The dominant-negative mitochondrial calcium uniporter subunit MCUb drives macrophage polarization during skeletal muscle regeneration

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ABSTRACT

Skeletal muscle can regenerate after damage due to the coordinated action of immune cells with muscle stem cells, called satellite cells. Pro-inflammatory macrophages infiltrate skeletal muscle transcriptional regulator of the macrophage pro-inflammatory soon after injury to sustain the proliferation of satellite cells. These macrophages later acquire the anti-inflammatory phenotype and promote the differentiation and fusion of satellite cells. Here, we showed that MCUb, the dominant-negative subunit of the mitochondrial calcium uniporter (MCU) complex, promotes muscle regeneration by controlling macrophage responses. Macrophages lacking MCUb lost the ability to efficiently acquire the anti-inflammatory profile and mice with MCUb-deficient macrophages showed delayed regeneration through exhaustion of the satellite cell pool. MCUb ablation altered macrophage metabolism by promoting glycolysis and the accumulation of TCA cycle intermediates, which was accompanied by the stabilization of HIF-1a, the master program. Together, these data demonstrate that MCUb abundance is tightly controlled in macrophages to enable satellite cell functional differentiation and recovery of tissue homeostasis after damage.

INTRODUCTION

Tissue regeneration is an evolutionarily conserved process in which the interaction between infiltrating and resident inflammatory cells must be tightly coordinated to restore tissue homeostasis and function (*1*). Skeletal muscle is the most abundant tissue in the body and is essential not only for breathing, posture maintenance and locomotion, but also for crucial homeostatic and metabolic roles such as heat production and carbohydrate and amino acid storage (*2*). Loss of muscle function due to trauma or chronic conditions results in diminished mobility and strength and may eventually lead to metabolic disorders that can have lethal consequences (*2*). Compared to other tissues that cannot regenerate after injury, skeletal muscle can fully regenerate after mechanical trauma, exposure to toxins or infections (*3*). The ability of muscle to regenerate primarily depends on a specific population of normally quiescent muscle stem cells called satellite cells that are intimately associated with muscle fibers (*4*). Satellite cells are myogenic precursor cells (MPCs) that reside in a quiescent state on the surface of fully differentiated myofibers, beneath the basal lamina that surrounds muscle fibers (*4*). Once activated, they start to proliferate and a subset of daughter cells continues to differentiate into myotubes to reconstitute damaged muscle structure, whereas others return to quiescence to replenish the reserve population of satellite cells (*5*). Although satellite cells and their progeny are essential for skeletal muscle regeneration, their presence alone is insufficient for the correct reorganization of muscle fibers (*5*). Key players in this complex scenario are the inflammatory cells that infiltrate the injured muscle and, together with satellite cells, appear to be critical for successful regeneration (*1*). Among inflammatory cells, monocytes/macrophages play the major role in this repair process (*1*). Indeed, macrophages provide a microenvironment for satellite cell proliferation and activation, orchestrating adult myogenesis during regeneration of damaged skeletal muscle (*6*). Neutrophil recruitment to the site of damage begins a few hours after injury, but after 24 hours, neutrophils begin to disappear from regenerating muscle, leaving only a monocyte-macrophage population recruited from blood (*5*, *7*). In damaged muscle, which is enriched with pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) released mainly by neutrophils, macrophages acquire a so called M1 pro-inflammatory profile (*5*). Pro-inflammatory macrophages, defined also as classically activated macrophages, peak in number 2 days post-injury and release pro-inflammatory

cytokines such as TNF-α, interleukin 6 (IL-6), interleukin 1β (IL-1β) and insulin-like growth factor 1 (IGF-1) that in turn increase MPC proliferation and further expand the MPC population (*5*, *6*). Indeed, during the early stages of skeletal muscle regeneration the inflammatory response occurs in parallel with the initial stages of myogenesis, when satellite cells are activated and begin to proliferate and differentiate (*5*). A common marker of pro-inflammatory macrophages is the inducible nitric oxide synthase (iNOS), which is required to efficiently metabolize L-arginine into the reactive free radical nitric oxide (NO) that in turn induces the apoptosis of damaged cells (*8*, *9*). Phagocytosis of apoptotic neutrophils and myogenic cells is instrumental to initiate the resolution phase of the inflammatory response. Signals received by macrophages during phagocytosis suppress the production of TNF-α and increase the expression of transforming growth factor beta (TGF-β) and interleukin 10 (IL-10), turning macrophages into anti-inflammatory cells (*10*). Anti-inflammatory M2 macrophages (defined also as alternatively activated macrophages) become the prevalent myeloid cell population approximately 3 to 5 days post-injury; in addition to their role in resolving inflammation, they sustain fiber reconstitution by releasing cytokines with trophic functions (*5*, *7*). The metabolic status of macrophages reflects their function. Indeed, pro-inflammatory macrophages are key players of the first line of defense against bacterial infections and obtain energy through glycolysis, a process that can rapidly provide energy and reducing equivalents (*11*). In addition, in pro-inflammatory macrophages, the Krebs cycle intermediate succinate promotes the stabilization of the hypoxia-inducible factor-1α (HIF-1α), which drives the sustained production of pro-inflammatory and glycolytic genes (*12*). In contrast, anti-inflammatory macrophages are involved in tissue repair and wound healing and use oxidative metabolism to fuel their longer-term functions that is orchestrated by signal transducer and activator of transcription 6 (STAT6) and peroxisome proliferator-activated receptor gamma coactivator-1β PGC-1β (*11*) Nevertheless, the mechanisms involved in the phenotypic transition of macrophages from an M1 to an M2 phenotype are only partially elucidated. Phagocytosis of tissue debris, which occurs during the first stages of skeletal muscle repair, crucially contributes the pro- to anti-inflammatory macrophage skewing through a metabolic pathway-dependent mechanism (*13*). Indeed, in a cardiotoxin (CTX)-induced muscle injury model, myeloid cell-specific deletion of AMP-activated protein kinase (AMPK) impairs alternative macrophage polarization, phagocytic capacity and thus skeletal muscle regeneration (*13*). In macrophages, transient increases in cytosolic Ca2+ ([Ca2+]cyt) are frequently observed during the course of phagosome formation (*14–17*), but the role of mitochondrial Ca2+ remains controversial (*14*, *18*). Mitochondrial Ca2+ regulates many cell functions, from stimulating aerobic metabolism and thus ATP production under normal conditions to the induction of cell death in pathological conditions (*19*). The discovery of the molecular identity of the mitochondrial calcium uniporter (MCU) complex (*20*, *21*), the highly selective channel responsible for mitochondrial Ca2+ entry, gave rise to studies that aimed to clarify not only the composition and the regulation of the MCU complex, but also the pathophysiological role of mitochondrial Ca2+ uptake (*22*). MCU exists in a large protein complex composed of pore-forming subunits (MCU itself, MCUb and EMRE) and of regulatory subunits (MICU1 and MICU2) that regulate MCU channel activity at both low and high [Ca2+]cyt (*23*). MCUb is a component of the pore region of the MCU complex that inhibits mitochondrial Ca2+ uptake by acting as a dominant-negative subunit (*24*). MCU and MCUb share a 50% protein sequence and structure similarity but their expression varies greatly between tissues (such as 3:1 in heart or lung and 40:1 in skeletal muscle), thus contributing to the spatiotemporal regulation of mitochondrial Ca2+ uptake in different tissues (*25*). Mitochondrial Ca2+ uptake is not only involved in ATP production but also promotes skeletal muscle trophism by regulating IGF-1/AKT/PKB and PGC-1a4 pathways (*26*). Here we showed that MCUb ablation affected the macrophage differentiation program by limiting the skewing from pro- to anti-inflammatory phenotype. This defect ultimately led to delayed regeneration of damaged fibers and exhaustion of the satellite cell pool.

RESULTS

MCUb is induced during skeletal muscle regeneration

Skeletal muscle regeneration after intramuscular injection of CTX provides a useful model for sterile inflammation (*13*, *27*) because it induces a regeneration process with highly reproducible pattern and kinetics. Indeed, CTX causes homogenous damage of the whole muscle, inducing the infiltration of many monocytes and macrophages participating in muscle repair (*13*, *27*). We found that *MCUb* was induced at mRNA level 3 days after CTX injection in tibialis anterior (TA) muscles of C57BL/6N mice (Fig. 1A). In contrast, the expression of the mRNAs encoding the pore-forming subunit *MCU* and the regulatory subunit of the MCU complex, *MICU1* were not induced during the progression of skeletal muscle regeneration, whereas the expression of the mRNA encoding the gatekeeper subunit *MICU2* was slightly increased at 3 days post-injury (fig. S1, A-C).

Muscle regeneration is a complex process characterized by the participation of many resident and infiltrating cell types (*5*). Thus, to determine the cell population responsible for the high *MCUb* expression levels in regenerating muscle, we analyzed the expression of *MCUb* in purified pro- and

anti-inflammatory macrophages, endothelial cells, fibro/adipogenic progenitors (FAPs), satellite and myeloid cells. At either 2 or 4 days post-injury, we found that *MCUb* mRNA was induced in the macrophage cell population to a much greater extent than in other cell types (Fig. 1B). To confirm these data, we performed fluorescence activated cell sorting (FACS) of macrophages purified as EGF-like module-containing mucin-like hormone receptor-like 1 positive cells (F4/80+) from regenerating TA muscles of wild-type (WT) animals 3 days post-CTX injection, a time point at which *MCUb* was maximally induced. qPCR analysis of F4/80+ cells extracted from regenerating muscles 3 days post-CTX injection showed that the mRNA for *MCUb* was highly expressed in macrophages compared to those encoding the other *MCU* complex components (Fig. 1C). Furthermore, we also performed a PrimeFlow RNA detection assay that, by coupling the classical antibody-based surface staining with intracellular staining for specific mRNA molecules, confirmed that *MCUb* expression was specifically increased in macrophages and not in satellite cells (fig. S1, D-I).

Next, we asked whether *MCUb* was expressed by a specific subpopulation of macrophages. To perform this experiment, bone marrow-derived macrophages (BMDMs) were stimulated for 24 hours with either pro-inflammatory stimuli, interferon-g (IFN-g) and lipopolysaccharide (LPS), to induce the pro-inflammatory M1 phenotype, or with an anti-inflammatory cytokine, interleukin 4 (IL-4), to induce the anti-inflammatory M2 phenotype (*28*). We found that *MCUb* was induced in pro-inflammatory macrophages at 24 hours after polarization (Fig. 1D). In contrast, the expression of the other components of the *MCU* complex did not significantly change in pro- and anti-inflammatory macrophages (fig. S2, A-C). Analysis of the expression of multiple pro- and anti-inflammatory markers confirmed the efficacy of the polarization process (fig. S2, D-H). Our data agree with a previous study showing the expression of *MCUb* in primary cultures of human monocyte-derived macrophages (*18*).

MCUb affects macrophages skewing during skeletal muscle regeneration

We then generated a total MCUb knockout (*MCUb-/-*) mouse model based on the 'knockout-first' technology (*29*), and analyzed the expression of pro-inflammatory and anti-inflammatory markers in regenerating TA muscles of both WT and *MCUb-/-* mice. As control, we used non-injected TA muscles from both WT and *MCUb-/-* animals. 3 days after CTX injection, qPCR analysis demonstrated a significant reduction in the expression level of the mRNAs encoding the mannose receptor type 1 (*CD206*) and arginase I (*ARG1*), which are typical anti-inflammatory markers (Fig. 2, A and B), and a significant induction in the expression of the pro-inflammatory markers *TNF-α* and *iNOS* (Fig. 2, C and D). These differences disappeared at 7 and 14 days after CTX injection (Fig. 2, A-D).

To extend these results, we characterized the inflammatory cellular infiltrate during the early stages of skeletal muscle regeneration in CTX-injured TA muscles of both WT and *MCUb-/-* mice by FACS analysis (fig. S3). At 1, 3, and 5 days post-injury, there was no difference in the percentage of monocytes (Ly6G-/Ly6C+) and total macrophages (Ly6G-/Ly6C-) recruited to regenerating muscles in either WT and *MCUb-/-* animals (Fig. 2, E and F), thus excluding a defect in leukocyte recruitment in *MCUb-/-* mice. However, as expected, the percentage of M1 pro-inflammatory macrophages (CD206-) decreased as regeneration progressed in WT muscles, whereas in *MCUb-/-* muscles, the pro-inflammatory infiltrate persisted at 1, 3, and 5 days post-injury (Fig. 2, G and H). Concomitantly, 5 days after muscle damage, the percentage of anti-inflammatory M2 macrophages (CD206+) was greatly reduced (Fig. 2, G and H). In contrast, we found a significant increase in the percentage of M1 macrophages 1, 3 and 5 days after injury (Fig. 2, G and H). Together, these results suggest that MCUb ablation does not affect monocyte/macrophage recruitment in the regenerating muscle, but it dampens or delays macrophage switching towards the anti-inflammatory phenotype.

