ROLE OF MACROPHAGIC HIFs IN THE RESOLUTION OF INFLAMMATION DURING SKELETAL MUSCLE REGENERATION: A COMBINED IN VITRO, EX VIVO AND MULTIMODAL MRI INVESTIGATION

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Target audience: physiologists; physiopathologists, experts in the field of skeletal muscle function

Purpose: The critical role of macrophages (MPs) and the corresponding inflammatory response associated to the muscle repair process has been recently highlighted[1]. Several studies have demonstrated that HIF-1α could play a key role in inflammation[2,3]. It has been recently claimed that myeloid HIF-1α was essential for skeletal muscle regeneration after a soft trauma[4]. However, the deletion of HIF-1α in MPs actually induced only a short delay at the onset of the repair process. Furthermore, the resolution of inflammation, which is indispensable for skeletal muscle regeneration[5], was not investigated. In the present study, we aimed at carefully addressing the role of myeloid HIFs in the resolution of inflammation during skeletal muscle regeneration on the basis of *in vitro* and *ex vivo* experiments combined to *in vivo* multimodal magnetic resonance imaging (MRI), including T_2 mapping and diffusion tensor imaging (DTI)[6].

Methods: ***Mice***: Experiments were conducted in adult male animals (8-16 week-old) from LysM-CRE:HIF-1$\alpha^{fl/fl}$ (LysM-HIF-1$\alpha^{-/-}$; n=7)[3], LysM-CRE:HIF-2$\alpha^{fl/fl}$ (LysM-HIF-2$\alpha^{-/-}$; n=3)[7], HIF-2$\alpha^{fl/fl}$ and HIF-1$\alpha^{fl/fl}$ (WT; n=6). ***Muscle injury***: Acute muscle injury was induced by the *i.m.* injection of cardiotoxin (CTX ; 50 µl) in the *tibialis anterior* (TA) muscle. ***MRI***: Measurements were performed before (D0) and 1 (D1), 2 (D2), 3 (D3), 7 (D7) and 21 days (D21) after the CTX injection. Investigations were performed at 11.75 T on a vertical Bruker Avance 500 MHz/89mm wide-bore imager (Bruker, Ettlingen, Germany)[8]. *T_2 mapping*: a 4-shot SE-EPI sequence was used with the following parameters: FOV: 2.0 × 2.0 cm², matrix: 128 × 128, slice thickness: 1 mm, 15NEX, TR: 3500 ms, TEs ranging from 8 to 48 ms. *DTI parameters*: a segmented (4 shots) SE-EPI readout sequence was used: b-values = (0 and 450) sec/mm^2; 12 diffusion-encoding directions; δ/Δ =5/10 ms; BW = 400 kHz; TR/TE = 3500/20 ms. *Data processing:* T_2 maps were generated and the DTI reconstruction was performed with the manufacturer software (Bruker; Paravision 5). DTI metrics including diffusivities (λ_1, λ_2 and λ_3), apparent diffusion coefficient (ADC) and fractional anisotropy (FA) were calculated[8]. For both T_2 maps and DTI metrics, a region of interest was selected in the TA muscle. ***Isolation of leukocytes and macrophages from muscle***: TA muscles were minced and digested in RPMI medium containing collagenase B 0.2% (Roche Diagnostics GmbH) at 37°C for 1h. The resulting homogenate was filtered and cells were counted. CD45$^+$ cells were isolated using magnetic sorting (Miltenyi Biotec) and stained with APC-conjugated anti-Gr1 (Ly6C/G) and PE-conjugated anti-F4/80 antibodies (eBioscience). Cells were analyzed using a FC-500 flow cytometer (Beckman Coulter). Analysis was performed with CXPTM Cytometer[5]. ***Histological analysis***: TA muscles were removed, snap frozen in nitrogen-chilled isopentane and kept at –80°C until use. 8 µm-thick cryosections were prepared for hematoxylin-eosin staining. Myofiber cross-sectional area (CSA) analysis was performed at D21 as recently described[5]. ***Statistical analyses.*** Two-factor analysis of variance with repeated measures on time and Newman–Keuls post-hoc analysis were used to compare MRI variables while t-tests were used for other analyses.

Results:

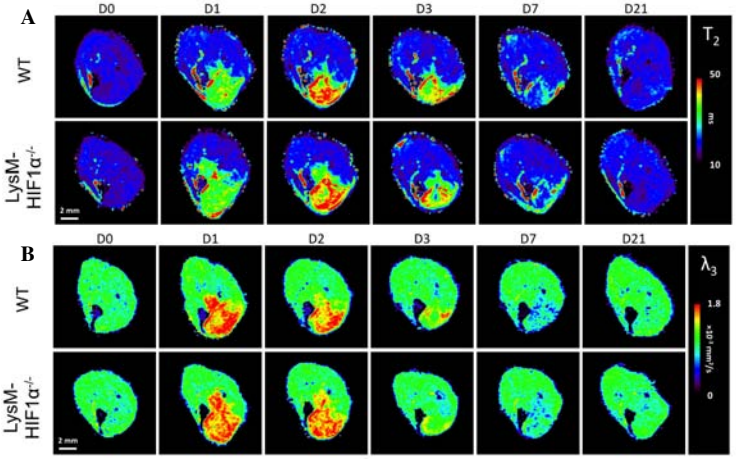

Figure 1. Typical representative axial T_2 maps (A) and axial maps of the third eigenvalue (λ_3) (B) obtained from WT and LysM-HIF-1$\alpha^{-/-}$ muscles at the respective time points.

