Anti-inflammatory macrophages improve skeletal muscle recovery from ischemia-reperfusion

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Hammers DW, Rybalko V, Merscham-Banda M, Hsieh PL, Suggs LJ, Farrar RP. Anti-inflammatory macrophages improve skeletal muscle recovery from ischemia-reperfusion. J Appl Physiol 118: 1067–1074, 2015. First published February 12, 2015; doi:10.1152/japplphysiol.00313.2014.—The presence of macrophages (MPs) is essential for skeletal muscle to properly regenerate following injury. The aim of this study was the evaluation of MP profiles and their importance in skeletal muscle recovery following tourniquet-induced ischemia-reperfusion (I/R). Using flow cytometry, we identified two distinct CD11b+ MP populations that differ in expression of the surface markers Ly-6C and F4/80. These populations are prominent at 3 and 5 days of reperfusion and molecularly correspond to inflammatory and anti-inflammatory MP phenotypes. Sorted MP populations demonstrated high levels of IGF-I expression, and whole muscle post-I/R IGF-I expression strongly correlates with F4/80 expression. This suggests MPs largely influence postinjury IGF-I upregulation. We additionally demonstrate that direct intramuscular injection of FACs-isolated CD11b+Ly-6C–F4/80hi MPs improves the functional and histological recovery of I/R-affected muscle. Taken together, these data further support the substantial influence of the innate immune system on muscle regeneration and suggest MP-focused therapeutic approaches may greatly facilitate skeletal muscle recovery from substantial injury.

Skeletal muscle response to injury involves a complex program of degenerative and regenerative activities to repair the damaged tissue and restore contractile muscle function. Coordination of these processes is achieved by an orchestration of molecules, including intracellular components, cytokines/chemokines, growth factors, and protease byproducts, which create a milieu that directs the cellular constituents of the injury site to their proper state of activation to promote efficient skeletal muscle regeneration. The body of literature that pertains to skeletal muscle regeneration is heavily dominated by studies focused on myogenic stem cell populations, primarily resident satellite cells; however, cellular components of the immune system, especially macrophages (MPs), are becoming increasingly known for their essential and intriguingly complex roles in the muscle regenerative process.

Acute skeletal muscle injury is immediately coupled with the release of intracellular signals and subsequent expression of chemotactic cytokines that attract neutrophils to the damaged tissue within hours (16, 39). This event is followed by the infiltration of circulating monocytes into the region, which differentiate into a proinflammatory and phagocytotic MP phenotype (1, 27, 45). The prevalence of this MP phenotype is gradually superceded by the emergence of a more anti-inflammatory, preregenerative phenotype (1, 45). An emerging model of the origin of this dual-MP profile from a common infiltrate, eloquently described by Arnold et al. (1), entails an immature population of “sentinel” monocytes expressing high levels of chemokine (C-C motif) receptor-2 (CCR2) and Ly-6C and low levels of fractalkine receptor (CX3CR1) chemotactically migrating to damaged tissue to become inflammatory MPs that characterize the degenerative phase of muscle repair (1, 18, 27). Subsequent phagocytosis of tissue debris then induces a phenotype shift to Ly-6C–CX3CR1hi anti-inflammatory MPs, which dominate the regenerative phase (1).

The absolute necessity of MPs in skeletal muscle regeneration has been repeatedly demonstrated by studies that have either depleted monocytes or impaired their migration through genetic or pharmacological means (1, 4, 27, 28, 46). In these models, the regeneration of muscle following injury is dramatically hindered, largely characterized by delayed myogenesis with highly disorganized fibers and substantial adipose deposition. This regenerative decrement found with the exclusion of MPs from the site of injury is likely a multifaceted process: prevention of the initial population results in the failed removal of necrotic tissue and impaired extracellular matrix modification, while eliminating anti-inflammatory MPs negates the supportive role in regeneration. This suggests a delicate coordination of this infiltration and transition must occur to achieve efficient regeneration of muscle (1, 41).