We then decided to study the polarization ability of BMDMs from these animals in a controlled environment to verify that the observed unbalanced skewing was due to properties intrinsic to *MCUb-/-* macrophages. We therefore analyzed the expression of pro- and anti-inflammatory genes in BMDM exposed to IFN-g together with either LPS or IL-4 in vitro. In *MCUb-/-* macrophages, we observed a trend towards increased expression of pro-inflammatory cytokines and a significant up-regulation of inducible *iNOS* expression (Fig. 3, A-C), which was paralleled by reduced expression of anti-inflammatory markers (Fig. 3, D-F). Consistently with the expression data, ELISAs showed increased levels of pro-inflammatory cytokines in macrophages polarized with pro-inflammatory stimuli (fig. S4, A-C) and decreased levels of an anti-inflammatory cytokine in macrophages polarized with anti-inflammatory stimuli (fig. S4D).

MCUb ablation affects phagocytosis

Our data suggested that MCUb ablation caused the accumulation of classically activated macrophages. We next analyzed the phagocytic capacity of macrophages from *MCUb-/-* mice because phagocytosis of cellular debris during muscle regeneration drives the skewing from pro- to anti-inflammatory macrophages that, in turn, orchestrates satellite cell differentiation and tissue integrity restoration (*7*, *13*). To assess our hypothesis that the lack of MCUb would not only alter macrophage

polarization but also impair their phagocytic ability, we measured the ability of macrophages to engulf apoptotic cells, a process known as efferocytosis. *MCUb-/-* macrophages were impaired in calcein-loaded apoptotic myocyte phagocytosis (Fig. 3G). Similarly, phagocytosis of bioparticles recognized either by the Toll-like receptor 4 (TLR4) or by Dectin-1/TLR2/TLR6 was significantly impaired in *MCUb-/-* macrophages (Fig. 3H and fig. S4E), suggesting a general defect in particle internalization and processing.

Cytosolic and mitochondrial Ca2 + measurements in *MCUb- / -* macrophages

Ca2+ ions are fundamental second messengers that play key roles in different macrophage signaling pathways, and are involved not only in the innate immune response but also in macrophage polarization (*30–32*). In addition, cytosolic Ca2+ may play a role during phagocytosis of extracellular material by macrophages (*15*, *33*, *34*). To evaluate whether the impairment of macrophage phagocytic capacity and macrophage polarization toward an anti-inflammatory phenotype observed in *MCUb-/-* macrophages was linked to the MCUb-mediated regulation of mitochondrial Ca2+ uptake, we performed mitochondrial and cytosolic Ca2+ measurements. We first assessed whether MCUb absence affects mitochondrial Ca2+ levels ([Ca2+]mt) in BMDMs polarized with pro- and anti-inflammatory cytokines, using the fluorescent mitochondrial Ca2+ indicator Rhod2-AM. No difference was observed in basal mitochondrial Ca2+ levels between WT or *MCUb-/-* pro- and anti-inflammatory macrophages (Fig. 4A). Conversely, upon ATP stimulation, [Ca2+]mt was significantly increased in *MCUb-/-* M1 and M2 macrophages compared to WT macrophages (Fig. 4B), consistent with the dominant-negative role of MCUb (*24*). There was also no difference in the loading of the fluorescent dye tetramethylrhodamine methyl ester (TMRM) (fig. S5), thus ruling out changes in mitochondrial membrane potential (ΔΨm) and thus in the driving force for mitochondrial Ca2+ uptake in macrophages lacking MCUb.

We then asked whether these changes in mitochondrial Ca2+ uptake would impinge on [Ca2+]cyt. Consistent with mitochondrial Ca2+ measurements, imaging of the ratiometric fluorescent dye FURA 2-AM revealed no significant differences in basal [Ca2+]cyt between WT and *MCUb-/-* pro-and anti-inflammatory macrophages (Fig. 4C). Conversely, after ATP stimulation cytosolic Ca2+ was significantly decreased in *MCUb-/-* pro-inflammatory macrophages compared to WT ones (Fig. 4D).

On the contrary, we did not observe any difference in [Ca2+]cyt in *MCUb-/-* anti-inflammatory macrophages compared to WT macrophages (Fig. 4D).

MCUb affects skeletal muscle repair by modulating macrophage plasticity

Because our in vitro and in vivo results showed that MCUb ablation induces the accumulation of pro-inflammatory macrophages, we asked whether MCUb ablation could impinge on skeletal muscle repair after damage. Histological analysis on regenerating muscles showed persistent inflammation and the presence of necrotic cells 7 days after CTX injury in *MCUb-/-* muscles compared to WT mice (Fig. 5A). Moreover, we observed a delay in the disappearance of cellular infiltrate and altered muscle structure in *MCUb-/-* mice 14 days after CTX injection (Fig. 5A), suggesting a delay in the progression of skeletal muscle regeneration. Nevertheless, neither the number of newly formed regenerating myofibers (fig. S6A) nor the cross-sectional area (CSA) of the regenerating fibers (fig. S6B) differed between genotypes at 14 days post-CTX injection.

We then analyzed whether the expression levels of the different myogenic regulatory factors (MRFs), involved in satellite cell proliferation and differentiation (*35*), was affected during skeletal muscle regeneration in *MCUb-/-* animals. Three days after CTX injection, *MCUb-/-* muscles showed a significant reduction in the expression of the mRNA encoding the paired-box transcription factor (*Pax7*) (Fig. 5B), which marks both quiescent and activated satellite cells (*36*). In addition, at the same time point after injury, *MCUb-/-* mice showed decreased expression of the mRNA encoding myoblast determination protein 1 (*MyoD*) (Fig. 5C), which marks both activated and proliferating satellite cells that are committed to differentiate (*35*). Overall, these data suggest an impairment of the capacity of satellite cells to proliferate and become committed to differentiation. Consistent with the latter result, regenerating muscles of *MCUb-/-* mice showed reduced numbers of MyoD positive nuclei 3 days after muscle damage (Fig. 5D), suggesting a defect in the commitment of satellite cells. Another important transcription factor required for full myocyte differentiation and survival is myogenin (MyoG), which is principally involved in the terminal stages of myogenesis (*35*). We observed a trend towards an increase in *MyoG* expression in *MCUb-/-* mice in untreated muscles and no changes in regenerating muscles (Fig. 5E). Overall, the alteration in the expression level of *Pax7* and *MyoD* caused by MCUb ablation suggests an impairment in the early phases of muscle repair.

We then performed triple regeneration experiments to analyze whether MCUb ablation could deplete the satellite cell pool. After injuring TA muscles with CTX three times every 16 days, we observed a reduction in the CSA of regenerating muscle fibers of *MCUb-/-* mice compared to those of WT mice (Fig. 5, F-H), consistent with the exhaustion of satellite cell pool and/or the depletion of pro-regenerative anti-inflammatory macrophages.

We next sought to validate the results obtained with mice with a global MCUb deficiency using chimeric mice with WT muscle but *MCUb-/-* bone marrow-derived leukocytes, as previously performed (*37*). Irradiated C57BL/6J CD45.1 mice were transplanted with C57BL/6N CD45.2 WT or *MCUb-/-* bone marrow cells, generating trWT and trMCUb-/- chimeric mice. TA muscles were injected with CTX eight weeks after the transfer. Analysis of blood cell populations at 6 weeks after transplant showed less than 1% of CD45.1 native myeloid cells and more than 90% of CD11b were CD45.2 both for WT and *MCUb-/-* cells. Bone marrow populations at the time of euthanasia (10 weeks) showed a complete and efficient engraftment of *MCUb-/-* cells (96.2%) and a less efficient but sufficient engraftment of WT cells (77.5%) (fig. S6C).

The lack of MCUb specifically in bone marrow-derived cells resulted in increased cellular infiltrate at 3 days post-injury that was maintained at 14 days post-injury in trMCUb-/- compared to trWT mice (Fig. 6A), confirming the delay in muscle regeneration observed in the total knockout mice (Fig. 5A). This was confirmed also by a significant decrease of the fiber CSA in trMCUb-/- as compared to trWT muscles (Fig. 6, B and C) and further corroborated by the increased percentage of embryonic myosin-positive fibers in trMCUb-/- muscles (Fig. 6D). Moreover, the number of Pax7-positive satellite cells was reduced in trMCUb-/-as compared with trWT muscles at 14 days post-CTX injury (Fig. 6E).

***MCUb-/-* anti-inflammatory macrophages fail to promote myotube formation in vitro**

The inflammatory profile of macrophages is related to their active role in promoting both MPC proliferation and differentiation (*6*, *10*). Indeed, macrophages release mitogenic growth factors required for MPCs to exert their role in muscle regeneration. In particular, pro-inflammatory macrophages stimulate MPC proliferation, whereas anti-inflammatory macrophages promote their differentiation and fusion (*6*, *10*). We performed co-culture experiments in which satellite cells derived from WT mice were incubated with medium conditioned by macrophages from either WT or *MCUb-/-* mice. Although there was no difference in satellite cell proliferation (Fig. 6F), medium conditioned by *MCUb-/-* M2 macrophages significantly impaired MPC fusion into myotubes, an effect not seen with medium conditioned by *MCUb-/-* M1 macrophages (Fig. 6, G and H). To determine whether *MCUb-/-* satellite cells could contribute to the phenotype observed in vivo, we analyzed the fusion index of WT- and *MCUb-/-*-derived myotubes and found no difference between genotypes (fig. S6D). We repeated the co-culture experiment using *MCUb-/-*-derived myotubes incubated with medium conditioned by macrophages from either WT or *MCUb-/-* mice to further exclude differences in satellite cells properties between genotypes. As for the experiment using WT-derived myotubes, only the medium conditioned by *MCUb-/-* M2 macrophages impaired MPC fusion into myotubes obtaining the same results (fig. S6E). These results demonstrated that *MCUb-/-* macrophages, which failed to acquire an anti-inflammatory phenotype, lose their ability to promote satellite cell fusion and myotube formation.

MCUb ablation enhances "M1-like" metabolic features of macrophages, which is accompanied by HIF-1a stabilization

Having established that the polarization status in *MCUb-/-* macrophages is shifted towards the pro-inflammatory M1 phenotype, we asked whether HIF-1α stabilization was involved because increased mitochondrial Ca2+ uptake caused by MCU overexpression promotes HIF1-α signaling in MDA-MB-231 cancer cells (*38*). HIF-1α was stabilized to a greater extent in M1 *MCUb-/-* compared to WT macrophages (Fig. 7, A and B), suggesting that this event could contribute to the persistent pro-inflammatory profile in *MCUb-/-* macrophages.

We next analyzed macrophage metabolic state. Metabolically, classically activated macrophages display enhanced glycolysis rates paralleled by reduced oxidative phosphorylation (OXPHOS), which is associated with accumulation of TCA cycle intermediates such as citrate, itaconate, malate and succinate and increased lipid synthesis (*39*, *40*). These events are orchestrated by HIF-1α, which is further stabilized by succinate accumulation (*41*). In contrast, alternatively activated macrophages obtain much of their energy from oxidative metabolism (*42*). We metabolically characterized BMDMs from WT and *MCUb-/-* mice stimulated either with INF-g and LPS, to induce polarization toward a M1 phenotype, or with IL-4, to induce polarization toward a M2 phenotype. As expected, anti-inflammatory macrophages displayed an increased oxygen consumption rate (OCR) compared to pro-inflammatory macrophages (Fig. 7, C and D and (*43*)), although MCUb ablation did not promote any further changes in the OCR of either M1 or M2 macrophages (Fig. 7, C and D). However, glycolysis and the maximum glycolytic capacity were enhanced in M1 and M2 *MCUb-/-* macrophages compared to WT ones (Fig. 7, E and F), confirming the ability of MCUb deletion to enhance the effect of pro-inflammatory stimuli. In line with this finding, *MCUb-/-*pro-inflammatory macrophages displayed higher levels of citrate, itaconate and succinate compared to WT ones (Fig. 8, A-C). Furthermore, the levels of malate were higher and those of citrulline were lower in *MCUb-/-* compared to WT M1 macrophages (Fig. 8, D and E). These results suggest that the arginosuccinate shunt flux, which recycles citrulline generated by iNOS by condensing it with aspartate, was enhanced in *MCUb-/-* compared to WT M1 macrophages, leading

to higher malate synthesis (*40*). Malate transported into mitochondria in exchange with citrate sustains the shunt by providing oxaloacetate that is transaminated to aspartate. 2-oxoglutarate (2-OG) produced from glutamate is channeled into the TCA cycle and sustains succinate production. This process is also supported by the increased glycolytic flux in *MCUb-/-*pro-inflammatory macrophages, because pyruvate can be converted into oxaloacetate by pyruvate carboxylase (PC), which is controlled by HIF-1α (*44*). Together, these data suggest that *MCUb-/-*pro-inflammatory macrophages display enhanced HIF-1α stabilization, accompanied by increased glycolytic flux, accumulation of TCA cycle intermediates and argininosuccinate flux shunt compared to WT pro-inflammatory macrophages, suggesting the acquisition of persistent metabolic "M1-like" characteristics (Fig. 8F).