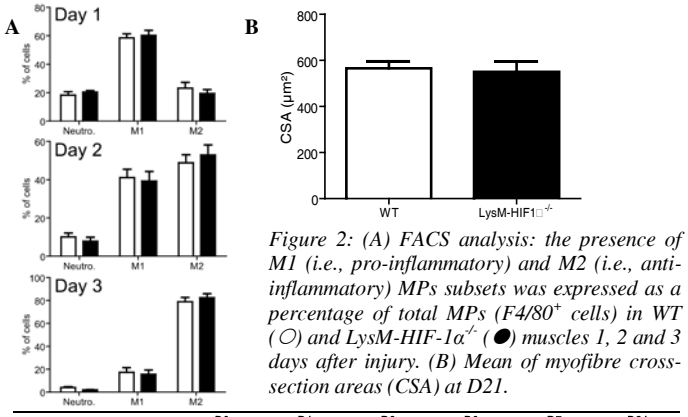

Figure 2: (A) FACS analysis: the presence of M1 (i.e., pro-inflammatory) and M2 (i.e., anti-inflammatory) MPs subsets was expressed as a percentage of total MPs (F4/80$^+$ cells) in WT (○) and LysM-HIF-1$\alpha^{-/-}$ (●) muscles 1, 2 and 3 days after injury. (B) Mean of myofibre cross-section areas (CSA) at D21.

		D0	D1	D2	D3	D7	D21
λ_1 (×10^{-3} mm²/sec)	WT	1.77 ± 0.04	2.09 ± 0.07***	2.04 ± 0.05***	1.84 ± 0.05	1.72 ± 0.10	1.76 ± 0.04
	LysM-HIF1α-/-	1.80 ± 0.07	2.08 ± 0.03***	2.05 ± 0.04***	1.80 ± 0.10	1.75 ± 0.05	1.72 ± 0.06
λ_2 (×10^{-3} mm²/sec)	WT	1.30 ± 0.02	1.82 ± 0.04***	1.76 ± 0.06***	1.47 ± 0.06***	1.17 ± 0.04***	1.31 ± 0.06
	LysM-HIF1α-/-	1.33 ± 0.06	1.84 ± 0.03***	1.77 ± 0.05***	1.41 ± 0.10***	1.18 ± 0.06***	1.30 ± 0.03
ADC (×10^{-3} mm²/sec)	WT	1.36 ± 0.03	1.84 ± 0.03***	1.80 ± 0.05***	1.53 ± 0.06***	1.25 ± 0.06***	1.35 ± 0.04
	LysM-HIF1α-/-	1.39 ± 0.05	1.83 ± 0.03***	1.81 ± 0.04***	1.46 ± 0.10***	1.28 ± 0.05***	1.34 ± 0.03
FA	WT	0.28 ± 0.01	0.14 ± 0.02***	0.13 ± 0.01***	0.19 ± 0.01***	0.34 ± 0.02***	0.29 ± 0.02
	LysM-HIF1α-/-	0.28 ± 0.02	0.13 ± 0.01***	0.13 ± 0.01***	0.22 ± 0.03***	0.33 ± 0.02***	0.27 ± 0.03

Table 1: The first two eigenvalues (λ_1 and λ_2), ADC and FA values obtained from WT and LysM-HIF-1$\alpha^{-/-}$ muscles at the respective time points. Significantly different from values recorded at D0: $^{***}P< 0.001$.

As illustrated in Figure 1, CTX injection largely modified both the DTI metrics and T_2 values, but these changes were strictly similar in LysM-HIF-1 $\alpha^{-/-}$ and WT groups. λ_2, λ_3 and ADC values peaked at D1 and remained elevated at D2 and D3 as compared to baseline values (Table 1 & Figure 1). These parameters were further reduced at D7 and returned to the baseline values at D21. As compared to λ_2 and λ_3 changes, FA evolved in an opposite direction (Table 1). We demonstrated that the recently reported CTX-induced increase of FA at D7[6] was actually related to the decreased λ_2 and λ_3 values, reflecting the well-known presence of small-diameter regenerating muscle fibres[1]. The time course of CTX-induced T_2 changes was clearly different from that highlighted by the DTI metrics. T_2 values were significantly elevated at D1, peaked between D2 and D3, remained elevated at D7 and fully recovered at D21 (Figure 1). While it has been suggested that T_2 variations represent a marker of muscle inflammation[6], T_2 changes may also be related to edema and/or necrosis, as illustrated by the early increase in T_2 values (*i.e.*, at D1). Figure 2A shows that the distribution of the two MPs subsets did not differ between the two groups during the MPs skewing period[5], indicating that M1 MPs were able to skew in M2 MPs at the time of resolution of inflammation even in the absence of HIF-1α. In agreement with the full recovery of MRI parameters, myofibre CSA was similar between the two groups at D21 (Figure 2B).

Discussion: Our multiscale methodological approach clearly demonstrated that neither HIF-1α nor HIF-2α (data not shown) are involved in the muscle regeneration process resulting from an acute injury. The discrepancies between our findings and those recently reported[6] might be related, at least in part, to the model of muscle injury (sterile injury *vs.* soft trauma), indicating that the lack of HIFs expression in myeloid cells might lead, at worst, to a short delay at the onset of the repair process. Overall, we concluded that myeloid HIFs are not essential for skeletal muscle regeneration and that multimodal MRI is a suitable diagnostic tool for monitoring skeletal muscle injury and repair.

References: [1]Arnold *et al. J. Exp. Med.* 2007; [2]Cramer & Johnson *Cell* 2003; [3]Peyssonnaux *et al. J. Clin. Invest.* 2005; [4]Scheerer *et al. J. Immunol.* 2013; [5] Mounier *et al. Cell Metab* 2013; [6]Esposito *et al. PloS One* 2013; [7]Imtiyaz *et al. J. Clin. Invest.* 2010; [8]Gineste *et al. PloS One* 2013.