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Myeloid HIFs Are Dispensable for Resolution of Inflammation during Skeletal Muscle Regeneration

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Besides their role in cellular responses to hypoxia, hypoxia-inducible factors (HIFs) are involved in innate immunity and also have anti-inflammatory (M2) functions, such as resolution of inflammation preceding healing. Whereas the first steps of the inflammatory response are associated with proinflammatory (M1) macrophages (MPs), resolution of inflammation is associated with anti-inflammatory MPs exhibiting an M2 phenotype. This M1 to M2 sequence is observed during postinjury muscle regeneration, which provides an excellent paradigm to study the resolution of sterile inflammation. In this study, using in vitro and in vivo approaches in murine models, we demonstrated that deletion of hif1a or hif2a in MPs has no impact on the acquisition of an M2 phenotype. Furthermore, using a multiscale methodological approach, we showed that muscles did not require macrophagic hif1a or hif2a to regenerate. These results indicate that macrophagic HIFs do not play a crucial role during skeletal muscle regeneration induced by sterile tissue damage. The Journal of Immunology, 2015, 194: 000-000.

B esides its role in hypoxia, hypoxia-inducible factor 1 (HIF-1) has recently emerged as a major transcriptional regulator of immunity and inflammation (1). HIF-1α can be stabilized in normoxia by a number of cytokines, growth factors, and other circulating molecules (2). HIF-1 has been shown to be involved in the regulation of macrophage (MP) function in normoxic conditions. Indeed, HIF-1 is induced in phagocytes by bacteria even under normal levels of oxygen (3, 4), and the activation of the HIF-1α pathway supports myeloid cell production of defense factors and improves bactericidal capacity (5). Conversely, some pathogens interfere with HIF activity to improve their survival and proliferation (6, 7). Inactivation of HIF-1α decreases MP motility, invasiveness, and adhesion, whereas hyperaccumulation of HIF-1α causes hyperinflammation in vivo (8). Proinflammatory (M1) stimuli such as LPS or IL-1 induce HIF-1α expression in MPs (9–11). Furthermore, levels of HIF-1α are high in inflammatory conditions such as rheumatoid arthritis (9). In turn, HIF-1α promotes the production of inflammatory cytokines (TNF-α, IL-1, IL-6, and IL-12) (12). These studies showed that HIF-1α is involved in the first step of innate immunity, which is the M1 activation of MPs. However, recent studies have proposed that HIF-1α is also involved in anti-inflammatory (M2) functions. These have been reported during acute inflammation (5, 13, 14) and later stages of the innate immune response. Indeed, Rosenberger et al. (15) showed that HIF-1 stimulates the expression of nitric oxide, which damps the inflammation by attenuating neutrophil transendothelial migration (through the synthesis and activation of adenosine receptor A2BAR on leukocytes). They have proposed a role of HIF-1 according to an endogenous mechanism, which attenuates the acute inflammation during mucosal inflammation. Moreover, HIF-1 is responsible to the selective induction of fibroblast integrin β1 required at time of wound repair in a model of intestinal submucosal hypoxia (16). In accordance with the prohealing properties of HIF, recent studies have shown less fibrotic activity in hepatic fibrotic models in HIF KO mice (17). Inversely, overexpression of HIF-1α induces fibrosis in white adipose tissue (18). These studies have suggested that beyond its classical inflammatory action, HIF-1α (particularly MP HIF-1α) may be implied in the resolution of inflammation preceding the healing. Finally, it has been further confirmed that HIF-2α regulates tumor-associated MP function and is an important immune regulator in these cells by mediating some of the M2 responses (19).