DISCUSSION

The efficiency of skeletal muscle repair relies on the interaction between resident and infiltrating cell types such as satellite cells, FAPS, endothelial cells and inflammatory cells (*45*). Among the inflammatory cells, macrophages not only coordinate the initial inflammatory response but also drive muscle growth (*1*, *5*, *45*). Indeed, both the classically activated pro-inflammatory M1 and the alternatively activated anti-inflammatory M2 macrophages participate to all phases of skeletal muscle regeneration, namely inflammation, myogenesis, fibrosis, revascularization and return to homeostasis (*45*). Although different regulatory pathways have been associated with macrophage skewing from pro- to anti-inflammatory phenotype (*13*, *46*), the signals modulating macrophage activation state in vivo are still under investigation. Here we identified an additional mechanism, mitochondrial Ca2+. Indeed, we found that the expression of *MCUb*, the dominant-negative subunit of the mitochondrial calcium uniporter (MCU) (*24*), was specifically induced during skeletal muscle regeneration (Fig. 1A and fig. S1, A-C) and that its upregulation specifically occurred in macrophages and not in the other cell populations involved in muscle regeneration (Fig. 1B and fig. S1, D-I). In addition, our in vitro results showed that *MCUb* was highly and specifically expressed in murine BMDMs polarized with pro-inflammatory cytokines (Fig. 1D and fig. S2, A-C). The induction of the expression of pro-inflammatory genes and the concomitant inhibition of anti-inflammatory genes in regenerating muscles of *MCUb-/-* mice 3 days after CTX injury (Fig. 2, A-D) led us to hypothesize that the lack of MCUb could impinge on pro- to anti-inflammatory skewing. This hypothesis was supported by the characterization of the mononuclear cellular infiltrate in regenerating muscles after CTX injection (Fig. 2, E-G), which showed an accumulation of pro-inflammatory macrophages in *MCUb-/-* muscles (Fig. 2, G and H), although the percentage of monocytes and macrophages was unaffected, thus ruling out defect in leukopoiesis (Fig. 2, E and F). Furthermore, MCUb ablation in BMDMs prevented the acquisition of an M2 phenotype in vitro, as assessed by the increased expression of pro-inflammatory markers (Fig. 3, A-C) and the decreased expression of anti-inflammatory markers (Fig. 3, D-F) upon IFN-γ +LPS and IL-4 treatment, respectively. Consistently, cytokines released in the same experimental model further confirmed that MCUb ablation impaired the acquisition of the anti-inflammatory phenotype (fig. S4, A-D).

Phagocytosis of tissue debris, which occurs during skeletal muscle regeneration, is involved in the mechanisms by which macrophages operate the transition from the M1 to M2 phenotype (*13*, *47*, *48*). Consistent with the block of pro- to anti-inflammatory skewing, we observed that *MCUb-/-* BMDMs showed decreased phagocytic capacity of both apoptotic cells and bioparticles (Fig. 3G and H and fig. S4E), suggesting a defect in both extracellular material internalization and processing. Increases in cytosolic Ca2+ levels promote phagocytosis (*14*, *15*), and cytosolic Ca2+ ions participate in intracellular signaling pathways involved in alternative macrophage polarization (*49*, *50*). However, nothing is known about the involvement of mitochondrial Ca2+ in the polarization potential of macrophages. Therefore, we investigated the effect of MCUb ablation on mitochondrial and cytosolic Ca2+ handling. Consistent with the dominant-negative role of MCUb on mitochondrial Ca2+ uptake (*24*), [Ca2+]mt was not altered under basal conditions (Fig. 4A), but was increased in both classically and alternatively activated *MCUb-/-* BMDMs upon ATP stimulation (Fig. 4B). Consistently, we observed no changes in resting [Ca2+]cyt (Fig. 4C) and decreased [Ca2+]cyt in pro-inflammatory *MCUb-/-* macrophages upon ATP addition but not in anti-inflammatory *MCUb-/-* macrophages (Fig. 4D). The increased buffering capacity of *MCUb-/-* mitochondria and the consequent decrease of [Ca2+]cyt might explain the inhibition of the phagocytic capacity in *MCUb-/-* BMDMs. Indeed, in murine BMDMs, decreased [Ca2+]cyt affects macrophage phagolysosome formation and maturation (*14*, *15*, *51*), which, in turn, could affect alternative macrophage polarization. Our and others' findings (*14*, *15*, *51*) contrast with a report showing that MCU is required for phagocytosis in human macrophages (*18*). This discrepancy might be due to the use of primary cultures of human monocyte-derived macrophages, which can behave differently from murine BMDMs. Furthermore, *MCU* expression was silenced, and thus residual MCU activity could have been present. Based on the role of macrophages on muscle regeneration (*6*), we investigated the effects of MCUb ablation on the progression of muscle repair. Muscle regeneration was delayed in muscles of *MCUb-/-* mice. Indeed, inflammation persisted 7 days post-CTX injury, leading to an alteration of the overall muscle

structure at 14 days post-injury (Fig. 5A). Despite these abnormalities, CSA and the number of regenerating fibers were not affected in regenerating muscles of *MCUb-/-* mice at 14 days post-CTX injury (fig. S6, A and B). Analysis of the expression level of MRFs in regenerating muscles showed decreased expression of *Pax7* and *MyoD* (Fig. 5, B and C) and a concomitant decrease in the number of MyoD+ nuclei (Fig. 5D) in *MCUb-/-* muscles 3 days post-CTX injection. However, the expression of *MyoG* did not change between WT and *MCUb-/-* mice during skeletal muscle regeneration (Fig. 5E). After damage, satellite cells enter the cell cycle, generating daughter cells that can either replenish the original pool of satellite cells necessary for future rounds of skeletal muscle repair that will therefore express *Pax7* but not *MyoD* (Pax7+; MyoD-) or can differentiate and lose their self-renewal capacity. In the latter case, satellite cells will express both Pax7 and MyoD (Pax7+; MyoD+) (*5*). Our results suggest that by affecting the expression level of both *Pax7* and *MyoD*, MCUb ablation might affect both the reconstitution of the pool of satellite cells and the ability of satellite cells to become activated and committed to differentiate into new myofibers. Consistent with this hypothesis, the CSA of muscle fibers was decreased in triple regeneration experiments (Fig. 5, F-H), supporting the idea that the persistence of pro-inflammatory macrophages and a decreased number of pro-regenerative macrophages affect muscle growth, possibly leading to the exhaustion of the pool of satellite cells.

Experiments with chimeric mice confirmed that, in our experimental settings, the expression of MCUb in immune cells enable muscle repair (Fig. 6, A-E and fig. S6C). In this model, the reduction of Pax7-positive cells at 14 days post-injury supports the hypothesis that MCUb ablation in macrophages affects satellite cell self-renewal (Fig. 6E). These data indicate that the defective regeneration in *MCUb-/-* muscle is a consequence of an intrinsic defect in immune, rather than stromal, cells. Because about 75% of leukocytes in post-CTX regenerating muscle are macrophages (*13*), and because MCUb was expressed mainly by macrophages in regenerating muscle (Fig. 1B), these results suggest that defects in muscle regeneration in both *MCUb-/-* and trMCUb-/- animals is due to the lack of MCUb in macrophages. The stronger phenotype of trMCUb-/- mice compared to global *MCUb-/-* mice may be due to adaptation during embryonic development in global *MCUb-/-* mice.

The decrease in the number of anti-inflammatory macrophages, which release cytokines and growth factors like IL-10 and TGF-β (*7*) that promote muscle fiber growth and regeneration, might contribute to the phenotype observed after multiple rounds of regeneration in global *MCU-/-* mice and after one round in trMCUb-/- mice. In line with this hypothesis, WT satellite cells cultured with media conditioned by WT M1 macrophages resulted in MPC proliferation (Fig. 6F), whereas those cultured with media conditioned by WT M2 macrophages resulted in MPC fusion into myotubes (Fig. 6G). MCUb ablation decreased MPC fusion (Fig. 6, G and H), although we did not observe any difference in MPC proliferative capacity (Fig. 6F). Overall, these results further confirm that *MCUb-/-* macrophages fail to acquire an anti-inflammatory phenotype.

Mitochondrial Ca2+ has been recognized as a main factor promoting aerobic metabolism (*52*). Less is known about the role of Ca2+ in modulating macrophage metabolism, which is a critical hallmark and regulator of the polarization process (*53*). Indeed, specific metabolic programs in macrophages can be perturbed, leading to the rewiring of function from one polarization state to another (*54*, *55*). A factor that orchestrates the acquisition of pro-inflammatory features is HIF-1α, which initiates the transcription of genes necessary to survive hypoxia, including those encoding glucose transporters, glycolytic enzymes and pro-inflammatory factors, and which enhances the pro-inflammatory phenotype (*56*, *57*). However, the involvement of HIF-1α in skeletal muscle regeneration is still debated (*58*, *59*). Increased glycolytic capacity, even in the presence of high oxygen concentration, can not only enhance ATP production, but also can feed reducing equivalents through the pentose phosphate pathway to NADPH oxidase, the most potent bactericidal effector of phagocytes (*11*). Additionally, OXPHOS is reduced, slowing down the TCA cycle (*60–62*). The subsequent accumulation of citrate, itaconate, malate and succinate are important signaling mediators and precursors in biosynthetic pathways (*39*, *40*). In contrast, alternatively activated macrophages rely mainly on mitochondrial OXPHOS that, in turn sustains their intense phagocytic activity and long-term anti-inflammatory and pro-regenerative functions (*53*).

Our hypothesis is that mitochondrial Ca2+ influences macrophage polarization, possibly through a HIF-1α-dependent mechanism. Indeed, *MCUb-/-* macrophages, in which mitochondrial Ca2+ is increased, show higher HIF-1α protein levels than the corresponding WT (Fig. 7, A and B). Therefore, the typical metabolic “M1-like” features were enhanced in M1 macrophages and only marginally in M2 macrophages. Although no changes were detected in OCR (Fig. 7, C and D), glycolysis was enhanced in *MCUb-/-*pro- and anti-inflammatory macrophages (Fig. 7, E and F). HIF-1α promotes the re-routing of increased pyruvate to lactate through LDH or to oxaloacetate through PC (*44*), leading to aspartate synthesis. 2-OG derived from glutamate transamination enters the TCA cycle leading to succinate. These events that replenish the TCA cycle in different ways are consistent with the increased levels of citrate and succinate (Fig. 8, A and C) in *MCUb-/-*pro-inflammatory

macrophages resulting from the two breakpoints in the TCA cycle. Citrate can be further metabolized into itaconate, an anti-microbial molecule abundant in pro-inflammatory macrophages, which was increased in *MCUb-/-*pro-inflammatory macrophages (Fig. 8B and (*63*, *64*)). Succinate levels increase (*41*), which may further support to the persistent M1 phenotype of *MCUb-/-* macrophages. In support of this hypothesis (Fig. 8F), *MCUb-/-* macrophages showed high levels of malate and lower levels of citrulline (Fig. 8, D and E).

The link between MCUb and HIF-1a may be due to intramitochondrial Ca2+ accumulation following MCUb ablation (Fig. 4B). Indeed, increased intramitochondrial Ca2+ levels due to the overexpression of MCU enhances HIF-1a expression in cancer cells (*38*). Because MCUb ablation mimics the effect of MCU overexpression in terms of intracellular Ca2+ level, it is conceivable that a persistent pro-inflammatory state, possibly linked to increased HIF-1a stabilization, takes place in macrophages lacking MCUb.

In summary, this study suggests a mechanism by which the MCUb-mediated regulation of mitochondrial Ca2+ uptake governs the conversion of pro-inflammatory macrophages to a pro-resolving phenotype, with a major impact on the progression of skeletal muscle regeneration. The clarification of this pathway has potential relevance for targeting pathological conditions characterized by chronic, nonresolving inflammation.