In the study of traumatic muscle injuries, the importance of this immunological component cannot be ignored, especially in evaluating novel therapeutic strategies (21, 31) or studying conditions of impaired regeneration, such as aging (8, 20, 50). The purpose of the present work was to utilize flow cytometry to characterize MP profiles in skeletal muscle following tourniquet-induced ischemia-reperfusion (I/R). The analysis of infiltrating CD11b+ cells for the expression of Ly-6C and F4/80 surface markers reveals distinct CD11b+Ly6C–F4/80hi and CD11b+Ly6C–F4/80hi MP populations that display divergent gene profiles and shift in prevalence during the reperfusion time course. Furthermore, we demonstrate that whole muscle insulin-like growth factor I (IGF-I) expression correlates with F4/80 expression and intramuscular injection of CD11b+Ly6C–F4/80hi cells drastically improves the functional recovery of skeletal muscle from I/R.
METHODS AND MATERIALS

Animals. Male C57BL/6 mice (6 mo; Jackson Laboratories) were used for this study. Animals were housed individually with ad libitum access to food and water and maintained on a 12-h light-dark cycle. All experimental procedures were approved and conducted in accordance with the guidelines set by The University of Texas at Austin Institutional Animal Care and Use Committee.

Tourniquet application. As previously described (19), mice were anesthetized with 2% isoflurane gas, and a single, randomly selected hindlimb was elevated. A pneumatic tourniquet (D. E. Hokanson) was wrapped snugly against the proximal portion of the limb and inflated to 250 mmHg by the Portable Tourniquet System (Delfi Medical Innovations) to ensure complete occlusion of blood flow to the limb (49). Body temperature was maintained at 37 ± 1°C with the use of a heat lamp during this procedure. After the 2 h, the pneumatic tourniquet was removed, and the mouse was returned to its cage for recovery. Where indicated, muscles from the uninjured contralateral limb served as internal controls, as performed in other studies (20, 47, 48).

MP isolation. Lateral gastrocnemius (LG) muscles (~60–100 mg of tissue) were harvested from euthanized mice, minced finely, and incubated in 10 vol/muscle weight of 1% type II collagenase (Invitrogen; dissolved in DMEM) at 37°C. Following 20 min of incubation, suspensions were gently triturated and incubated for another 20 min. Samples were filtered through 40-um nylon cell strainers (BD) into media containing 10% fetal bovine serum, centrifuged at 300 g for 6 min, and resuspended in 2 ml of media. The concentration of viable, Trypan blue negative cells, with the exclusion of red blood cells (RBCs), was determined using a hemacytometer. Total cells and total cells per milligram of muscle were calculated from this value. For control and 1-day reperfusion samples, bulk lower hindlimb muscles (consisting of the gastrocnemius, plantaris, tibiaalis anterior, extensor digitorum longus, flexor hallucis longus, and quadriceps) from each animal were pooled to obtain enough cells for analysis.

Flow cytometry and fluorescence-activated cell sorting. Immediately following isolation, two aliquots of 2.5–5 × 10⁶ cells each were harvested from each muscle sample and were washed in 1% BSA in PBS, blocked in 2% BSA in PBS, and stained with either a cocktail containing PE-conjugated anti-CD11b (BD), FITC-conjugated anti-Ly-6C (Biotrend), and APC-conjugated anti-F4/80 (Biotrend) or a cocktail of the corresponding isotype controls (antibody concentration of 1 μl per 2.5 × 10⁶ cells). Samples were run on the BD Fortessa Flow Cytometer at The University of Texas at Austin Institute of Cell and Molecular Biology core facility (ICMB) with the forward scatter (FSC) settings gated to exclude RBC-sized cells. The data were analyzed using Flowing Software 2.

For fluorescence-activated cell sorting (FACS), MPs were similarly isolated and stained from bulk hindlimb muscles of 1-, 3-, and 5-day reperfusion animals to obtain adequate amounts or cells for experiments. Stained cells were sorted using the BD FACS Aria at The University of Texas at Austin ICMB. For injections, cells were counted, checked for viability, and injected at a concentration of ~1.5 × 10⁶ cells/100 μl PBS into the affected gastrocnemius muscles (50 μl into each gastrocnemius head) of 3-day reperfusion animals (n = 5). Saline-injected animals served as controls for this experiment (n = 6).

RT-PCR. RNA was extracted from either freshly sorted cells (n = 3 per experiment) or snap-frozen gastrocnemius muscles (n = 3–5 per time period) using Trizol Reagent (Invitrogen), treated with RNase-free DNase I (Ambion), and reverse transcribed using SuperScript III Kit (Invitrogen) according to manufacturers’ instructions. Resulting cDNA was subjected to real-time PCR analysis run on the Bio-Rad iCycler IQ5 using corresponding primers (Table 1) and SYBR-green probe. Relative gene expression was determined using the ΔΔCt method with B2m and Gapdh as the loading controls for isolated cells and whole muscle, respectively. PCR products were confirmed on 2% agarose gels.