The inflammatory response is a spatially and temporally coordinated process. Although the first steps of the inflammatory response are associated with M1 MPs, which secrete microbialic and M1 compounds, resolution of inflammation is associated with M2 MPs exhibiting alternative and/or anti-inflammatory phenotypes (20). Both in vitro and in vivo studies have demonstrated that...
MPs can undergo dynamic transitions between M1 and M2 states of activation, which is called polarization skewing (20, 21). After an injury, skeletal muscle regenerates ad integrum due to the properties of the main adult muscle stem cells (satellite cells). MPs are essential for skeletal muscle regeneration (22, 23). Effectively, when the recruitment of circulating monocytes is totally prevented during the first 24 h after injury, muscle regeneration is totally inhibited, with the persistence of necrotic fibers until 7 d after injury (22). Moreover, a series of works using mice deficient for either the chemokine receptor CCR2 or its main ligand CCL2/MCP1 shows impaired muscle regeneration characterized by formation of fat, decrease in the diameter of new myofibers, and reduced capillary number (23). This was associated with a dramatic decrease of MP infiltration in muscle (24–26). Other molecular systems involved in cell migration are required for a proper regeneration because they regulate monocyte/MP entry into damaged muscle (23). Urokinase-type plasminogen activator is required for MP chemotaxis and is necessary for MP migration into skeletal muscle to ensure an efficient regeneration (27). Altogether, these studies demonstrate that MPs are indispensable for efficient muscle regeneration. Soon after injury, muscle-associated MPs exhibit an M1 profile and stimulate myogenic precursor proliferation. From 24 to 72 h later, these MPs skew into M2 MPs that stimulate terminal differentiation of myogenic precursor cells, their fusion into myotubes, and the growth of the new regenerating myofibers (22, 23, 28, 29). Thus, postinjury skeletal muscle regeneration provides an excellent paradigm to study events related to the resolution of sterile inflammation. Recently, Scheerer et al. (30) claimed that myeloid HIF-1α is essential for skeletal muscle regeneration, using a model of soft-tissue trauma of the mouse hindlimb. However, according to the relevant markers for evaluation of skeletal muscle regeneration presented in this study, deletion of HIF-1α in MPs induces only a delay in short-term period of skeletal muscle regeneration (30). Furthermore, the resolution of inflammation, which is essential for efficient skeletal muscle regeneration (22), was not investigated. Indeed, authors asserted that MPs showed an M2 phenotype already 24 h after injury based on the analysis of only two M2 markers, which is not sufficient to characterize MP phenotype at this time point (31).

Our general hypothesis was that the macrophagic HIFs play a role during the resolution of inflammation occurring during skeletal muscle regeneration. We hypothesized that HIFs may control the resolution of inflammation through endogenous regulation despite participation in the promotion of the M1 signals associated with the first steps of muscle remodeling. Despite a high degree of sequence homology, HIF-1 and HIF-2 have been described to have nonoverlapping, and even sometimes opposing, roles, particularly regarding inflammation. Therefore, in line with a previous work showing in vitro that HIF-1α and HIF-2α mRNA are differentially expressed in M1 and M2 MPs (32), we investigated whether HIFs play a role at the time of MP phenotype skewing toward M2 profile and later during muscle regeneration and healing. We originally combined in vitro investigations with in vivo multimodal magnetic resonance imaging (MRI), including transverse relaxation time (T2) mapping and diffusion tensor imaging (DTI), which has been recently introduced for noninvasive quantitative monitoring of the different physiological steps associated with skeletal muscle injury and regeneration (33, 34).

Materials and Methods

Mice
Experiments were conducted on adult male animals (8- to 16-wk-old animals) from LysM-CRE+/−:HIF-1αfl/fl (5), LysM-CRE+/−:HIF-2αfl/fl (19), HIF-2αfl/fl, and HIF-1αfl/fl (named wild-type [WT] in this article).

Muscle injury
Muscle injury was first induced by i.m. injection of cardiotoxin (CTX) in tibialis anterior (TA; 12 μl, 50 μl/Ta; Latoxan, Valence, France). Skeletal muscle regeneration after a toxic injury, which causes massive myofiber death, is a useful model for sterile inflammation. This process, which provides homogenous damage in the whole muscle (31), is tightly associated with the infiltration of a great number of monocytes/MPs in the regenerating muscle until the end of the regeneration (22). The earliest stage of muscle injury is characterized by a robust infiltration of Ly6C/G F4/80+/CD11b+ monocytes/MPs (M1 MPs) and Ly6C/G F4/80−/CD11b− neutrophils to the site of the injury (22). Rapidly (i.e., between 1 and 3 d after CTX injury), M1 MPs skew into Ly6C/G F4/80+/CD11b+ MPs (M2 (29)). In other words, muscle M2 MPs are generated from M1 MPs, not from Ly6C/G MCP1+ monocytes/MPs (22, 29). Mice were anesthetized with isoflurane and were injected in the TA muscle. Muscles were harvested for analysis at different time points postinjury (1–3, 7, 21 d). We used ischemia-reperfusion as a second model of skeletal muscle injury. In brief, hindlimbs of LysM-CRE+/−:HIF-1αfl/fl, LysM-CRE+/−:HIF-2αfl/fl, and WT mice have been submitted to ischemia during 2 h (35) and TA muscles were sampled 21 d after injury.

Detection of hypoxia
In brief, 2, 4, and 8 d after CTX lesion (12 μl, 50 μl/Ta; Latoxan, Valence, France), mice were injected with Hypoxyprobe (Hypoxyprobe-1 [HP-1], 60 mg/kg), and TA muscles were collected. Single-cell suspensions were obtained from muscles by enzymatic digestion (collagenase B [Roche Diagnostics] 10 mg/ml and Dispase II [Dutscher Dominique] 2.4 U/ml) for cytometry analysis. Immune cells were isolated by CD45 Ab (anti-mouse-CD45, BD Biosciences) and stained with anti-CD45 PerCP-Cyanine5.5; eBioscience). Pimonidazole forms adducts with thiol-containing proteins at pO2 ≤ 10 mm Hg. Adducts were detected with primary mouse anti–HP-1, and specific staining (i.e., “hypoxia status”) was evaluated with an anti–HP-1/PE Ab (Mouse Dylight549-MAb; Hypoxyprobe).