MATERIAL AND METHODS

Mouse experiments

Adult male C57BL/6N mice (8 weeks old) from WT and *MCUb-/-* mouse strains were used in all experiments. For the generation of the total *MCUb-/-* mouse model, the strategy provided by the European Conditional Mouse Mutagenesis (EUCOMM) and the National Institute of Health Knockout Mouse (KOMP) was used. EUCOMM uses promoterless and promoter-driven targeting cassettes for the generation of a 'knockout-first' allele (*29*) in C57BL/6N embryonic stem cells (*65*). For the generation of a total *MCUb-/-* mouse model, mice containing a promoterless selection cassette were used. Mice have been provided in heterozygous form and were bred to obtain the homozygous genotype. As controls, age, and sex matched WT C57BL/6N were used. In vivo experiments were performed in accordance with the Italian law D. L.vo n.26/2014.

Mouse Cardiotoxin Muscle Regeneration Model

Myonecrosis was induced by intramuscular injection of 50 µl of 10 µM CTX (Merck) in TA muscles, as previously performed (*6*, *13*). Muscles were collected for analysis at different time points post-injury (3, 7, 14 days). In the triple regeneration protocol, TA muscles of both WT and *MCUb-/-* animals were injected three times every 16 days with CTX and muscles were collected 16 days after the last injury, as previously performed (*66*).

Isolation of leukocytes, satellite cells, FAPs, endothelial cells and M1 and M2 macrophages by cell sorting

TA muscles were enzymatically and mechanically digested using the skeletal muscle dissociation kit (Miltenyi Biotec) and the gentleMACS™ Dissociator (Miltenyi Biotec), following manufacturer instructions. In the experiments in figures 2G and 2H, a total of 0.5 x 106 cells derived from TA muscles were extracellularly stained with the following antibodies: Ly6C-efluor 450 (BD Biosciences), Ly6G APC-Cy7 (BD Biosciences), F4/80-Fitc (BD Biosciences), CD206-Alexa Fluor 647 (BD Biosciences). Dead cells were excluded by incorporation of the Live/Dead Stain Aqua (Thermo Fisher Scientific). Non-specific binding was blocked using CD16/32 Blocker (BD Bioscience). The gating strategy is shown in figure S3. Cells were analyzed with FACS Canto (BD Biosciences) and data were analyzed using FlowJo software.

In the experiment in figure 1B, muscles were harvested 2, and 4 days post-CTX injury. Muscles were dissociated and cell populations were isolated as described (*67*). Briefly, muscles were digested with 10 mg/mL collagenase B and 2.4 U/mL Dispase (Roche Diagnostics GmBH) at 37° C for 30 minutes and passed through a 30 µm cell strainer. Cells were separated into CD45+ and CD45- using magnetic beads (Miltenyi Biotec). CD45+ cells were incubated with anti-mouse FcgRII/III (2.4G2) for 20 minutes at 4° C in PBS 2% FBS. Neutrophils (CD64-/Ly6C/G+) and CD64+/Ly6C/G+ and CD64+/Ly6C/G- macrophages were isolated using PE-Cy7-conjugated anti-CD45 (25-0451-82, eBioscience), Alexa Fluor 647-conjugated anti-CD64 (558539, BD Pharmigen) and PE-conjugated anti-Ly6C (RM3004, Invitrogen) antibodies. Endothelial cells (CD31+), satellite cells (CD31-/Sca1-/int-α7+), FAPs (CD31-/Sca1+/CD34+) were isolated from the CD45- fraction using PE-Cy7-conjugated anti-CD45, PerCP-Cy5.5-conjugated anti-Sca1 (45-5981-82, eBioscience), Alexa Fluor 647-conjugated anti integrin-α7 (AB0000538, AB lab, University British Columbia), PE-conjugated anti-CD31 (12-0311-82, eBioscience) and FITC-conjugated anti-CD34 antibodies (11-0341-82, eBioscience). Cells were sorted using a FACS Aria II cell sorter (BD Biosciences).

Isolation and maintenance of bone-marrow derived macrophages in culture

Macrophages were obtained from bone-marrow precursor cells isolated from 8 weeks

C57BL/6N WT and *MCUb-/-* mice. Briefly, total bone marrow was extracted from mice by flushing femur and tibiae bone marrow with IMDM (Gibco) with 10% Superior Fetal Bovine Serum (FBS; Merk-Millipore) and 1% of Penicillin/Streptomycin (Lonza). Red blood cells were lysed by osmotic shock by resuspending them in 10 ml ACK lysing buffer (Lonza). After 5 minutes, an equal amount of complete medium was added to the cell suspension. Cells were then filtered using a 70 μm Cell strainer (BD Falcon) and centrifuged. Finally, cells were seeded in complete medium in Suspension culture dish (Corning) in the presence of 40 ng/ml of macrophage colony stimulating factor (M-CSF; Miltenyi Biotec). 7 days after differentiation, BMDMs were treated either with IFN-g (100 ng/ml; Miltenyi Biotec) and LPS (100 ng/ml; Merck) or IL-4 (20 ng/ml; Miltenyi Biotec) for 24 hours to trigger the polarization into pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes, respectively.

Bone marrow transfer

Recipient male mice (6 week of age) were irradiated with two lethal doses of 4,5 Gy (a total of 9 Gy) with a 4 hours interval, using a RS2000 Irradiator (Rad Source Technologies) set at 160 kV, 25 mA for 4 minutes each at ca 45 cm. Donor mice (C57BL/6N CD45.2 WT or *MCUb-/-*) were sacrificed and bone marrow cells were collected as described above. 18 hours after irradiation, recipient mice were intravenously injected with 5 x 106 cells in tail veins using a 27-gauge needle. The donor/recipient combinations were CD45 WT ® CD45.1 (trWT) and CD45 *MCUb-/-* ® CD45.1 (trMCUb-/-). Irradiated but not transplanted control mice died 9 days after irradiation. Irradiated mice were housed in individually ventilated cages and kept in sterile conditions. After 6- and 10-weeks, engraftment of donor cells was checked in TrWT and trMCUb-/- mice. Briefly, at 6 weeks after transplant, blood was collected from the retro-orbital sinus of trWT and trMCUb-/- , and red-blood cells were lysed with ACK (Lonza) and incubated with anti-mouse FcgRII/III (2.4G2) for 20 minutes at 4° C in PBS with 2% FBS. Cells were stained with BV-421-cojugated CD11b, PerCP-Cy5.5-conjugated anti-CD45 and PE-Cy7-conjugated CD45.1 (all antibodies are from BD-bioscience). At 10 weeks, after CTX injection, bone marrow cells were isolated from trWT and trMCUb-/- mice as described above. Cells were then incubated with anti-mouse FcgRII/III (2.4G2) for 20 minutes at 4° C in PBS with 2% FBS and stained with BV-421-cojugated CD11b, PerCP-Cy5.5-conjugated anti-CD45 and PE-Cy7-conjugated CD45.1 as above. Data were acquired with the instrument BD FACSCelesta and data were analyzed with FlowJo.

RNA extraction, reverse transcription and quantitative qPCR

Total RNA was extracted trough mechanical tissue homogenization or lysis in TRIZOL reagent (Thermo Fisher Scientific) from mouse skeletal muscle and BMDMs, respectively, following the manufacturer's instructions. RNA was quantified with Nanodrop (Thermo Fisher Scientific) and 1 µg of each sample was retrotranscribed with the cDNA synthesis kit SuperScript II (Thermo Fisher Scientific), following manufacturer instructions. Oligo (dT)12-18 primer (Thermo Fisher Scientific) was used as the primer for first stand cDNA synthesis with reverse transcriptase together with dNTPs (Thermo Fisher Scientific), following the manufacturer's instructions. The obtained cDNA was analyzed by qPCR using the IQ5 thermocycler and SYBR green chemistry (Bio Rad). The primers were designed and analyzed with Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) and their efficiency was between 95 and 100%. The housekeeping gene *GAPDH* was used for cDNA normalization of cDNA samples derived from both BMDMs and TA muscles of WT and *MCUb-/-* animals. For quantification, expression levels were calculated by using the 2-ΔΔCT Method (*68*).

To quantify MCUb expression as shown in Figure 1B, total RNA was isolated using NucleoSpin RNA Plus XS kit (Macherey-Nagel). RNAs were retrotranscribed into cDNA using Superscript II Reverse Transcriptase and qPCR was carried out in triplicate on a CFX ConnectTM qPCR Detection System (Bio-Rad). Reaction mixtures had a final volume of 10 µL, consisting of 2 µL of cDNA, 5µL of LightCycler 480 SYBR Green I Master and 0.5 mM primers. After initial denaturation, amplification was performed at 95° C (10 seconds), 60° C (5 seconds) and 72° C (30 seconds) for 45 cycles. Relative expression was determined by the Bio-Rad CFX ManagerTM software and fold change was normalized as Normalized Relative Quantity (or ΔΔCq) for each series, as NRQ = ! Δ$%&'$%$() !Δ$%*'$%$() where T is the target sample, Cal the calibrator value (or the mean of all sample Cqs of the series) and R is the housekeeping gene GAPDH. Log2-NRQ values were used to perform statistical analyses. cDNAs were amplified with the primers listed in table S1.

Detection of Cytokines and Chemokines in Culture Supernatants

Culture supernatants were collected, and the amount of IL-6, IL-1β, TNF- a and TGF-b was quantified by ELISA (R&D Systems) according to the manufacturer's instructions. Data were normalized on not-treated cells and represented as n-fold of control.

Prime Flow RNA assay on macrophages and satellite cell population

The expression of *MCU* and *MCUb* in both macrophages and satellite cells purified from

regenerating muscle were analyzed using the PrimeFlow RNA Assay (Thermo Fisher Scientific) as described before (https://bio-protocol.org/e2892). Different combinations of the following probes were used to stain cells: mouse MCU type 10 probe (AF568), mouse MCUb type 1 probe (AF647), mouse ARG1 type 4 probe (AF488), mouse iNOS type 6 probe (AF750), mouse PAX7 type 4 probe (AF488) and mouse ActB type 4 probe (AF488) as positive control (Thermo Fisher Scientific). Cells were also stained for extracellular markers F4/80 PE-Cy7 (BD-Bioscience) and integrin-a7 PE (AB lab) for macrophages and satellite cells respectively. Cells were analyzed with FACS Aria and data analyzed using FlowJo software.

Phagocytosis assay

Phagocytosis assay was performed using pHrodo™ Green E. coli Bioparticles™ Conjugate for phagocytosis (Thermo Fisher Scientific). Data were analyzed with FlowJo and expressed as n-fold of negative control. 7 days after differentiation, BMDMs from WT or *MCUb-/-* mice were harvested with PBS with 2 mM EDTA. Cells were then starved in IMDM 0,2% BSA for 40 minutes at 37° C. After starvation, 105 macrophages were seeded into pre-chilled 96-well tissue culture treated plate (Round bottom; FALCON®) and kept at 4° C for 30 minutes. To analyze the phagocytosis of apoptotic cells, cells were prepared as described (*15*) and stained with the far-red dye 1,3-dichloro-9,9-dimethylacridin-2-one-7-yl (DDAO) (Thermo Fisher Scientific). Apoptotic cells were prepared as follows. C2C12 muscle cells were stained with Calcein green (Thermo Fisher Scientific) and apoptosis was induced by heath shock (10 minutes at 56° C) followed by treatment with staurosporin (Merck). A ratio of 20:1 apoptotic cells were added to macrophages and cells were placed at 37° C. Cells pre-treated with Cytochalasin D (CytD) (Merk-Milliore) were used as negative control. At the indicated time points cells were collected, internal fluorescence (not-internalized particles) was quenched with Trypan-blue (Merk-Millipore), and phagocytosis was assessed by flow cytometry using a FACS Canto (BD Bioscience). The macrophage gate was designed based on DDAO positivity and the percentage of Calcein green positive (specifically, AC phagocyting macrophages) inside macrophages gate was calculated.

For TLR-mediated phagocytosis (*69*), cells were prepared as before. After 30 minutes on ice, pHrodo™ Bioparticles™ (prepared following the manufacturer's instructions) were added to the cells and plate was placed at 37° C. Cells pre-treated with cytochalasin D (CytD) were used as negative control. At the indicated time points cells were collected and phagocytosis was assessed by flow cytometry. Mean fluorescence intensity (M.F.I.) was measured in the FITC positive (phagocyting cells); phagocytic index (P.I.) was determined by multiplying the percentage of cells that had ingested the cells and the M.F.I. of the phagocytic gate.

For phagocytosis assay in microscopy, BMDMs were seeded on a glass cover slip and after 3 hours of adhesion, were treated with Zymosan *S. cerevisiae* BioParticles™, Texas Red™ conjugate (Thermo Fisher Scientific) at a ratio cell:beads of 1:20. After 30 minutes, cells were washed with PBS and fixed with 4% paraformaldehyde (Merck). Actin filament and nuclei were stained with AF488 conjugated Phalloidin and Hoechst 33342 (Thermo Fisher Scientific) respectively. Images were acquired with Leica SP5 confocal microscope using a 63X objective and elaborated using FIJI software.