Force measurements. Following 14 days of reperfusion, cell- and saline-injected gastrocnemius muscles were surgically isolated from all other muscles and connective tissue and subjected to in situ functional measurements. The Achilles tendon was secured to the muscle lever arm of a servomotor (model 305B; Cambridge Technologies) interfaced with a computer equipped with an A/D board (National Instruments). The muscle was stimulated to contract using an Isolated Pulse Stimulator (Model 2100; A-M Systems) with leads applied to the sciatic nerve. Muscle temperature was kept constant at 37°C with warm mineral oil and a radiant heat lamp throughout the procedure. Optimal length of the muscle was determined by measuring maximal twitch tension at a stimulation of 0.5 Hz. At optimal length, the muscle was stimulated at 150 Hz to elicit the peak tetanic tension (P0) and was allowed 2 min of rest between each contraction. Data were stored and analyzed using LabView software (National Instruments).

Histology. Formalin-fixed gastrocnemius muscles were paraffin embedded, sectioned at 10 μm, and stained with hematoxylin and eosin, as previously described (31). The slides were observed with a light microscope (Nikon Diaphot) with the ×20 objective lens, and images were taken using a mounted digital camera (Optronix Microfire). Myofiber cross-sectional area (CSA) was measured by an investigator blind to the experimental design and sample designations using ImageJ software (195–350 fibers/group).

Statistical analysis. Data were analyzed using Student’s t-tests, one-way ANOVA (Tukey’s post hoc tests), or linear regression, where appropriate (α = 0.05). Values are represented as means ± SE or means ± SD, where indicated.

RESULTS

MP profiles and phenotypes following reperfusion injury. Flow cytometric analysis of the post-I/R injury infiltrate profiles in skeletal muscle was achieved by labeling freshly isolated cells with anti-CD11b, a marker of neutrophils and cells of monocytes lineage; anti-Ly-6C, a surface marker found on inflammatory monocytes/MPs and neutrophils; and anti-F4/80, a marker associated with mature MPs. Following 3 days of reperfusion, the pool of CD11b⁺ cells from the LG contained three distinct subpopulations that varied in expression of Ly-6C and F4/80 (Fig. 1A), which included Ly-6Cmed/F4/80⁻/quadrant 1 (Q1), Ly-6Cmed/F4/80⁺ (Q4), and Ly-6Chi/F4/80⁺ (Q2) cells, in agreement with previous reports detailing distinctive infiltrate profiles in postinjury muscle (1). Additionally, we observed the clear temporal transition from primarily Ly-6Cmed/F4/80⁺ at 1 day of reperfusion towards Ly-6Cmed/F4/80⁺ at 7 days (Fig. 1B). In agreement with total cell numbers (Fig. 1C), we found that the peak prevalence of CD11b⁺, CD11b⁺Ly-6Cmed/F4/80⁺, and CD11b⁺Ly-6Chi/F4/80⁺ popula-

![Table 1. RT-PCR primers](image)
upregulation of the proregenerative growth factor IGF-I occurs during the recovery time course. Interestingly, expression of inflammatory MP markers was highest at day 5 (Fig. 2A). Total cell numbers (C) in the LG peak following 3 days of reperfusion and decline during the course of recovery are shown. Analysis of individual cell populations (D) from days 3 and 5 show the selective decline in Ly-6C^hiF4/80lo cells. Values are expressed as means (B) or means ± SE (C and D); *P < 0.05 vs. previous day population size.

Macrophages Influence Regeneration • Hanners DW et al. • J Appl Physiol 1069

Fig. 1. Flow cytometry was used to analyze macrophage (MP) infiltrate profiles following 2-h tourniquet-induced ischemia-reperfusion (I/R) injury of mouse lateral gastrocnemius (LG) muscle. By gating for CD11b^+ cells, 3 distinct subpopulations were evident based on expression of Ly-6C and F4/80, including Ly-6C^hiF4/80lo (Q4) and Ly-6C^loF4/80hi (Q2) MP populations (A). The time course of recovery from I/R displayed distinctive MP profiles, shifting from predominantly Ly-6C^hiF4/80lo MPs at 1 day of reperfusion to Ly-6C^hiF4/80lo by day 7 (B). Total cell numbers (C) in the LG peak following 3 days of reperfusion and decline during the course of recovery are shown. Analysis of individual cell populations (D) from days 3 and 5 show the selective decline in Ly-6C^hiF4/80lo cells.
treated muscle (Table 2). This also translated to significantly better recovery (in terms of % of contralateral limb) of maximum force and muscle mass, while mass-normalized force trended towards significance (Fig. 4C). In agreement, MP-treated muscles displayed an improved histological morphology over saline treatment (Fig. 4A), as well as significantly larger fiber size (Fig. 4B). This result demonstrates that an increased CD11b⁺ Ly-6C⁺ F4/80hi population improves muscle recovery from I/R and suggests that MP modifying therapies are worth investigation for clinical facilitation of muscle regeneration.