Magnetic resonance imaging
MRI measurements were conducted on LysM-CRE+/−:HIF-1αfl/fl (n = 7), WT (n = 8), and LysM-CRE+/−:HIF-2αfl/fl in mice before injury (1 D1), 2, 3, 7, and 21 d after CTX injury (D21) (27). Investigations were performed at 11.75 T on a vertical Bruker Avance 500-MHz/89-mm-wide-bore imager (Bruker, Ettlingen, Germany), equipped with high-performance actively shielded gradients (1 T/m maximum gradient strength, 9 kT/m maximum slew rate) and interfaced with Paravision 5.1. A transmit/receive volume radiofrequency coil (birdcage, diameter Ø = 3 cm, homogenous length L = 5 cm; Micro 2.5 Probe; Bruker) was used for image acquisition.

Animal preparation. Mice were initially anesthetized in an induction chamber using 2% isoflurane. Each anesthetized mouse was placed in a home-built cradle, which has been specially designed for the strictly noninvasive high-field MRI investigation of the left hindlimb muscles (36). Mice lay supine in the cradle with the head up and were maintained using a teeth holder and tape at the pelvis level. The left foot was placed on a pedal and the leg was firmly immobilized by a small piece of Teflon placed above the knee joint. Then, the cradle was inserted into the 30-mm coil with the hindlimb muscles positioned at the magnetic center. Throughout a typical experiment, the anesthesia was maintained under spontaneous respiration (room air flow ~270 m3/min, regular breathing ~90 breaths/min) using 1.7% isoflurane (vaporizer Univeron 400 anesthesia unit; Univeron, Zejua, Malta). The respiration rate was controlled throughout the experiment using a compatible monitoring and gating system (SA Instruments, Stony Brook, NY).

MRI sequences. Seven contiguous axial imaging slices (thickness = 1 mm) were selected across the left lower hindlimb based on a set of scout images. T2-weighted images were obtained using a 4-shot spin echo-echo planar imaging sequence with the following parameters: field of view, 2,0 × 2,0 cm2; matrix, 128 × 128; slice thickness, 1 mm; number of excitation, 15; repetition time, 350 ms; echo times (TEs), 8–48 ms; acquisition time, ~20–30 min. DTI experiments were performed with a Stejskal-Tanner preparation and a segmented (4 shots) spin echo-echo planar imaging readout technique. The following acquisition parameters were used: diffusion gradient duration (Δ) = 10 ms; b values = (0 and 450) sec/mm2 and 12 diffusion-encoding directions. Imaging parameters were: bandwidth = 400 kHz; TE = 30 ms; b = 0; number of excitation 15; field of view, 2,0 × 2,0 cm2; matrix size = 128 × 128. The total acquisition time was ~45 min. For both T2-weighted and DTI acquisitions, a fat suppression module was used.
Data processing. T2 maps were generated by fitting on a pixel-by-pixel basis the logarithm of the data to the following linear equation: ln(S(TE)) = ln(S0) − TE/T2, where S(TE) is the signal at time = TE and S0 is the equilibrium magnetization. The DTI reconstruction was performed with the manufacturer software (ParaVision 5; Bruker), and the corresponding data were processed using an in-house program developed with Interactive Data Language (Research Systems, Boulder, CO). DTI metrics, including the three eigenvalues (longitudinal diffusivity, first eigenvalue (λ1), axial diffusivity, second eigenvalue (λ2), and axial diffusivity, third eigenvalue (λ3)), fractional anisotropy (FA), and apparent diffusion coefficient (ADC) were obtained. For both T2 maps and DTI metrics, a region of interest was selected around the injured TA muscle. These measurements were done and averaged for the three slices with the largest sections.

Histological analysis
TA muscles were removed, snap-frozen in nitrogen-chilled isopentane, and kept at −80°C until use. For H&E staining, 8-μm-thick cryosections were prepared.

Isolation of leukocytes and MPs from muscle
Fascia of the TA muscles was removed. The muscles were minced and digested in RPMI 1640 medium containing collagenase B 0.2% (Roche Diagnostics GmbH) at 37°C for 1 h. The resulting homogenate was filtered and cells were counted. CD45− cells were isolated using magnetic sorting (Miltenyi Biotec) and stained with allophycocyanin-conjugated anti-Gr1 (Ly6C/G; eBioscience) and PE-conjugated anti-F4/80 Abs (eBioscience). Cells were analyzed using a FC-500 flow cytometer (Beckman Coulter). Analysis was performed with CXP Cytometer.