Histochemical and Immunohistochemical staining

6 µm-thick TA muscle cryosections were prepared and stained by H&E staining kit (BioOptica), following the manufacturer's instructions. For immunofluorescence analysis, 20 µm-thick TA muscle cryosections were fixed in 4% paraformaldehyde for 20 minutes, quenched with 50 mM NH_4Cl in PBS and blocked in PBS containing 10% goat serum and 0.5% BSA for 20 minutes, as previously performed (*26*). Sections were then incubated with α-laminin rabbit primary antibody (Merck) at 1:100 in PBS with 0.5% BSA and 2% goat serum over night at 4° C to label the sarcolemma of muscle fibers. Muscle cryosections were washed 3 times with PBS. The sections were incubated for 1 hour at room temperature with an anti-rabbit Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher Scientific) at 1:500 in PBS with 0.5% BSA and 2% goat serum.

MyoD detection was performed using the standard immunofluorescence protocol decribed above. Muscle sections were incubated with an α-MyoD rabbit primary antibody (Santa Cruz) and α-laminin mouse primary antibody (Merck) at the dilution of 1:100 for 1 hour at room temperature. Muscle cryosections were washed 3 times with PBS and then incubated with an anti-rabbit Alexa Fluor 555-conjugated secondary antibody (Thermo Fisher Scientific) and an anti-mouse Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher Scientific) at 1:500 for α-MyoD and α-laminin respectively. The number of MyoD positive nuclei/total myofibers was calculated as the number of MyoD positive nuclei divided by the total number of myofibers.

Immunofluorescence detection of eMyHC positive fibers

To detect eMyHC, muscle sections were blocked in M.O.M. blocking solution (Vector Laboratory) for 1 hour at room temperature. Muscle sections were incubated for 1 hour at 37° C with an α-BF-G6 IgG (eMyHC) mouse primary antibody diluted in a PBS solution containing 0,5% BSA.

After three 5 minutes washes in PBS with 0,1% Triton X-100 (Merk), sections were incubated with an anti-mouse Alexa Fluor 555-conjugated secondary antibody (Thermo Fisher Scientific) at the dilution of 1:500 for 30 minutes at 37° C. After three 5 minutes washes in PBS with 0,1% Triton X-100 (Merk), sections were incubated with α-laminin rabbit primary antibody (Merck) at the dilution of 1:100 for 1 hour at 37° C and then after three 5 minutes washes an anti-rabbit Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher Scientific) was added and sections incubates for 30 minutes at 37° C. The number percentage of eMyHC positive myofibers/total myofibers was calculated as the percentage of eMyHC positive fibers in a regenerated area on the total number of myofibers inside the regenerated area.

Muscle fiber characterization

20 µm-thick TA muscle cryosections were labelled with α-laminin primary antibody following the protocol described above. CSA of TA muscles was manually measured by using IMAGE software (Scion, Frederick, MD). At least 500 fibers were measured for each muscle. The number of regenerating muscle fibers was calculated 14 days post-CTX injury and was expressed as the percentage of centro-nucleated myofibers divided by the total number of myofibers.

MPC culture

TA mouse muscles were enzymatically and mechanically digested using the Skeletal Muscle Dissociation kit (Miltenyi Biotec) and the gentleMACS™ Dissociator (Miltenyi Biotec), following manufacturer instructions. Mouse satellite cells were isolated from TA, gastrocnemius, and quadriceps muscles by depletion of non-target cells with the Satellite Cell Isolation Kit (Miltenyi Biotec). Non target cells were magnetically labelled with a cocktail of monoclonal antibodies conjugated with MACS® MicroBeads (Miltenyi Biotec). The magnetically labelled non-target cells were depleted by retaining them within a MACS Column in the magnetic field of a MACS Separator, whereas the unlabeled satellite cells passed through the column. Murine satellite cells were cultured using standard conditions in DMEM/F12 (Thermo Fisher Scientific) containing 20% FBS and 1% Penicillin-Streptomycin (Euroclone) and 2,5 ng/ml FGF-2 (Merck). For proliferation studies, 10.000 cell/cm2 satellite cells were seeded on laminin pre-treated slides (Merck) and incubated for 1 day with MPC-conditioned medium supplemented with 2.5% FBS as previously performed (*6*, *13*). Cells were then incubated with anti-ki67 antibodies (19679, BD PharmingenTM) and counterstained with Hoechst 33342 (Merck) for the detection of nuclei. For differentiation and fusion studies, 30.000 cell/cm2 MPCs were seeded on laminin pre-treated slides and incubated for 2 days with macrophage-conditioned medium containing 2% horse serum. Finally, cells were incubated with antibodies against desmin (D1033, Merck) and counterstained with Hoechst 33342 (Merck) for the detection of nuclei. For proliferation studies we calculated the number of Ki67+ fibers divided by the total number of nuclei. Fusion index has been calculated as the number of nuclei inside myotubes divided by the total number of nuclei.

Mitochondrial and Cytosolic Ca2 + measurements

BMDMs were plated in 24-mm round glass coverslips at 60% confluence in 6-well plates and polarized for 24 hours either with 100 ng/ml IFN-γ and LPS 100 ng/ml towards the pro-inflammatory M1 phenotype or 20 ng/ml IL-4 towards the anti-inflammatory M2 phenotype before mitochondrial and cytosolic Ca2+ measurements. For mitochondrial Ca2+ measurements cells were loaded with 3 µM of the fluorescent calcium indicator Rhod-2 AM (Thermo Fisher Scientific) for 20 minutes. For baseline mitochondrial Ca2+ measurements, M1 and M2 macrophages were then imaged for Rhod-2 fluorescence (F) on a Nikon inverted confocal microscope (552 nm excitation/600 nm detection/40X objective) at room temperature (22° C - 24° C). 500 µM ATP (Merck) was added when indicated to elicit Ca2+ release from intracellular stores. Changes in mitochondrial Ca2+ level were expressed as F/F0, where F are the fluorescent values registered at different time points and F0 is the mean of values registered at the beginning of the experiment. Ca2+ peak was expressed as mean of F/F0 values registered after ATP addition. Analysis was performed with the Fiji distribution of ImageJ (*70*).

For cytosolic Ca2+ measurements, the ratiometric fluorescent indicator Fura-2 (Fura 2-AM) was used. M1 and M2 macrophages were imaged for Fura-2AM on a Nikon inverted confocal microscope. Measurement of Ca2+ -induced fluorescence at both 340 nm and 380 nm allows the calculation of calcium concentrations based on 340/380 nm ratio (R) (*71*). Changes in cytosolic Ca2+ (340/380 nm) were expressed as R/R0, where R are the fluorescent values registered at different time points and R0 is the mean of basal values registered at the beginning of the experiment before ATP stimulation. Baseline cytosolic Ca2+ measurements were expressed as mean of R0 values registered before ATP addition. Ca2+ peak was expressed as mean of R/R0 values registered after ATP addition. Analysis was performed with the Fiji distribution of ImageJ (*70*). For cytosolic and mitochondrial Ca2+ measurements, images were background corrected frame by frame by subtracting the mean pixel value of a cell-free region of interest.

Seahorse analysis

Metabolic parameters were calculated by using the Seahorse XF24, as previously described (*72*). Briefly, polarized BMDMs were seeded in a 24-well Seahorse XF24 cell culture microplate in complete medium. To evaluate the oxygen consumption rate (OCR), the medium was replaced with Seahorse medium (DMEM D5030 Merck supplemented with 33 mM NaCl, 5 mM glucose, sodium pyruvate, 15 mg/l phenol red, 2 mM glutamine pH 7.4) 1 hour before the experiment. OCR analysis was performed by sequentially injecting electron transport chain inhibitors: oligomycin A (oligo, 1.5 μM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 1.5 μM), antimycin A (AA, 2.5 μM) and rotenone (R, 1.25 μM). OXPHOS parameters were calculated as follows: Basal respiration = average OCR before Oligo – average OCR after Rot/AA; ATP production = average OCR before Oligo – average OCR after Oligo; Maximal respiration = average OCR after FCCP – average after Rot/AA; Non-mitochondrial respiration = average after Rot/AA. To measure the extracellular acidification rate (ECAR), the medium was replaced with Seahorse medium (DMEM D5030 Merck supplemented with 33 mM NaCl, 15 mg phenol red, 2 mM glutamine pH 7.4) 1 hour before the experiment. Sodium pyruvate (1 mM) together with glucose (25 mM), Oligomycin A (1.5 μM), FCCP (1.5 μM), antimycin A (2.5 μM) and rotenone (1.25 μM) were sequentially added. Glycolytic parameters were calculated as follows: Non-Glycolytic Acidification (Non-Glyc AC) = Average of ECAR before injecting glucose, Glycolysis = average ECAR after injecting glucose – average ECAR before injecting glucose, Maximal Glycolytic capacity (Max Glyc Cap) = average ECAR after injecting Oligo – average ECAR before injecting glucose, Glycolytic Reserve (Glyc Res) = average ECAR after injecting Oligo – average after injecting glucose. All the drugs, media and chemicals used in the Seahorse experiments were purchased from Merck. Data were normalized to the total protein content, measured with BCA assay following the manufacturer's instructions (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific). Data were analyzed to calculate metabolic parameters, as previously reported (*72*).

Western Blotting of HIF-1α

For protein extraction, both WT and *MCUb-/-* murine BMDMs were grown at 70-80% confluency in 6-well plates and polarized either with 100 ng/ml IFN-γ together with 100 ng/ml LPS, to induce polarization toward the M1 phenotype, or with 20 ng/ml IL-4, to induce polarization toward the M2 phenotype. Two replicates for each condition were prepared and combined together after the lysis procedure. 24 hours after polarization, cells were washed once with PBS and then 40 μl of NuPAGE LDS Sample Buffer (4X) (Thermo Fisher Scientific) containing complete EDTA-free proteases inhibitor (Complete™ Roche Applied Science) and phosphatase inhibitors (PhosSTOP™ Roche Applied Science) and 1 mM phenylmethylsulfonyl fluoride inhibitors of serine proteases (PMSF Roche Applied Science) were added to each well plate. Cells were first scraped and then collected into microcentrifuge tubes. Samples were briefly sonicated until the viscosity was reduced. Crude extracts were centrifuged at 15000x g for 10 minutes to remove debris, and protein supernatants were collected. This lysis method allows to minimize protein degradation because HIF-1α is rapidly degraded under normoxia conditions. For each protein sample,1,4-dithiothreitol (DTT) at 100 mM concentration was added and proteins were denatured for 10 minutes at 70° C. After electrophoretic separation, proteins were transferred to nitrocellulose membranes and probed with a specific antibody for HIF-1α (Cell signaling Technology #3434) and for actin (Santa Cruz Technology SC56459 #) as loading control. Isotype-matched, horseradish peroxidase-conjugated secondary antibodies (BioRad) and chemiluminescent regeantsreagents (Super-Signal Pico, Pierce) were used. The chemiluminescent signal was detected by UVITEC Imaging Systems. Densitometry was performed to quantify the amount of HIF-1α protein level normalized to actin levels using the ImageQuantsoftware.

TMRM analysis

Polarized BMDMs were seeded in 96-well Cell Imaging Plate (Eppendorf) at 0.3 x 105 cells/well in complete medium. On the day of experiment, medium was completely removed and 180 μl/well of IMDM w/o FBS and Phenol Red containing TMRM (20 nM) and CsH (1 μM) were added. The plate was incubated at 37° C (5% CO2) for 20 minutes to allow the loading of TMRM into the macrophages. TMRM fluorescence was measured using Operetta High-Content Imaging System (PerkinElmer). After 10 minutes of basal reading, 2 μM Oligomycin was added to the wells and fluorescence was recorded for 40 minutes. Finally, 10 μM FCCP was added, and fluorescence was recorded for further 15 minutes. To assess the analysis of TMRM fluorescence in macrophages, cells were highlighted using the digital phase contrast using the Harmony® Content Imaging and Analysis software.

Metabolic characterization of WT and *MCUb-/-* macrophages

For mass spectrometry analysis, cells were extracted with 80% methanol and phase separation was achieved by centrifugation at 4° C. The methanol-water phase containing polar metabolites was separated and dried using a vacuum concentrator. For media extraction, 50 µl of medium was

extracted with methanol and treated as above. The dried metabolite samples were stored at −80° C. Metabolite levels were measured with a Quattro Micro GCMS (Waters) or with a UPLC system (Acquity, Waters), interfaced with a Quattro Premier mass spectrometer (Waters) (*73*). Calibration curves were established using standards, processed under the same conditions as the samples, at 5 concentrations (*74*). The best fit was determined using regression analysis of the peak analyte area. Chromatographic resolution was achieved as indicated (*75*, *76*).