DISCUSSION

The recent emergence of the immune system as a fascinating and absolutely critical component of muscle regeneration has created additional considerations in the field of myogenic regulation and regeneration. In the present study, we employed flow cytometry to investigate MPs at the tissue level in skeletal muscle regenerating from I/R injury. Using this method, we quantified individual inflammatory and anti-inflammatory MP populations, respectively. Interestingly, we found that expression of Igf1 peaks at 5 days of reperfusion in the CD11b⁺ F4/80⁺ cell population despite a reduction of the modestly higher expressing Ly-6C⁺ F4/80hi population isolated from 3-day reperfusion muscle. Whole muscle Igf1 expression levels from 1-, 3-, 5-, and 7-day reperfusion gastrocnemius muscles show strong correlation to Emr1 (F4/80) expression (C; n = 3–5).

Table 3. Gastrocnemius function following 14 days of reperfusion

<table>
<thead>
<tr>
<th>Muscles</th>
<th>Pmax, N</th>
<th>Normalized Pmax, N/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 11)</td>
<td>152 ± 2</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Saline (n = 6)</td>
<td>112 ± 3*</td>
<td>1.4 ± 0.1*</td>
</tr>
<tr>
<td>Macrophage (n = 5)</td>
<td>143 ± 6†</td>
<td>2.2 ± 0.1*†</td>
</tr>
</tbody>
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Values are means ± SE; n = no. of animals. Pmax, maximum isometric tetanic force production. *P < 0.05 vs. control. †P < 0.05 vs. saline.

Fig. 2. Gene expression analysis of CD11b⁺ F4/80⁺ cells sorted from 1-, 3-, 5-, and 7-day reperfusion muscles (A) and the Ly-6C⁺ F4/80hi and Ly-6C⁺ F4/80lo subsets of CD11b⁺ cells isolated from 3-day reperfusion muscles (B) reveals divergent inflammatory and anti-inflammatory phenotypes. AU, arbitrary units. Values are expressed as means ± SD; *P < 0.05 vs. previous day (A) or Ly-6C⁺ F4/80hi (B) value; n = 3 for each experiment.

Fig. 3. Igf1 gene expression was analyzed in CD11b⁺ F4/80⁺ cell populations from 1-, 3-, 5-, and 7-day reperfusion muscles (A), and Ly-6C⁺ F4/80hi and Ly-6C⁺ F4/80lo populations from 3-day reperfusion muscles (B). Whole muscle Igf1 expression levels from 1-, 3-, 5-, and 7-day reperfusion gastrocnemius muscles show strong correlation to Emr1 (F4/80) expression (C; n = 3–5). Values are expressed as means ± SD; *P < 0.05 vs. previous day value.
expression within similarly sorted cells across the recovery time course, which is likely dictated by surrounding regenerative environment. We cannot, however, rule out the possibility that this shift in gene expression is linked to changes in intrapopulation surface marker distinctions that were not investigated in this study.

While much of the necessary nature of MPs in muscle regeneration can likely be attributed to their acting as demolition specialists in the phagocytosis of necrotic debris, a more sophisticated role of supporting and coordinating myogenic events has emerged. The proproliferative and prosurvival effects of MPs, as a general population, on myoblasts is well documented (5, 7, 14, 30, 44) and has been attributed to both diffusible factors (5, 14) and cell-to-cell contact (7, 44). With the data described herein and in other recent reports (14, 27, 28), MPs are emerging as being highly influential to the postinjury expression of IGF-I. Additionally, muscle IGF-I expression is severely reduced with pharmacological ablation of MPs (13). However, although isolated MPs do demonstrate high levels of IGF-I expression, it cannot be ruled out that MPs influence IGF-I expression by other cell types in vivo, thus contributing to...
the upregulation indirectly. Regardless, given the robust benefit of increased IGF-I levels on muscle regeneration (21, 38), the importance of MPs to the IGF-I landscape is undeniable for the field of regenerative medicine.

