL-10 or IL-10–activated macrophages influenced myoblast proliferation in vitro. Consistent with previous findings (32), we found that the direct application of IL-10 to myoblasts in vitro did not affect myoblast proliferation but that coculture of myoblasts with IL-10–activated M2 macrophages caused significant increases in myoblast proliferation (myoblasts alone increased 5.5-fold in 48 h [SEM = 0.28]; myoblasts cocultured with IL-10–stimulated macrophages increased 7.4-fold [SEM = 0.20]; p < 0.01). We then tested whether direct stimulation of myoblasts with IL-10 affected early stages of muscle differentiation but found no detectable changes in the expression of MyoD or myogenin when muscle cell extracts were assayed by Western blots (Fig. 7B). Finally, we tested whether the lack of effect of direct stimulation of muscle with exogenous IL-10 in vitro could result from a saturation of IL-10 effects on muscle by endogenous IL-10 by assaying whether muscle cells in vitro expressed IL-10. However, no IL-10 mRNA was detectable in cultures of subconfluent myoblasts, confluent myoblasts, or differentiated myotubes (Fig. 7C).

Because perturbations of IL-10 expression in vivo influenced expression of myogenin, but direct application of IL-10 to muscle cells in vitro did not affect MyoD or myogenin expression, we assayed whether coculturing muscle cells with IL-10–stimulated M2 macrophages affected the expression of MyoD or myogenin. Macrophages were stimulated with IL-10 to drive them to an M2 phenotype or stimulated with both IFN-γ and TNF-α to drive them to an M1 phenotype before coculturing with C2C12 cells. Western blot analysis of CD163 confirmed that IL-10 stimulation promoted the M2 phenotype and showed that maximum induction of CD163 was achieved by 10 ng/ml (Fig. 7D). Western blot analysis of iNOS expression confirmed induction of the M1

FIGURE 5. Levels of expression of transcripts related to the Th2 cytokine response during muscle unloading and reloading. Expression levels of CD163 (A), IL-10 (B), IL-10R1 (C), and Arg1 (D) were elevated at 4-d of reloading in wild-type muscles, reflecting a Th2 cytokine response, although HMOX-1 (E) was not elevated at that stage. Null mutation of IL-10 attenuated the Th2 cytokine response at 4 d of reloading, reflected by significant decreases in CD163 and Arg1. Each bar represents the mean and SEM for the muscles collected from six mice in each data set. All data in each set were normalized relative to expression levels in ambulatory, wild-type muscles, which were set at 1.0. *p < 0.05, versus ambulatory control muscle of same genotype, †p < 0.05 versus 1-d reloaded muscle under the same treatment conditions, ‡p < 0.05 versus 1-d reloaded muscle of same genotype.
muscle regeneration was substantial; muscle fiber growth was significantly slowed, and muscle regeneration was reduced, which emphasize the important role of IL-10–activated macrophages in modulating muscle repair.

The results of the present investigation show that IL-10 plays a dominant role in regulating macrophage transition to a CD163+ M2 phenotype in regenerative muscle. In the absence of IL-10, no significant increase in CD163 expression occurred in regenerative muscle, and the increase in CD163+ M2 cells during the transition from 1 to 4 d of reloaded muscle was nearly abolished. Our data also indicate that IL-10 is required in regenerative muscle for induction of Arg1 expression, another specific indicator of activation of the M2 phenotype. We also observed that the expression of some transcripts associated with the M1 phenotype, in particular, CCL2 and IL-6, was greatly amplified by IL-10 ablation at 1 d of muscle reloading, indicating that IL-10 has a suppressive effect on the Th1 cytokine response in injured muscle. The impact on muscle of perturbing induction of the M2 macrophage phenotype was substantial; muscle fiber growth was significantly slowed, muscle membrane lesions persisted, and muscle regeneration was reduced, which emphasize the important role of IL-10–activated macrophages in modulating muscle repair.