MP cell culture
MPs were obtained from bone marrow (BM) precursor cells as previously described (31). In brief, total BM was obtained from mice by flushing femurs and tibiae BM with DMEM. Cells were cultured in DMEM medium containing 20% FBS and 30% conditioned medium of L929 cell line (enriched in CSF-1) and prepared for 7 d as described previously (37). Purity of differentiated MPs was estimated by flow cytometry after F4/80–PECy7 labeling (eBioscience). MPs were seeded at 50,000 cell/cm² for all experiments and were activated with cytokines to obtain various activation states: IFN-γ (50 ng/ml), IL-4 (10 ng/ml), IL-10 (10 ng/ml) to obtain M1, M2a, and M2c MPs, respectively, in DMEM containing 10% FBS medium for 3 d.

Phagocytosis assay
Activated MPs were incubated in culture media (previously described) with 20 × 10⁶ GFP-conjugated beads (1-μm diameter; Invitrogen) during 2 h. After three PBS washes, MPs nuclei were labeled with Hoechst (Fluka). H&E-stained muscle sections were recorded with an E800 microscope (Nikon) at 20× magnification and averaged for the three slices with the largest sections.

Quantitative RT-PCR
Quantitative RT-PCRs were performed as described previously (31). In brief, total RNAs were extracted using the RNAasy Mini Kit (Qiagen). One microgram of total RNA was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen). Quantitative PCR was carried out on a Real-Time PCR System (LightCycler 480; Roche Applied Science). Reaction mixtures had a final volume of 20 μl containing 1× SYBR Green I Master (LightCycler 480), and 0.5 μM of each primer. After initial denaturation, amplification was performed at 95°C (10 s), 60°C (5 s), 72°C for 3 d.

Histological analysis
Cross-sectional area (CSA; expressed in μm²) of regenerating myofibers was quantified using the MetaMorph software (Molecular Devices).

Statistical analyses
All experiments were performed using at least three independent primary cultures or at least three animals for in vivo analyses. Statistical analyses of MRI were performed with the Statistica software version 9 (StatSoft, Tulsa, OK). Normality was checked using a Kolmogorov–Smirnov test. Two-factor (group × time) ANOVAs with repeated measures on time were used to compare T2 values and DTI metrics. When a main effect or a significant interaction was found, Newman–Keuls post hoc analysis was used. Statistical analyses of in vivo and in vitro studies were performed with Student t test. Significance was accepted when p < 0.05.

Results
Deletion of macrophagic HIFs does not affect skeletal muscle inflammation and regeneration
To determine whether the tissue injured with CTX was hypoxic, we used pimonidazole labeling (30). Eleven, 67, and 55% of CD45− cells extracted from regenerating WT muscle were hypoxic 2, 4, and 8 d after injection of CTX in the TA muscle, respectively (Supplemental Fig. 1). Multimodal MRI investigations, including T2 mapping and DTI, were performed to investigate in vivo the effects of myeloid HIFs in muscle damage and regeneration. In brief, recent studies demonstrated that T2 changes can be used as an index of muscle inflammation (33, 34) even though other biological phenomena (edema, necrosis) can also affect T2 values (38). In addition, diffusion-weighted MRI provides information about the water molecules self-diffusion, which is restricted by physical barriers such as membranes, cytoskeleton, mitochondria, and sarcoplasmic reticulum, thereby leading to anisotropic diffusion. Diffusion indices including the mean ADC, eigenvalues (λ1, λ2, and λ3), and diffusion anisotropy (FA) can be derived from DTI. λ1 represents diffusive transport along the long axis of a muscle fiber (39), whereas λ2 and λ3 have been proposed to reflect the long and short cross-sectional axes of the muscle fibers (40), respectively. A significant time effect (p < 0.001) was observed for all the recorded variables indicating that both T2 values and DTI metrics were significantly modified as a result of CTX injection for all three groups. Indeed, T2 values were significantly elevated at D1 (+101 ± 12%), peaked between D2 and D3 (+139 ± 15 and +131 ± 15%, respectively), remained elevated at D7 (+36 ± 9%), and recovered at D21 (Fig. 1A).