A critical finding in this work is the improvement in muscle recovery found with the direct IM injection of sorted CD11b+Ly-6Ch(F4/80hi) cells into temporally matched I/R-affected muscle. While the beneficial effect of increased MPs on tissue recovery has been suggested by previous studies that either impair MP migration into muscle (1, 28, 29, 36, 46) or increase MP prevalence by upregulating of extracellular matrix remodeling enzymes (34), this result directly demonstrates that increasing the anti-inflammatory subset improves functional recovery of skeletal muscle following traumatic injury. Ex vivo-stimulated MP therapies have been utilized in both rodents and humans to improve outcomes of myocardial infarction (24), sternal wounds (37), and skin ulcers (10, 12, 17, 51). Although important physiological value can be gleaned from these data, they also indicate that MP-emphasized approaches represent potential therapies for muscle injuries.

The recently emerging paradigm entails temporally and environmentally specific roles of the inflammatory and anti-inflammatory MP phenotypes: the former selectively promote myoblast proliferation and inhibit differentiation, while the latter induce myoblast differentiation (1, 2, 42). During the course of this work, an intriguing report exploiting these characteristics was published demonstrating enhanced engraftment of cultured, donor-derived myoblasts into immune-compromised, dystrophin-deficient mice with coinjection of inflammatory MPs (2). Not only does this underscore the importance of MPs in myogenic regulation, it also suggests that the limited efficacy of myoblast engraftment can be attributed to premature differentiation caused by the improper environment. In our study, we specifically chose to temporally match (3 days of reperfusion) the donor cells and recipient muscle to avoid potential misregulation via temporal imbalance. However, purposely sustaining a high percentage of the inflammatory phenotype for a longer duration may bolster the myogenic population. In fact, a recent report demonstrated that treatment with culture-derived, classically activated (M1) MPs improves muscle recovery from laceration injury (35). Because our study did not include a CD11b+Ly-6Csh(F4/80lo) treatment group, we cannot discern which population is more beneficial for skeletal muscle recovery from I/R.

In this study, we used the clinically relevant tourniquet-induced I/R model of muscle injury. I/R injury involves the progressive accumulation of metabolites and ATP depletion during the ischemic phase followed by a more damaging free radical burst from reperfusion, resulting in severe damage that can ultimately lead to dramatic dysfunction or death of both affected muscle fibers and vasculature (3, 22). Severe decrement in muscle recovery from ischemia occurs with genetic deletion of CCR2 (9) and monocyte chemoattractant protein-1 (MCP-1) (43), which both substantially impair MP recruitment. Much of this regenerative deficit has been attributed to impairments in neovascularization as a consequence of MP reduction (36); therefore, in addition to facilitating postinjury myogenesis, our treatment of I/R injury with CD11b+Ly-6Csh(F4/80lo) MPs may also hasten vascular recovery. Indeed, this potential vascular improvement may even result from the direct transdifferentiation of CD11b+Ly6Chlo cells into vasculature (23). To address this potential mechanism, future efforts should focus on this vascular aspect, as it has many important implications for vascular disease.

A major importance of the current work extends beyond normal, regeneration competent muscle but additionally towards conditions where skeletal muscle regeneration is impaired, such as aging (19, 20) and metabolic disorders (33). While no thorough flow cytometric studies have been reported for the analysis of MP profiles of aged muscle, elderly human subjects exhibit reduced exercise-induced accumulation of CD11b+ cells (40), and aged rodents have lower leukocyte mobilization and infiltration following myocardial I/R (25, 26). This evidence suggests an age-associated decrease in MPs or their activity may account for the severe regenerative impairment seen in aged muscle and, possibly, other tissues. In fact, the drastic improvement in wound healing following heterochronic MP transfer (young donor; old recipient) (11) suggests MPs may be the systemic factor accounting for the strikingly improved regeneration in heterochronic muscle transplantation (6) and parabiosis (8) models. A recent and interesting line of work from the Koh laboratory suggests regenerative deficits in diabetic models can also be attributed to reduced MP numbers (32, 33). Coupled with the currently reported findings, these lines of evidence suggest that methods to increase MPs in regenerating muscles of elderly or diabetic individuals, two rapidly growing populations of enormous clinical concern, could be of large translational benefit in the near future.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


Macrophages Influence Regeneration • Hammers DW et al. 

1073