Statistical Analysis

All in vitro experiments were replicated at least three times and at least three animals for each condition were used for in vivo analyses. Isolation of macrophages from regenerating muscle required at least three mice for each condition (1, 3 and 5 days post-CTX injury). Statistical data are presented as mean ± SEM, unless otherwise specified. Depending on the experiments, a parametric Student's t test, a parametric one-way ANOVA with *post hoc* Tukey's multiple comparison test were applied. Whenever the sample size was lower than 5, non-parametric Mann-Whitney or non-parametric Krustal-Wallis tests were performed to confirm the statistical analysis. $p \leq 0.05$ was considered significant.

SUPPLEMENTARY MATERIALS

Fig. S1-S6.

Table S1.

REFERENCES AND NOTES

1. Y. Kharraz, J. Guerra, C. J. Mann, A. L. Serrano, P. Muñoz-Cánoves, Macrophage Plasticity and the Role of Inflammation in Skeletal Muscle Repair. *Mediators Inflamm.* 2013, 1–9 (2013).
2. S. Schiaffino, K. A. Dyar, S. Ciciliot, B. Blaauw, M. Sandri, Mechanisms regulating skeletal muscle growth and atrophy. *FEBS J.* 280, 4294–4314 (2013).
3. J. G. Tidball, Mechanisms of muscle injury, repair, and regeneration. *Compr. Physiol.* 1, 2029–62 (2011).
4. S. B. P. Chargé, M. A. Rudnicki, Cellular and molecular regulation of muscle regeneration. *Physiol. Rev.* 84, 209–38 (2004).
5. J. G. Tidball, Regulation of muscle growth and regeneration by the immune system. *Nat. Rev. Immunol.* 17, 165–178 (2017).
6. M. Saclier, H. Yacoub-Youssef, A. L. Mackey, L. Arnold, H. Ardjoune, M. Magnan, F. Sailhan, J. Chelly, G. K. Pavlath, R. Mounier, M. Kjaer, B. Chazaud, Differentially activated macrophages orchestrate myogenic precursor cell fate during human skeletal muscle regeneration. *Stem Cells*. 31, 384–96 (2013).
7. E. Rigamonti, P. Zordan, C. Sciorati, P. Rovere-Querini, S. Brunelli, Macrophage plasticity in skeletal muscle repair. *Biomed Res. Int.* 2014, 560629 (2014).
8. Y. Yoshioka, A. Yamamuro, S. Maeda, Nitric oxide at a low concentration protects murine macrophage RAW264 cells against nitric oxide-induced death via cGMP signaling pathway. *Br. J. Pharmacol.* 139, 28–34 (2003).
9. J. G. Tidball, S. A. Villalta, Regulatory interactions between muscle and the immune system during muscle regeneration. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 298, R1173-87 (2010).
10. L. Arnold, A. Henry, F. Poron, Y. Baba-Amer, N. van Rooijen, A. Plonquet, R. K. Gherardi, B. Chazaud, Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J. Exp. Med.* 204, 1057–69 (2007).
11. S. Galván-Peña, L. A. J. O'Neill, Metabolic reprogramming in macrophage polarization. *Front. Immunol.* 5, 1–6 (2014).
12. C. Diskin, E. M. Pålsson-McDermott, Metabolic modulation in macrophage effector function. *Front. Immunol.* 9, 1–17 (2018).
13. R. Mounier, M. Théret, L. Arnold, S. Cuvellier, L. Bultot, O. Göransson, N. Sanz, A. Ferry, K. Sakamoto, M. Foretz, B. Viollet, B. Chazaud, AMPKα1 regulates macrophage skewing at the time of resolution of inflammation during skeletal muscle regeneration. *Cell Metab.* 18, 251–64 (2013).
14. Y. Wang, M. Subramanian, A. Yurdagul, V. C. Barbosa-Lorenzi, B. Cai, J. de Juan-Sanz, T. A. Ryan, M. Nomura, F. R. Maxfield, I. Tabas, Mitochondrial Fission Promotes the Continued Clearance of Apoptotic Cells by Macrophages. *Cell*. 171, 331-345.e22 (2017).
15. S. Zumerle, B. Calì, F. Munari, R. Angioni, F. Di Virgilio, B. Molon, A. Viola, Intercellular Calcium Signaling Induced by ATP Potentiates Macrophage Phagocytosis. *Cell Rep.* 27, 1-10.e4 (2019).
16. T. Hishikawa, J. Y. Cheung, R. V. Yelamarty, D. W. Knutson, Calcium transients during Fc receptor-mediated and nonspecific phagocytosis by murine peritoneal macrophages. *J. Cell Biol.* 115, 59–66 (1991).
17. M. Vaeth, I. Zee, A. R. Concepcion, M. Maus, P. Shaw, C. Portal-Celhay, A. Zahra, L. Kozhaya, C. Weidinger, J. Philips, D. Unutmaz, S. Feske, Ca 2+ Signaling but Not Store-Operated Ca 2+ Entry Is Required for the Function of Macrophages and Dendritic Cells . *J. Immunol.* 195, 1202–1217 (2015).
18. S. Tedesco, V. Scattolini, M. Albiero, M. Bortolozzi, A. Avogaro, A. Cignarella, G. P. Fadini,

Mitochondrial calcium uptake is instrumental to alternative macrophage polarization and phagocytic activity. *Int. J. Mol. Sci.* 20 (2019), doi:10.3390/ijms20194966.
19. S. Feno, R. Rizzuto, A. Raffaello, D. Vecellio Reane, The molecular complexity of the Mitochondrial Calcium Uniporter. *Cell Calcium*. 93, 102322 (2021).
20. D. De Stefani, A. Raffaello, E. Teardo, I. Szabò, R. Rizzuto, A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. *Nature*. 476, 336–340 (2011).
21. J. M. Baughman, F. Perocchi, H. S. Girgis, M. Plovanich, C. A. Belcher-Timme, Y. Sancak, X. R. Bao, L. Strittmatter, O. Goldberger, R. L. Bogorad, V. Koteliansky, V. K. Mootha, Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. *Nature*. 476, 341–345 (2011).
22. C. Mammucari, A. Raffaello, D. Vecellio Reane, G. Gherardi, A. de Mario, R. Rizzuto, Mitochondrial calcium uptake in organ physiology: from molecular mechanism to animal models. *Pflugers Arch. Eur. J. Physiol.* (2018), doi:10.1007/s00424-018-2123-2.
23. C. Mammucari, A. Raffaello, D. Vecellio Reane, R. Rizzuto, Molecular structure and pathophysiological roles of the Mitochondrial Calcium Uniporter. *Biochim. Biophys. Acta - Mol. Cell Res.* 1863, 2457–2464 (2016).
24. A. Raffaello, D. De Stefani, D. Sabbadin, E. Teardo, G. Merli, A. Picard, V. Checchetto, S. Moro, I. Szabò, R. Rizzuto, The mitochondrial calcium uniporter is a multimer that can include a dominant-negative pore-forming subunit. *EMBO J.* 32, 2362–76 (2013).
25. F. Fieni, S. B. Lee, Y. N. Jan, Y. Kirichok, Activity of the mitochondrial calcium uniporter varies greatly between tissues. *Nat. Commun.* 3, 1317 (2012).
26. C. Mammucari, G. Gherardi, I. Zamparo, A. Raffaello, S. Boncompagni, F. Chemello, S. Cagnin, A. Braga, S. Zanin, G. Pallafacchina, L. Zentilin, M. Sandri, D. De Stefani, F. Protasi, G. Lanfranchi, R. Rizzuto, The mitochondrial calcium uniporter controls skeletal muscle trophism in vivo. *Cell Rep.* 10, 1269–79 (2015).
27. G. Juban, B. Chazaud, Metabolic regulation of macrophages during tissue repair: insights from skeletal muscle regeneration. *FEBS Lett.* 591, 3007–3021 (2017).
28. F. O. Martinez, S. Gordon, The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 6, 13 (2014).
29. W. C. Skarnes, B. Rosen, A. P. West, M. Koutsourakis, W. Bushell, V. Iyer, A. O. Mujica, M. Thomas, J. Harrow, T. Cox, D. Jackson, J. Severin, P. Biggs, J. Fu, M. Nefedov, P. J. de Jong, A. F. Stewart, A. Bradley, A conditional knockout resource for the genome-wide study of mouse gene function. *Nature*. 474, 337–42 (2011).
30. S. Feske, H. Wulff, E. Y. Skolnik, *Ion Channels in Innate and Adaptive Immunity* (2015), vol. 33.
31. X. Zhou, W. Yang, J. Li, Ca2+- and protein kinase C-dependent signaling pathway for nuclear factor-κB activation, inducible nitric-oxide synthase expression, and tumor necrosis factor-α production in lipopolysaccharide-stimulated rat peritoneal macrophages. *J. Biol. Chem.* 281, 31337–31347 (2006).
32. H. Kang, K. Zhang, D. S. H. Wong, F. Han, B. Li, L. Bian, Near-infrared light-controlled regulation of intracellular calcium to modulate macrophage polarization. *Biomaterials*. 178, 681–696 (2018).
33. N. Demaurex, D. Guido, in *Advances in Experimental Medicine and Biology* (2017), pp. 277–296.
34. D. Guido, N. Demaurex, P. Nunes, Junctate boosts phagocytosis by recruiting endoplasmic reticulum Ca2+ stores near phagosomes. *J. Cell Sci.* 128, 4074–4082 (2015).
35. N. Zanou, P. Gailly, Skeletal muscle hypertrophy and regeneration: interplay between the myogenic regulatory factors (MRFs) and insulin-like growth factors (IGFs) pathways. *Cell. Mol. Life Sci.* 70, 4117–4130 (2013).
36. V. D. Soleimani, V. G. Punch, Y. ichi Kawabe, A. E. Jones, G. A. Palidwor, C. J. Porter, J. W. Cross, J. J. Carvajal, C. E. M. Kockx, W. F. J. van IJcken, T. J. Perkins, P. W. J. Rigby, F. Grosveld, M. A. Rudnicki, Transcriptional Dominance of Pax7 in Adult Myogenesis Is Due to High-Affinity Recognition of Homeodomain Motifs. *Dev. Cell*. 22, 1208–1220 (2012).
37. S. McArthur, G. Juban, T. Gobbetti, T. Desgeorges, M. Theret, J. Gondin, J. E. Toller-Kawahisa, C. P. Reutelingsperger, B. Chazaud, M. Perretti, R. Mounier, Annexin A1 drives macrophage skewing to accelerate muscle regeneration through AMPK activation. *J. Clin. Invest.* 130, 1156–1167 (2020).
38. A. Tosatto, R. Sommaggio, C. Kummerow, R. B. Bentham, T. S. Blacker, T. Berecz, M. R. Duchen, A. Rosato, I. Bogeski, G. Szabadkai, R. Rizzuto, C. Mammucari, The mitochondrial calcium uniporter regulates breast cancer progression via HIF -1α . *EMBO Mol. Med.* 8, 569–585 (2016).
39. D. G. Ryan, L. A. J. O'Neill, Krebs cycle rewired for macrophage and dendritic cell effector functions. *FEBS Lett.* 591, 2992–3006 (2017).
40. A. K. Jha, S. C.-C. Huang, A. Sergushichev, V. Lampropoulou, Y. Ivanova, E. Loginicheva, K. Chmielewski, K. M. Stewart, J. Ashall, B. Everts, E. J. Pearce, E. M. Driggers, M. N. Artyomov, Network Integration of Parallel Metabolic and Transcriptional Data Reveals Metabolic Modules that Regulate Macrophage Polarization. *Immunity*. 42, 419–430 (2015).
41. G. M. Tannahill, A. M. Curtis, J. Adamik, E. M. Palsson-McDermott, A. F. McGettrick, G. Goel, C.