The course of DTI changes was clearly different from that highlighted by the T2 values. λ3, λc, and ADC values peaked at D1 (+37 ± 6, +56 ± 9, and +32 ± 6%, respectively) and remained elevated at D2 (+32 ± 8, +52 ± 12, and +29 ± 8%, respectively) and D3 (+9 ± 9, +20 ± 12, and +8 ± 8%, respectively) as compared with values obtained at D0 (Fig. 1B and Table 1). These parameters were further reduced at D7 (−12 ± 4, −13 ± 5, and −9 ± 5%, respectively) and returned to the baseline values at D21. The FA evolved in an opposite way with a significant reduction at D1 (−52 ± 6%), D2 (−53 ± 5%), and D3 (−27 ± 10%), an increase at D7 (−18 ± 8%), and a full recovery at D21 (Table 1). λ1 only increased at D1 (+15 ± 6%) and D2 (+12 ± 7%), and returned to the baseline values at D3 (Table 1).

Importantly, it should be noted that both DTI and T2 changes were similar for WT, LysM-CRE−/−:HIF-1a−/−, and LysM-CRE−/−:Mice, indicating that macrophagic HIFs are not involved in the CTX-induced MRI changes.

HIF-1α−/− MPs acquire M2 phenotype in vivo
To determine the specific contribution of MP-derived HIF-1α−/−, we performed loss-of-function experiments by using LysM-CRE−/−:
FIGURE 1. Multimodal MRI investigations of the effects of myeloid HIFα1 and HIFα2 in muscle regeneration. (A) Typical representative axial T2 maps from WT and LysM-CRE<sup>−/−</sup>:HIF-1α<sup>fl/fl</sup> mouse hindlimb muscles (top panels) and quantitative analysis of T2 values (bottom panel) obtained from WT (n = 6), LysM-CRE<sup>−/−;HIF-1α<sup>fl/fl</sup> (n = 7), and LysM-CRE<sup>−/−;HIF-2α<sup>fl/fl</sup> (n = 3) mouse TA muscles before (D0) and 1 (D1), 2 (D2), 3 (D3), 7 (D7), and 21 d (D21) after injury. Values are presented as mean ± SD. Significantly different from values recorded at D0: ***p < 0.001.

(B) Typical representative axial maps of the third eigenvalue (λ<sub>3</sub>) from WT and LysM-CRE<sup>−/−;HIF-1α<sup>fl/fl</sup> mouse hindlimb muscles (top panels) and quantitative analysis of λ3 values (bottom panel) obtained from WT (n = 6), LysM-CRE<sup>−/−;HIF-1α<sup>fl/fl</sup> (n = 7), and LysM-CRE<sup>−/−;HIF-2α<sup>fl/fl</sup> (n = 3) mouse TA muscles at the same respective time points than those mentioned in (A). Values are presented as mean ± SD. Significantly different from values recorded at D0: ****p < 0.0001.
HIF-1αfl/fl (5). The capacity of HIF-1α−/− MPs to switch their phenotype was assessed in vivo during normal skeletal muscle regeneration. Thanks to flow cytometry analysis coupled with Ly6C and F4/80 labeling, cell population analysis was performed among CD45+ cells extracted from regenerating WT and HIF-1α−/− muscles at different time points (Fig. 2A). First, the number of infiltrating neutrophils and MPs was similar in WT and HIF-1α−/− muscles 1 d after injury (Fig. 2A), excluding a difference of leukodiapedesis of WT and HIF-1α−/− myofibers. Second, the distribution of the two MPs subsets did not differ between WT and HIF-1α−/− regenerating muscles during the MP skewing period in this model of sterile inflammation (i.e., D1, D2, and D3 after CTX injury) (31), showing that LysM-CRE+/−:HIF-1αfl/fl mice had an unaltered M2 MP development (Fig. 2B). Thus, these results show that in absence of HIF-1α, M1 MPs were able to skew in M2 MPs at the time of resolution of inflammation.

**HIF-1α−/− MPs acquire M2 phenotype in vitro**

Next, BM-derived MPs (BMDMs) from WT and LysM-CRE+/−; HIF-1α−/−; and WT and LysM-CRE+/−; HIF-2α−/− mice before (i.e., D0) and 1 (D1), 2 (D2), 3 (D3), 7 (D7), and 21 d (D21) after injury

### Table I. The first two eigenvalues (λ1 and λ2), ADC, and FA obtained from WT, LysM-CRE+/−; HIF-1α−/−, and LysM-CRE+/−; HIF-2α−/− mice before