Previous observations already established that shifts in macrophage phenotype coincide with transitions in the stage of myogenesis in regenerating muscle following acute injury. For example, CD68+ macrophages reach maximum numbers at ∼2 d following muscle injury and then decline (7, 38). The number of CD163+ macrophages begins to increase in regenerative muscle at ∼2 d post-injury and reaches maximum levels at ∼4 d (7, 10). Suggestively, that shift in macrophage phenotype coincides with changes in expression of myogenic regulatory transcription factors. Injection of cardiotoxin into soleus muscle increases MyoD expression at 2 d postinjury, and myogenin expression is elevated in muscle fibers at ∼1 d following the increase in MyoD (21). Other muscle-injury models show a similar time course in the increase in MyoD at ∼2 d postinjury, followed by the elevated expression of other, developmentally regulated transcripts (40).

The transition during myogenesis to a stage at which myogenin expression is elevated is developmentally important, because it represents the transition from a proliferative population of myogenic cells that is unable to undergo terminal differentiation to a population that has permanently withdrawn from the cell cycle and enters early stages of differentiation (41). We had anticipated that if shifts in macrophage phenotype and the stage of myogenesis that occur in injured muscle in vivo were linked, then disruptions in macrophage phenotype switching would prevent the transition to early differentiation of myogenic cells that would be reflected in lower myogenin expression. Although our findings show that obstructing the shift to the M2 phenotype disrupts normal patterns of muscle differentiation and slows muscle growth, we unexpectedly found that amplifying the Th1 cytokine response

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*National Center for Biotechnology Information.
at 1 d of reloading by ablating IL-10 expression increased the number of myogenin-expressing satellite cells. In addition, our data and previous findings (32) show that IL-10–stimulated M2 macrophages increase the proliferation of myoblasts in cocultures, also indicating a role for M2 macrophages in promoting the proliferative stage of myogenesis, rather than mediating the transition to early differentiation. Furthermore, our data show that the significant reduction of myogenin expression that occurs between 1 and 4 d of reloading in wild-type muscles coincides with the large increase in CD163+ M2 macrophages and increase in IL-10 expression, supporting the interpretation that M2 macrophages suppress myogenin expression in vivo. These findings contrast with a previous report showing that muscle reloading is repaired between days 1 and 4 of unloading (Unl.) and 1-d and 4-d reloaded muscles indicates that membrane lesions caused by muscle reloading are repaired between days 1 and 4 of reloading in wild-type, but not IL-10 mutant, muscles. (D) Quantification of the proportion of central-nucleated muscle fibers in reloaded muscles shows that the increase in regenerative muscle fibers that occurs in wild-type muscles between 1 and 4 d of reloading is diminished in IL-10–null mutants. (E) Muscle fiber growth during reloading was assayed by measuring changes in cross-sectional area of the muscle fibers in soleus muscle sections. Note that fiber size in ambulatory controls (Amb.) did not differ between wild-type and IL-10 mutant muscles and that mutation of IL-10 did not affect the atrophy of fibers that occurred during unloading (Unl.). However, fiber growth that occurred during 4 d of reloading did not occur in IL-10 mutants. Each bar represents the mean and SEM for the muscles collected from six mice in each data set. *p < 0.05 versus 1-d reloaded muscle of the same genotype. †p < 0.05 versus 4-d reloaded wild-type muscle.