Frezza, N. J. Bernard, B. Kelly, N. H. Foley, L. Zheng, A. Gardet, Z. Tong, S. S. Jany, S. C. Corr, M. Haneklaus, B. E. Caffrey, K. Pierce, S. Walmsley, F. C. Beasley, E. Cummins, V. Nizet, M. Whyte, C. T. Taylor, H. Lin, S. L. Masters, E. Gottlieb, V. P. Kelly, C. Clish, P. E. Auron, R. J. Xavier, L. A. J. O'Neill, Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature*. 496, 238–42 (2013).
42. L. Zhu, Q. Zhao, T. Yang, W. Ding, Y. Zhao, Cellular metabolism and macrophage functional polarization. *Int. Rev. Immunol.* 34, 82–100 (2015).
43. J. Van den Bossche, J. Baardman, N. A. Otto, S. van der Velden, A. E. Neele, S. M. van den Berg, R. Luque-Martin, H.-J. Chen, M. C. S. Boshuizen, M. Ahmed, M. A. Hoeksema, A. F. de Vos, M. P. J. de Winther, Mitochondrial Dysfunction Prevents Repolarization of Inflammatory Macrophages. *Cell Rep.* 17, 684–696 (2016).
44. K. L. Eales, K. E. R. Hollinshead, D. A. Tennant, Hypoxia and metabolic adaptation of cancer cells. *Oncogenesis*. 5, e190–e190 (2016).
45. J. Dort, P. Fabre, T. Molina, N. A. Dumont, Macrophages Are Key Regulators of Stem Cells during Skeletal Muscle Regeneration and Diseases. *Stem Cells Int.* 2019 (2019), doi:10.1155/2019/4761427.
46. T. Lawrence, G. Natoli, Transcriptional regulation of macrophage polarization: Enabling diversity with identity. *Nat. Rev. Immunol.* 11, 750–761 (2011).
47. C. G. Freire-de-Lima, Q. X. Yi, S. J. Gardai, D. L. Bratton, W. P. Schiemann, P. M. Henson, Apoptotic cells, through transforming growth factor-β, coordinately induce anti-inflammatory and suppress pro-inflammatory eicosanoid and NO synthesis in murine macrophages. *J. Biol. Chem.* 281, 38376–38384 (2006).
48. A. M. Johann, A. von Knethen, D. Lindemann, B. Brüne, Recognition of apoptotic cells by macrophages activates the peroxisome proliferator-activated receptor-γ and attenuates the oxidative burst. *Cell Death Differ.* 13, 1533–1540 (2006).
49. L. Racioppi, P. K. Noeldner, F. Lin, S. Arvai, A. R. Means, Calcium/calmodulin-dependent protein kinase kinase 2 regulates macrophage-mediated inflammatory responses. *J. Biol. Chem.* 287, 11579–11591 (2012).
50. A. Yarilina, K. Xu, J. Chen, L. B. Ivashkiv, TNF activates calcium-nuclear factor of activated T cells (NFAT)c1 signaling pathways in human macrophages. *Proc. Natl. Acad. Sci. U. S. A.* 108, 1573–8 (2011).
51. P. Nunes, N. Demaurex, The role of calcium signaling in phagocytosis. *J. Leukoc. Biol.* 88, 57–68 (2010).
52. A. Raffaello, C. Mammucari, G. Gherardi, R. Rizzuto, Calcium at the Center of Cell Signaling: Interplay between Endoplasmic Reticulum, Mitochondria, and Lysosomes. *Trends Biochem. Sci.* 41 (2016), doi:10.1016/j.tibs.2016.09.001.
53. A. Viola, F. Munari, R. Sánchez-Rodríguez, T. Scolaro, A. Castegna, The Metabolic Signature of Macrophage Responses. *Front. Immunol.* 10, 1462 (2019).
54. M. Mazzone, A. Menga, A. Castegna, Metabolism and TAM functions-it takes two to tango. *FEBS J.* 285, 700–716 (2018).
55. E. M. Palmieri, A. Menga, R. Martín-Pérez, A. Quinto, C. Riera-Domingo, G. De Tullio, D. C. Hooper, W. H. Lamers, B. Ghesquière, D. W. McVicar, A. Guarini, M. Mazzone, A. Castegna, Pharmacologic or Genetic Targeting of Glutamine Synthetase Skews Macrophages toward an M1-like Phenotype and Inhibits Tumor Metastasis. *Cell Rep.* 20, 1654–1666 (2017).
56. G. L. Semenza, Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene*. 29, 625–34 (2010).
57. J. Kim, I. Tchernyshyov, G. L. Semenza, C. V Dang, HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab.* 3, 177–85 (2006).
58. J. Gondin, M. Théret, G. Duhamel, K. Pegan, J. R. R. Mathieu, C. Peyssonnaux, S. Cuvellier, C. Latroche, B. Chazaud, D. Bendahan, R. Mounier, Myeloid HIFs Are Dispensable for Resolution of Inflammation during Skeletal Muscle Regeneration. *J. Immunol.* 194, 3389–3399 (2015).
59. N. Scheerer, N. Dehne, C. Stockmann, S. Swoboda, H. A. Baba, A. Neugebauer, R. S. Johnson, J. Fandrey, Myeloid hypoxia-inducible factor-1α is essential for skeletal muscle regeneration in mice. *J. Immunol.* 191, 407–14 (2013).
60. E. M. Palmieri, I. Spera, A. Menga, V. Infantino, V. Porcelli, V. Iacobazzi, C. L. Pierri, D. C. Hooper, F. Palmieri, A. Castegna, Acetylation of human mitochondrial citrate carrier modulates mitochondrial citrate/malate exchange activity to sustain NADPH production during macrophage activation. *Biochim. Biophys. Acta - Bioenerg.* 1847, 729–738 (2015).
61. E. L. Mills, B. Kelly, A. Logan, A. S. H. Costa, M. Varma, C. E. Bryant, P. Tourlomousis, J. H. M. Däbritz, E. Gottlieb, I. Latorre, S. C. Corr, G. McManus, D. Ryan, H. T. Jacobs, M. Szibor, R. J. Xavier, T. Braun, C. Frezza, M. P. Murphy, L. A. O'Neill, Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell*. 167, 457-470.e13 (2016).

62. K. Benmoussa, J. Garaude, R. Acín-Pérez, How Mitochondrial Metabolism Contributes to Macrophage Phenotype and Functions. *J. Mol. Biol.* 430, 3906–3921 (2018).
63. J. Naujoks, C. Tabeling, B. D. Dill, C. Hoffmann, A. S. Brown, M. Kunze, S. Kempa, A. Peter, H.-J. Mollenkopf, A. Dorhoi, O. Kershaw, A. D. Gruber, L. E. Sander, M. Witzenrath, S. Herold, A. Nerlich, A. C. Hocke, I. van Driel, N. Suttorp, S. Bedoui, H. Hilbi, M. Trost, B. Opitz, IFNs Modify the Proteome of Legionella-Containing Vacuoles and Restrict Infection Via IRG1-Derived Itaconic Acid. *PLOS Pathog.* 12, e1005408 (2016).
64. V. Lampropoulou, A. Sergushichev, M. Bambouskova, S. Nair, E. E. Vincent, E. Loginicheva, L. Cervantes-Barragan, X. Ma, S. C.-C. Huang, T. Griss, C. J. Weinheimer, S. Khader, G. J. Randolph, E. J. Pearce, R. G. Jones, A. Diwan, M. S. Diamond, M. N. Artyomov, Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation. *Cell Metab.* 24, 158–166 (2016).
65. S. J. Pettitt, Q. Liang, X. Y. Rairdan, J. L. Moran, H. M. Prosser, D. R. Beier, K. C. Lloyd, A. Bradley, W. C. Skarnes, Agouti C57BL/6N embryonic stem cells for mouse genetic resources. *Nat. Methods*. 6, 493–495 (2009).
66. D. Hardy, A. Besnard, M. Latil, G. Jouvion, D. Briand, C. Thépenier, Q. Pascal, A. Guguin, B. Gayraud-Morel, J.-M. Cavaillon, S. Tajbakhsh, P. Rocheteau, F. Chrétien, Comparative Study of Injury Models for Studying Muscle Regeneration in Mice. *PLoS One*. 11, e0147198 (2016).
67. G. Juban, M. Saclier, H. Yacoub-Youssef, A. Kernou, L. Arnold, C. Boisson, S. Ben Larbi, M. Magnan, S. Cuvellier, M. Théret, B. J. Petrof, I. Desguerre, J. Gondin, R. Mounier, B. Chazaud, AMPK Activation Regulates LTBP4-Dependent TGF-β1 Secretion by Pro-inflammatory Macrophages and Controls Fibrosis in Duchenne Muscular Dystrophy. *Cell Rep.* 25, 2163-2176.e6 (2018).
68. X. Rao, X. Huang, Z. Zhou, X. Lin, An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. *Biostat. Bioinforma. Biomath.* 3, 71–85 (2013).
69. G. D. Brown, Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol.* 6, 33–43 (2006).
70. J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, Fiji: an open-source platform for biological-image analysis. *Nat. Methods*. 9, 676–82 (2012).
71. M. . Roe, J. . Lemasters, B. Herman, Assessment of Fura-2 for measurements of cytosolic free calcium. *Cell Calcium*. 11, 63–73 (1990).
72. J. Van den Bossche, J. Baardman, M. P. J. de Winther, Metabolic Characterization of Polarized M1 and M2 Bone Marrow-derived Macrophages Using Real-time Extracellular Flux Analysis. *J. Vis. Exp.* (2015), doi:10.3791/53424.
73. E. M. Palmieri, A. Menga, A. Lebrun, D. C. Hooper, D. A. Butterfield, M. Mazzone, A. Castegna, Blockade of Glutamine Synthetase Enhances Inflammatory Response in Microglial Cells. *Antioxid. Redox Signal.* 26, 351–363 (2017).
74. A. Menga, E. M. Palmieri, A. Cianciulli, V. Infantino, M. Mazzone, A. Scilimati, F. Palmieri, A. Castegna, V. Iacobazzi, SLC 25A26 overexpression impairs cell function via mt DNA hypermethylation and rewiring of methyl metabolism. *FEBS J.* 284, 967–984 (2017).
75. E. M. Palmieri, I. Spera, A. Menga, V. Infantino, V. Iacobazzi, A. Castegna, Glutamine synthetase desensitizes differentiated adipocytes to proinflammatory stimuli by raising intracellular glutamine levels. *FEBS Lett.* 588, 4807–4814 (2014).
76. V. Iacobazzi, V. Infantino, F. Bisaccia, A. Castegna, F. Palmieri, Role of FOXA in mitochondrial citrate carrier gene expression and insulin secretion. *Biochem. Biophys. Res. Commun.* 385, 220–224 (2009).

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FIGURE LEGENDS

Figure 1. *MCUb* is highly expressed in macrophages during the progression of skeletal muscle regeneration and in BMDMs polarized with pro- and anti-inflammatory stimuli.

(**A**) – (**C**) In all experiments, adult WT C57BL/6N male animals were used. 50 µl of 10 µM CTX were injected in TA muscles. The expression of the different components of the MCU complex was evaluated by qPCR and normalized to *GAPDH*.

(**A**) qPCR analysis of *MCUb* expression on regenerating muscles 3, 7 and 14 days post-injury. n=4 mice/group.

(**B**) Regenerating muscles 2 and 4 days post-injury were dissociated and cell populations were isolated, as previously described (*27*). *MCUb* expression levels were evaluated by qPCR in M1 macrophages (CD64+/Ly6C/G+), M2 macrophages (CD64+/Ly6C/G-), satellite cells (CD31-/Sca1-/α7-int+), neutrophils (CD64-/Ly6C/G+), FAPs (CD31-/Sca1+/CD34+) and endothelial cells (CD31+). n=3 mice/group.

(**C**) Regenerating muscles 3 days post-injury were dissociated and the F4/80+ cell population was isolated. *MCU*, *MCUb*, *MICU1* and *MICU2* expression levels were evaluated by qPCR. n=4 mice/group.

(**D**) Murine BMDMs were treated either with 100 ng/ml IFN-γ and 100 ng/ml LPS (to induce polarization toward a M1 phenotype) or 20 ng/ml IL-4 (to induce polarization toward a M2 phenotype). 24 hours after polarization, total mRNA was extracted and subjected to qPCR. The expression of *MCUb* was assessed. qPCR results were normalized to control cells at time 0 and to *GAPDH*. n=3 mice/group.

Data are expressed as mean ± SEM. For data analysis, parametric one-way ANOVA was used with *post hoc* Tukey's multiple comparison test for each sample. Statistical analysis was confirmed with non-parametric Kruskal-Wallis test. * $p \leq 0.05$; *** $p \leq 0.001$.

Figure 2. Regenerating TA muscles of *MCUb-/-* mice display reduced expression of anti-inflammatory markers with a concomitant induction of pro-inflammatory markers.

(**A**) – (**D**) WT and *MCUb-/-* TA muscles were injured with CTX and harvested for analysis at 3, 7 and 14 days post-injury. The mRNA expression levels of both anti-inflammatory (*CD206* (A) and *ARG1* (B)), and pro-inflammatory (*TNF-α* (C) and *iNOS* (D)) markers were assessed by qPCR and normalized to *GAPDH*. n=5 mice/group.