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<tr>
<th></th>
<th>D0</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D7</th>
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<tr>
<td>λ1 (10^{-3} mm²/sec)</td>
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<tr>
<td>LysM-CRE+/−; HIF-1α−/−</td>
<td>1.80 ± 0.07</td>
<td>2.08 ± 0.03***</td>
<td>2.05 ± 0.04***</td>
<td>1.80 ± 0.10</td>
<td>1.75 ± 0.05</td>
<td>1.72 ± 0.06</td>
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<td>WT</td>
<td>1.77 ± 0.04</td>
<td>2.09 ± 0.07***</td>
<td>2.04 ± 0.05***</td>
<td>1.84 ± 0.05</td>
<td>1.72 ± 0.10</td>
<td>1.76 ± 0.04</td>
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<tr>
<td>LysM-CRE+/−; HIF-2α−/−</td>
<td>1.79 ± 0.05</td>
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<td>1.86 ± 0.09***</td>
<td>1.73 ± 0.09</td>
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<td>LysM-CRE+/−; HIF-1α−/−</td>
<td>1.33 ± 0.06</td>
<td>1.84 ± 0.03***</td>
<td>1.77 ± 0.05***</td>
<td>1.41 ± 0.10***</td>
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<td>LysM-CRE+/−; HIF-1α−/−</td>
<td>1.39 ± 0.05</td>
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<td>WT</td>
<td>1.36 ± 0.03</td>
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<td>1.42 ± 0.12***</td>
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<td>LysM-CRE+/−; HIF-1α−/−</td>
<td>0.28 ± 0.02</td>
<td>0.13 ± 0.01***</td>
<td>0.13 ± 0.01***</td>
<td>0.22 ± 0.03***</td>
<td>0.33 ± 0.02***</td>
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<td>WT</td>
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<td>LysM-CRE+/−; HIF-2α−/−</td>
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<td>0.16 ± 0.01***</td>
<td>0.21 ± 0.04***</td>
<td>0.34 ± 0.02***</td>
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***p < 0.001, significantly different from D0.

HIF-1α and HIF-2α display unique and sometimes opposing activities in regulating cellular functions in both physiological and pathophysiological context (19, 42, 43). As we did for HIF-1α−/− MPs, loss-of-function experiments were performed by using LysM-CRE+/−; HIF-2α−/− (19). Thus, cell population analysis was performed among CD45+ cells extracted from regenerating WT and HIF-2α−/− muscles at different time points (Fig. 4A) to determine the capacity of HIF-2α−/− MPs to switch their phenotype in vivo. The number of infiltrating neutrophils and MPs was similar in WT and HIF-2α−/− muscles 1 d after injury (Fig. 4A). Moreover, the distribution of the two MPs subsets did not differ between WT and HIF-2α−/− regenerating muscles during the MP skewing period, suggesting that the acquisition of M2 phenotype by MPs was unaffected in LysM-CRE+/−; HIF-2α−/− animals (Fig. 4B). Thus, these results show that in absence of HIF-2α, M1 MPs were able to skew into M2 MPs at the time of resolution of inflammation.

**Macrophagic HIFs are not required for proper skeletal muscle regeneration**

MPs are necessary for skeletal muscle regeneration, as inhibiting monocyte/MP infiltration impairs muscle regeneration (22). Twenty-one days after CTX injury, visual histological examination has shown the same pattern of muscle regeneration in mice deficient for HIF-1α−/− or for HIF-2α−/− in myofibers, as compared with WT mice (Fig. 5A). Furthermore, CSA of the new myofibers (an indicator of skeletal muscle regeneration efficiency) in WT, LysM-CRE+/−; HIF-1α−/−, and LysM-CRE+/−; HIF-2α−/− mice was similar at D21 (Fig. 5B), which is in accordance with our MRI findings. Finally, distribution of myofiber CSA of WT mice paralleled distribution of myofiber CSA of LysM-CRE+/−; HIF-1α−/− and LysM-CRE+/−; HIF-2α−/− mice (Fig. 5C). Finally, ischemia-reperfusion was used as another model of sterile skeletal muscle injury to clarify the role of macrophagic HIFs in skeletal muscle regeneration. We showed that CSAs of the new myofibers were similar among WT, LysM-CRE+/−; HIF-1α−/−, and LysM-CRE+/−; HIF-2α−/− mice at day 21 (Fig. 5D), considered as a late time point of skeletal muscle regeneration. Therefore, deletion of HIF-1α or HIF-2α in myeloid cells did not alter skeletal muscle
regeneration, at least in two different models of sterile injury (CTX and ischemia-reperfusion).