FIGURE 6. IL-10 mutants experience reductions in muscle fiber repair, regeneration, and growth during reloading. Cross-section of soleus muscles from wild-type (A) or IL-10–null mutant (B) mice from 4-d reloaded mice labeled with FITC-conjugated, anti-mouse IgG. The presence of IgG in the muscle fiber cytosol (arrows) indicates the presence of membrane lesions large enough to allow influx of IgG from the extracellular space. Scale bars, 100 μm. (C) Quantification of the number of IgG+ muscle fibers in unloaded (Unl.) and 1-d and 4-d reloaded muscles indicates that membrane lesions caused by muscle reloading are repaired between days 1 and 4 of reloading in wild-type, but not IL-10 mutant, muscles. (D) Quantification of the proportion of central-nucleated muscle fibers in reloaded muscles shows that the increase in regenerative muscle fibers that occurs in wild-type muscles between 1 and 4 d of reloading is diminished in IL-10–null mutants. (E) Muscle fiber growth during reloading was assayed by measuring changes in cross-sectional area of the muscle fibers in soleus muscle sections. Note that fiber size in ambulatory controls (Amb.) did not differ between wild-type and IL-10 mutant muscles and that mutation of IL-10 did not affect the atrophy of fibers that occurred during unloading (Unl.). However, fiber growth that occurred during 4 d of reloading did not occur in IL-10 mutants. Each bar represents the mean and SEM for the muscles collected from six mice in each data set. *p < 0.05 versus 1-d reloaded muscle of the same genotype. †p < 0.05 versus 4-d reloaded wild-type muscle.
influence of dexamethasone on M2 macrophages overwhelmed the influence of IL-10 on macrophage-mediated effects on muscle cells, at least in the muscle coculture experiments.

The effect of IL-10 ablation on myogenin expression in injured muscle in vivo may be mediated indirectly by changes in the expression of other cytokines that can influence myogenin expression. For example, the large increase in myogenin expression and increase in numbers of myogenin-expressing satellite cells that we observed in IL-10 mutants at 1 d of reloading coincided with large increases in IL-6 expression. However, the relationships between IL-6 and myoblast proliferation and differentiation are complex; IL-6 is well established as a muscle nitrogen (25) that plays a significant role in muscle fiber growth (42), but it can also induce muscle wasting (43). Nevertheless, IL-6 treatment of myoblasts in vitro was reported to increase myogenin expression (44), and a recent investigation showed a strong, positive effect of IL-6 on myogenin expression in vivo (45). Unlike in wild-type mice, myogenin expression did not increase in skeletal muscle of IL-6-null mutant mice at 1 d of muscle reloading (45), although interpretation of the finding is complicated by the higher baseline level of myogenin in the muscles of IL-6 mutants. Similarly, the increase in myogenin expression in reloaded muscles of IL-10–null mice may be attributable to the loss of increased TNF-α expression in the injured muscles of mutants. Application of TNF-α to muscle cells in vitro can greatly diminish myogenin expression (46–48) that can result from activation of c-Jun N-terminal kinase (49).

The mechanism through which IL-10 promotes the shift in muscle macrophages to the M2 phenotype is not known, although several observations indicate that activation of phagocytosis may be a component of the signaling pathway. Recent findings showed that IL-10 stimulation of macrophages isolated from skeletal muscles increases the phagocytic activity of macrophages (32), as shown previously for circulating macrophages (50). Furthermore, phagocytosis of cellular debris by M1 macrophages can promote their transition to the M2 phenotype in vitro (18). In addition, depletion of populations of phagocytic muscle macrophages in vivo by the administration of clodronate-containing liposomes specifically reduced the numbers of M2 macrophages (32), possibly reflecting a decrease in the M1-to-M2 phenotype switch caused by the depletion of phagocytes. However, based on in vitro observations by previous investigators, the induction of the M1-to-M2 phenotype transition by phagocytosis may be greatly influenced by the identity of the phagocytosed material, so that phagocytosis is not sufficient to drive the change in macrophage phenotype. For example, if the debris to be phagocytosed was generated by neutrophil lysis, then phagocytosis by macrophages causes great increases in IL-10 production, reflecting a switch to the M2 phenotype (51). However, an increase in IL-10 production does not occur if the debris results from lymphocyte death (51). These findings indicate that phagocytosis may promote the M2 macrophage phenotype during innate immune responses, which occurs in injured muscle, but perhaps not in an acquired immune response. Furthermore, phagocytosis of apoptotic neutrophils by M1 macrophages increased production of the Th2 cytokine TGF-β by the macrophages, while reducing expression of the Th1 cytokines IL-1β and TNF-α, reflecting a shift toward an M2 phenotype (52). However, if the neutrophils were opsonized with anti-CD45, their phagocytosis did not cause a shift in phenotype (52). Those observations indicate that phagocytosis of apoptotic neutrophils during an innate immune response can induce the M1-to-M2 phenotype switch in macrophages. Interestingly, these cues for macrophage phenotype switch are in place in reloaded skeletal muscle at the time of the M1-to-M2 transition in vivo. The peak of apoptosis and phagocytosis of inflammatory cells occurs 2 d after the onset of muscle reloading (53), which coincides with an increase in M2 macrophage populations.