(**E**) – (**H**) Cellular infiltrate was isolated from regenerating muscles of WT and *MCUb-/-* mice 1, 3 and 5 days after CTX injection. Four cell populations were detected in treated muscles using cytometer antibodies specific for CD11b (myeloid lineage cells), Ly6C and Ly6G (monocytes), F4/80 (macrophages) and CD206 (M2 macrophages). The bar diagrams represent the percentage of monocytes (Ly6C+/Ly6G+) (E), macrophages (Ly6C-/Ly6G-) (F), M1 (CD206-) and M2 macrophages (CD206+) (G), calculated within the gate of positive cell (F4/80+) (H) in regenerating muscles 1,3 and 5 days post-CTX injection. n=4 mice/group.

Data are expressed as mean ± SEM. For qPCR data, parametric one-way ANOVA was used with *post hoc* Tukey's multiple comparison test for each sample in (A) – (D). Statistical analysis was confirmed with non-parametric Kruskal-Wallis test. For FACS experiments parametric t-test was used (E) – (G). Statistical analysis was confirmed with non-parametric Mann-Whitney test. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 3. MCUb ablation causes a delay in alternative macrophages polarization and affects the phagocytic capacity of macrophages.

(**A**) – (**F**) Murine BMDMs from WT and *MCUb-/-* were treated either with 100 ng/ml IFN-γ and 100 ng/ml LPS (to induce polarization toward a M1 phenotype) or 20 ng/ml IL-4 (to induce polarization toward a M2 phenotype). 24 hours after polarization, total mRNA was extracted and subjected to qPCR. The expression levels of pro-inflammatory *IL-1*b (A), *IL-6* (B) and *iNOS* (C)) (A) – (C), and anti-inflammatory (*ARG1* (D), *FIZZ1* (E) and *CD206* (F)) markers (D) – (F) were assessed. qPCR results were normalized to control cells at time 0 and to *GAPDH*. n=5 sets of cells/group from 3 mice/group.

(**G**) and (**H**) Phagocytosis was assessed in murine macrophages from WT or *MCUb-/-* using Calcein loaded apoptotic cells (AC) (G) or pHrodo™ Green E. Coli Bioparticles™ Conjugate and Zymosan BioParticles™, Texas Red™ conjugate (H). In (G), macrophages were counter stained with D-DAO, and external fluorescence, due to binding without internalization of Calcein loaded-AC, was quenched using trypan blue. Percentage of double positive cells DDAO and Calcein, (macrophages that have engulfed AC) was evaluated and data were normalized with the respective CytD negative control. n=4. In (H), the Phagocytosis Index (P.I.) was calculated by multiplying the percentage of pHrodo Green positive cells (cells that have engulfed bioparticles) and the Mean Fluorescence Intensity (M.F.I.) of these cells. The M.F.I. of pHrodo is inversely proportional to pH and thus proportional to the phago-lysosome maturation; CytD was used as negative control (H). n=6 sets of cells/group from 3 mice/group.

In microscopy images, Zymosan BioParticles™, Texas Red™ conjugate and two representative images are shown. Scale bar: 20 μm.
Data are expressed as mean ± SEM. For all data, parametric one-way ANOVA was used with *post hoc* Tukey's multiple comparison test for each sample. Statistical analysis was confirmed with non-parametric Kruskal-Wallis test. * p≤0.05; ** p≤0.01; *** p≤0.001.

Figure 4. MCUb absence influences Ca2+ levels in pro- and anti-inflammatory macrophages.
Murine BMDMs were treated either with 100 ng/ml IFN-γ and 100 ng/ml LPS (to induce polarization toward a M1 phenotype) or 20 ng/ml IL-4 (to induce polarization toward a M2 phenotype).
(**A**) and (**B**) Cells were loaded with the fluorescent Ca2+ indicator Rhod-2 AM. Mitochondrial Ca2+ measurements were performed in WT and *MCUb-/-* M1 and M2 macrophages under resting conditions (A) or challenged with 500 μM ATP (B). In (B), representative traces are shown on the right. n ³ 20 cells/per group.
(**C**) and (**D**) Cells were loaded with the ratiometric fluorescent indicator Fura2-AM. Cytosolic Ca2+ measurements were performed in WT and *MCUb-/-* M1 and M2 macrophages under resting conditions (C) or challenged with 500 μM ATP (D). In (D), representative traces are shown on the right. n ³ 20 cells/per group.
Data are expressed as mean ± SEM; For data analysis, one-way ANOVA was used with *post hoc* Tukey's multiple comparison test for each sample. n.s.: not significant. ** p≤0.01, *** p≤0.001, ** p≤0.01.
non-injected TA muscles (Ctrl)

Figure 5. MCUb constitutive ablation affects muscle regenerative capacity.
(**A**) Hematoxylin-eosin (H&E) staining of WT and *MCUb-/-*
and 3, 7 and 14 days after CTX injection. Scale bar: 50 μm.
(**B**) – (**C**) mRNA expression levels of markers of myogenic precursor cells *Pax7* (B), *MyoD* (C) were analyzed by qPCR and normalized to *GAPDH* in WT and *MCUb-/-* TA muscles 3, 7 and 14 days post-CTX injection. n=4 mice/group.
(**D**) Regenerating muscles of WT and *MCUb-/-* at 3 days post-CTX injection were subjected to immunohistochemistry with MyoD (red) and laminin (green) to mark the sarcolemma. On the left, bar graph showing the quantification of number of MyoD positive nuclei on the total number of fibers. On the right, representative images of the experiment. n=3 mice/group. Scale bar: 100 μm.(**E**) *MyoG* was analyzed by qPCR and normalized to *GAPDH* as performed in (B) and (C). n=4 expression mice/group.
(**F**) H&E and immunofluorescence images of WT and *MCUb-/-* regenerating muscles after triple skeletal muscle injuries. The staining was performed at 16 days from the last injection. An antibody for laminin (green) was used to detect skeletal muscle fibers. Scale bar: 50 μm.
(**G**) Bar graph showing the quantification of the CSA of regenerating fibers of WT and *MCUb-/-* mice in (F). n=4 mice/group.
(**H**) Frequency histograms showing the distribution of CSA of ~1000 regenerating fibers of WT and *MCUb-/-* mice as in (G). n ³ 1000 fibers/per group.
Data are expressed as mean ± SEM. Parametric one-way ANOVA was used with *post hoc* Tukey's multiple comparison test for each sample in (B), (C) and (E). Statistical analysis was confirmed with non-parametric Kruskal-Wallis test. Parametric t-test was used for each sample in (D) and (G). Statistical analysis was confirmed with non-parametric Mann-Whitney test. * p≤0.05, ** p≤0.01, *** p≤0.001.

Figure 6. MCUb ablation in macrophages affects muscle regenerative capacity by inhibiting M2 macrophage ability to promote myogenic differentiation, thus affecting myotube formation in vitro.
(**A**) – (**D**) C57BL/6N CD45.1 mice were irradiated and then transplanted with bone marrow cells isolated from C57BL/6N CD45.2 WT or *MCUb–/–* mice. Transplanted recipient animals are indicated as trWT and trMCUb-/-
. Bone marrow engraftment was checked on blood samples after 6 weeks. TA muscles of these mice were then injected with CTX and muscles analyzed 3 or 14 days later. Engraftment was confirmed on the bone marrow of each animal on the day of sacrifice.
(**A**) staining of TA muscles of trWT or trMCUb-/-
non-injected and injected with CTX
for 3 and 14 days. Scale bar: 100 μm.
(**B**) Bar graph showing the quantification of the CSA of regenerating fibers of trWT and trMCUb-/- mice in (A). n=3 mice/group.
(**C**) Frequency histograms showing the distribution of CSA of ~2500 regenerating fibers of trWT and trMCUb-/- mice as in (A). n=3 mice/group.

(**D**) Regenerating muscles of trWT and trMCUb-/- at 14 days upon CTX injection were subjected to immunohistochemistry with embryonic myosin heavy chain (eMyHC, red) and laminin H&E (green) to mark the sarcolemma. On the left, bar graph showing the quantification of the percentage of eMyHC positive fibers. On the right representative images of the experiment. n=3 mice/group. Scale bar: 100 μm.
(E) Regenerating muscles of trWT and trMCUb-/- at 14 days upon CTX injection were subjected to immunohistochemistry with Pax7 (red) and laminin (green) to mark the sarcolemma. On the left, bar graph showing the quantification of number of Pax7 positive nuclei on the total number of fibers. On the right, representative images of the experiment. n=3 mice/group. Scale bar: 100 μm.(**F**) – (**H**) WT and *MCUb-/-* macrophages were treated either with 100 ng/ml IFN-γ and 100 ng/ml LPS (to induce polarization toward a M1 phenotype) or 20 ng/ml IL-4 (to induce polarization toward a M2 phenotype) and macrophage conditioned media (MP-CM) was added to WT satellite cells. (F) MPC proliferation was measured as number of Ki67+ cells on the total number nuclei. (G) and (H) The MPC fusion index was calculated after desmin and nuclei labelling (red, desmin; blue, Hoechst) as the number of nuclei inside myotubes on the total number of nuclei. n=3 mice/group. Scale bar: 50 μm.
Data are expressed as mean ± SEM. Parametric t-test was used for each sample in (B), (D) and (E). Statistical analysis was confirmed with non-parametric Mann-Whitney test. Parametric one-way ANOVA was used with *post hoc* Tukey's multiple comparison test for each sample in (F) and (G). Statistical analysis was confirmed with non-parametric Kruskal-Wallis test. * $p \leq 0.05$.

Figure 7. MCUb ablation induces the stabilization of HIF-1a and increases glycolytic capacity without affecting OXPHOS in macrophages.

(**A**) – (**F**) WT and *MCUb-/-* murine BMDMs were polarized for 24 hours either with 100 ng/ml IFN-γ and 100 ng/ml LPS (to induce polarization toward a M1 phenotype) or 20 ng/ml IL-4 (to induce polarization toward a M2 phenotype).
(A) Polarized BMDMs were harvested 24 hours after polarization and total proteins were extracted and subjected to western blotting analysis for HIF-1α and actin, used as loading control.
(B) Quantification of the levels of HIF-1α protein obtained as in (A) and normalized to actin. n = 3 independent experiments.
(**C**) – (**F**) Oxygen Consumption Rate (OCR) ((C) and (D)) and extracellular Acidification Rate (ECAR) ((E) and (F)) were measured in independent assays. (C) and (D) Serial injections were performed: Olygomycin (1,5 μM), FCCP (1.6 μM), rotenone and antimycin A (1.5 and 2.5 μM respectively). n=4 replicates.
(E) and (F) cells were cultured in glucose-free medium for two hours before running the assay. 25 mM glucose was added in the first injection, followed by Oligomycin (1,5 μM), FCCP (1,6 μM), rotenone and antimycin A (1.5 μM and 2.5 μM, respectively). n=5 replicates.
Results are presented as mean ± SEM. Statistical significance was determined by parametric one-way ANOVA with *post hoc* Tukey's multiple comparison test for each sample in (B), (D) and (F). Statistical analysis was confirmed with non-parametric Kruskal-Wallis test. * p 0.05; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 8. MCUb ablation forces metabolism toward a stronger pro-inflammatory state by increasing TCA cycle intermediate accumulation.

(**A**) – (**E**) LC-MS/MS quantification of metabolites in WT and *MCUb-/-* murine BMDMs polarized for 24 hours either with 100 ng/ml IFN-γ and 100 ng/ml LPS (to induce polarization to a M1 phenotype) or 20 ng/ml IL-4 (to induce polarization to a M2 phenotype). n=6 mice/group.
Data are presented means ± SEM. Statistical significance was calculated by ANOVA test with Tukey *post hoc* test. *$p \leq <0.05$; ** $p \leq <0.01$; *** $p \leq 0.001$.
(**F**) Schematic representation of the metabolic consequences of MCUb ablation in macrophages. *MCUb-/-* M1 macrophages display a higher HIF-1α level, which enhances glycolysis and pyruvate anaplerosis to oxaloacetate (OA). Transamination of OA leads to aspartate that, in the cytosol, condenses with citrulline, produced from arginine through NOS, leading to argininosuccinate. Fumarate and then malate are produced. Malate enters mitochondria in exchange with citrate and replenishes the TCA cycle, leading to OA and then citrate. 2-oxoglutarate (2-OG) derived from OA transamination can be channeled into the TCA cycle, leading to succinate accumulation, as a result of the first interruption of the TCA cycle occurring in M1 macrophages. Citrate accumulates as the result of the second interruption of the TCA cycle and leads to itaconate synthesis. PC, pyruvate carboxylase; NOS, nitric oxide synthase.