Discussion

The results of this study show that the absence of HIFs in MPs has no impact on the resolution of inflammation in two sterile models of skeletal muscle regeneration. Based on the earlier findings, we conclude that the same types of MPs were generated in the same number from an identical cell infiltrate in WT, LysM-CRE\textsuperscript{+/+}:HIF-1\textalpha\textsuperscript{fl/fl}, and LysM-CRE\textsuperscript{+/+}:HIF-2\textalpha\textsuperscript{fl/fl} muscles during the early stages of regeneration. In other words, the infiltration, differentiation, and kinetics of M1 and M2 muscle MPs were found to be identical in both examined mice strains. Moreover, HIF-1\textalpha\textsuperscript{−/−} and HIF-2\textalpha\textsuperscript{−/−} MPs showed no functional alterations in regenerating muscle. Therefore, although HIF-1\textalpha has been reported to influence MP polarization in various tissues (44–46), HIF-deficient MPs are able to acquire M2 phenotype and functions in sterile inflammation during skeletal muscle regeneration. Phagocytosis of tissue debris is a crucial function of MPs in the resolution of inflammation in skeletal muscle (22, 28). It has been shown previously that HIF-1\textalpha has an important role in phagocytic function of MPs in various experimental models (47, 48) that seems not to be the case in the present in vitro experiments.

Besides its role in hypoxia and in inflammation under normoxic conditions, HIF-1 is involved in skeletal muscle homeostasis and physiology (43, 49–52). Recently, it has been claimed that myeloid HIF-1\textalpha was essential for skeletal muscle regeneration after a soft trauma (30). Our results, showing no alteration of skeletal muscle regeneration in mice depleted for myeloid HIFs in two different models of sterile injury dispute this assertion. Discrep-

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** HIF-1\textalpha\textsuperscript{−/−} macrophages acquire M2 phenotype in vivo. CD45\textsuperscript{+} cells were isolated from muscle using magnetic sorting and stained with allophycocyanin-conjugated anti-Gr1 (Ly6C/G) and PE-conjugated anti-F4/80 Abs. (A) Number of MPs (Ly-6C/G\textsuperscript{+}F4/80\textsuperscript{+} and Ly-6C/G\textsuperscript{−}F4/80\textsuperscript{+} cells) and neutrophils (Ly-6C/G\textsuperscript{−}F4/80\textsuperscript{+} cells) was evaluated per milligram of muscle 1 d after the injury from WT and LysM-CRE\textsuperscript{+/+}:HIF-1\textalpha\textsuperscript{−/−} mouse. (B) The presence of M1 (Ly-6C/G\textsuperscript{−}F4/80\textsuperscript{+}) and M2 (Ly-6C/G\textsuperscript{−}F4/80\textsuperscript{+}) MPs subsets was expressed as a percentage of total MPs (F4/80\textsuperscript{+} cells) in muscle at days 1, 2, and 3 after injury. Results are means ± SEM. All experiments were performed using at least three animals.
FIGURE 3. HIF-1α−/− macrophages acquire M2 phenotype in vitro. (A and B) Analysis of M1 markers, M2 markers, and hif1a and hif2a gene expression in WT and HIF-1α−/− MPs after in vitro polarization. MPs were obtained from BM precursor cells (BMDMs). BMDMs were activated with cytokines to obtain various activation states: IFN-γ, IL-4, IL-10 to obtain M1, M2a (alternative), and M2c (anti-inflammatory) MPs, respectively, for 3 d. (C) WT and HIF-1α−/− MPs were polarized as in (A) and (B), incubated in culture media with 20 × 10⁶ GFP-conjugated beads during 2 h. After three PBS washing, MPs nuclei were labeled with Hoechst. The number of phagocytic MPs was expressed as a percentage of total cells. All experiments were performed using at least three independent primary cultures. Significantly different from WT: ***p < 0.001, *p < 0.05. Significantly different from M1: ###p < 0.001, ##p < 0.01, #p < 0.05. Significantly different from M2c: $p < 0.05.
ancies between the two studies might be related to the model of skeletal muscle injury and the techniques used to quantify myeloid cells (immunohistology versus flow cytometry) and myofiber parameters (different number of myofibers taken into consideration for CSA calculation). Nevertheless, several data appearing in Scheerer et al.’s (30) study strongly dampen the title assertion of “an essential” role of macrophagic HIFs in skeletal muscle regeneration. First, the analysis of CSA showed a significant difference between WT and HIF-1α−/− mice only at day 7 after the injury, whereas no significant difference was observed at 10 d. This is characteristic of a transient delay at the onset of regeneration process in this model of injury, discordant with an “essential” function of HIF in myeloid cells. Accordingly, the variations observed in myeloid cells between the two mice strains were observed only between days 2 and 7. To assess a definitive role of HIF1 in myeloid cells, further analysis at later time points of regeneration (i.e., 3 wk) is required (31). Moreover, the study by Scheerer et al. (30) reported no alteration of myeloid HIF-1α deletion on MP infiltration or on MP polarization, which is in agreement with the data presented in this article in two models of sterile inflammation. Scheerer et al.’s (30) study reported a transient delay in the number of F4/80+ cells, suggesting a defect in the proliferation of some MPs in the HIF1αKO strain. Because we did not observe such a delay in the two models we used and knowing that only M2 Ly6Cneg MPs are capable of proliferation (22), further investigations are required to understand which environmental changes the myotraumatic model is inducing on these cells. In conclusion, taking in consideration the fact that the pre-