The IL-10–mediated shift in macrophage phenotype in injured muscle may be amplified by activation of MAPK, especially p38 MAPK, through signaling that is antagonized by MAPK phosphatase-1 (MKP-1). Although IL-10 can function as either a potent activator of p38 in macrophages (54) or inhibitor of p38 (55, 56), activation of p38 by IL-10 or IL-10–independent pathways can cause further increases in IL-10 expression and contribute to macrophage phenotype switch from the M1 to M2 phenotype. For example, null mutation of MKP-1 caused supraphysiological levels of p38 activity in macrophages that induced increased expression of IL-10 and TNF-α (57, 58), and the increased levels of IL-10 and TNF-α are reduced by p38 inhibition (57). Furthermore, null mutation of MKP-1 increased the number of M2 macrophages in injured muscle, but that increase was reduced by MAPK inhibitor (59), indicating that p38 activation contributes to the macrophage phenotype switch in injured muscle, which could increase the production of IL-10 (60), creating positive feedback for the phenotype switch. However, if IL-10 activation of p38 promotes the switch to the M2 phenotype during the regeneration of wild-type muscle, the effect appears to be independent of HMOX-1 induction. Although IL-10 can suppress the M1 macrophage phenotype by p38 activation of HMOX-1 (54), no changes in HMOX-1 expression were observed in reloaded muscle in the present investigation, even at 4 d of reloading when IL-10 expression was greatly increased.

Although this investigation illustrates that IL-10 is required for normal growth and regeneration of muscle following injury, the findings are not sufficient to show that further increases in IL-10 in injured muscle will improve repair. As shown recently, direct injection of IL-10 into injured, wild-type muscle slowed the growth of regenerative fibers (59). Thus, either the loss of IL-10 or the application of superphysiological levels of IL-10 can impede muscle growth and repair following injury. However, the negative effect of supraphysiological levels of IL-10 may reflect the dose dependency of IL-10 effects on leukocyte activation. For example, IL-10 generally functions as an anti-inflammatory cytokine, but supraphysiological levels of IL-10 administered during sepsis exacerbate the inflammatory response (61); administration of exogenous IL-10 at low doses is protective against graft-versus-host disease, whereas higher dosages promote lethality of the disease (62). Thus, developing IL-10 treatments that are useful for promoting muscle repair and regeneration following injury will require identification of therapeutically appropriate dosing with exogenous IL-10 or precise manipulations of the levels of IL-10 expression by leukocytes in vivo (63).

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1.

Co-distribution of CD68+ and CD163+ macrophages in inflammatory lesions of injured skeletal muscle. Macrophages labeled with anti-CD68 (arrows in “A”) or anti-CD163 (arrows in “B”) within an inflammatory lesion in soleus muscle of IL-10 null mutant mouse after 4-days of muscle reloading. A and B are adjacent sections taken from the same muscle with identical muscle fibers appearing in the same muscle numbered for reference. Note the relatively large number of CD68+ macrophages distributed throughout the connective tissue surrounding muscle fibers (A) compared to the scant distribution of CD163+ macrophages in the same lesion (B). Bar = 80 µm.