FIGURE 4. HIF-2α−/− MPs acquire M2 phenotype in vivo. CD45+ cells were isolated from muscle using magnetic sorting and stained with allophycocyanin-conjugated anti-Gr1 (Ly6C/G) and PE-conjugated anti-F4/80 Abs. Cells were analyzed using a flow cytometer. (A) Number of MPs (Ly-6C/G+F4/80+ and Ly-6C/G+F4/80− cells) and neutrophils (Ly-6C/G+F4/80− cells) was evaluated per milligram of muscle 1 d after the injury from WT and LysM-CRE−/+;HIF-2α−/− mouse. (B) The presence of M1 (Ly-6C/G+F4/80+) and M2 (Ly-6C/G+F4/80−) MPs subsets was expressed as a percentage of total MPs (F4/80+ cells) in muscle at days 1 and 2 after injury. Results are means ± SEM. All experiments were performed using at least three independent primary cultures or at least three animals.
vious work done by Scheerer et al. (30) was carried out in a different model of muscle injury, complementary investigations are needed to determine the discrepancies between the different models of injuries.

Multimodal MRI investigations were performed to assess whether myeloid HIFs play a role during muscle regeneration. We found that the time course of both T2 and DTI changes resulting from CTX injection was similar among WT, LysM-CRE\(^{+/+}\):HIF-1\(\alpha^{+/+}\), and LysM-CRE\(^{+/+}\):HIF-2\(\alpha^{+/+}\) mice, indicating that both inflammation and muscle regeneration were not delayed in myeloid HIF-1\(\alpha\) and HIF-2\(\alpha\) knockout mice. These findings clearly showed that myeloid HIFs are not essential for skeletal muscle regeneration. Although the CTX-induced MRI changes observed in this study are in line with those recently reported in the literature (33), we further demonstrated that the increase of FA occurring 7 d after injury (33) was actually related to the decreased \(\lambda_2\) and \(\lambda_3\) values, thereby reflecting the well-known presence of small-diameter regenerating muscle fibers (22). Although it has been suggested that T2 variations represent a marker of muscle inflammation (33, 34), it should be pointed out that a linear relationship of T2 and intracellular volume has been previously reported (53). On that basis and considering the reduced second and

**FIGURE 5.** Deletion of myeloid HIFs did not affect skeletal muscle regeneration resulting from an acute injury. (A) H&E staining of regenerating muscle in WT, LysM-CRE\(^{+/+}\):HIF-1\(\alpha^{+/+}\), and LysM-CRE\(^{+/+}\):HIF-2\(\alpha^{+/+}\) mice. TA muscles were injured with CTX and analyzed 21 d postinjury. TA muscles were removed, snap-frozen in nitrogen-chilled isopentane, and kept at \(-80^\circ\)C until use. Scale bar, 50 \(\mu\)m. Mean (B) and distribution (C) of myofiber CSA. Significantly different between WT and LysM-HIF-1\(\alpha^{-/-}\) mice, *\(p < 0.05\). Significantly different between WT and LysM-HIF-2\(\alpha^{-/-}\) mice, $\hat{p} < 0.05$. All experiments were performed using at least three animals. (D) Macrophagic HIFs are not required for proper skeletal muscle regeneration after injury due to ischemia-reperfusion. CSA of the new myofibers of TA is calculated 21 d after the injury in LysM-CRE\(^{+/+}\):HIF-1\(\alpha^{+/+}\) LysM-CRE\(^{+/+}\):HIF-2\(\alpha^{+/+}\) and WT mice. All experiments were performed using at least three animals. ***\(p < 0.001\), significantly different from Nonischemic muscles.
third eigenvectors at day 7, the associated higher T2 values might actually be related to the expansion of the extracellular component, including, for instance, a widened interstitial space (54, 55), rather than to intracellular changes. Overall, the combination and/or comparison of multimodal MRI with the recently introduced bioluminescence imaging technique (56) would be of interest for monitoring noninvasively and quantitatively the pathophysiological events, such as chronic inflammation, which are associated with skeletal muscle diseases.

In conclusion, our multiscale methodological approach clearly showed that myeloid HIFs are not essential for skeletal muscle regeneration after a sterile injury in two different models.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Muscle injured with cardiotoxin is hypoxic.
Percentage of hypoxyprobe-positive CD45+ cells extracted from regenerating WT muscle after injection of cardiotoxin in the Tibialis Anterior muscle is quantified with flow cytometry and Hypoxyprobe. Results are represented as means ± SEM. All experiments were performed using at least 3 animals. Significantly different from Day 2: ** P< 0.05.