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**MACROPHAGE-DERIVED SUPEROXIDE PRODUCTION AND ANTIOXIDANT
RESPONSE FOLLOWING SKELETAL MUSCLE INJURY**

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Running title: Low levels of superoxide in macrophages following skeletal muscle injury

Key words: oxidative stress, tissue repair, sterile inflammation.

Highlights:

- Macrophage-derived ROS release is absent or weak after an acute skeletal muscle injury.
- Antioxidant gene expression is similar in pro and anti-inflammatory macrophages.
- Antioxidant supplementation is deleterious for skeletal muscle regeneration after an acute injury.

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ABSTRACT

Macrophages are key players of immunity that display different functions according to their activation states. In a regenerative context, pro-inflammatory macrophages (Ly6C^{pos}) are involved in the mounting of the inflammatory response whereas anti-inflammatory macrophages (Ly6C^{neg}) dampen the inflammation and promote tissue repair. Reactive oxygen species (ROS) production is a hallmark of tissue injury and of subsequent inflammation as described in a bacterial challenge context. However, whether macrophages produce ROS following a sterile tissue injury is uncertain. In this study, we used complementary *in vitro*, *ex vivo* and *in vivo* experiments in mouse to show that macrophages do not release ROS following a sterile injury in skeletal muscle. Furthermore, expression profiles of genes involved in the response to oxidative stress in Ly6C^{pos} and Ly6C^{neg} macrophage subsets did not indicate any antioxidant response in this context. Finally, *in vivo*, pharmacological antioxidant supplementation with N-Acetyl-cysteine (NAC) following skeletal muscle injury did not alter macrophage phenotype during skeletal muscle regeneration. Overall, these results indicate that following a sterile injury, macrophage-derived ROS release is not involved in the regulation of the inflammatory response in the regenerating skeletal muscle.

INTRODUCTION

Macrophages play various important roles in tissue homeostasis (Chazaud, 2014). They exhibit a wide spectrum of phenotypes or inflammatory status, which are associated with a diversity of functions (Chazaud, 2014). Upon an immune challenge, macrophages invading the infected area exhibit a pro-inflammatory phenotype, that support microbicidal activity, mainly through the activation of Toll-like receptors (TLRs) upon recognition of Pathogen Associated Molecular Patterns (PAMPs). Once the pathogens phagocytosed and inactivated, resolution of inflammation takes place to avoid too much tissue degradation (Chazaud, 2014). Inflammation is often associated with an increase of the production of reactive oxygen species (ROS) (Le Moal et al., 2017). It is largely accepted that macrophages produce and release ROS to support bactericidal activity and counteract with invader pathogens (West et al., 2011). Thus, ROS are important mediators of macrophage functions during immune challenges (Brune et al., 2013; Forman and Torres, 2001).

After a tissue damage that does not involve bacteria or virus (*i.e.* sterile inflammation), the activation of macrophages infiltrating the injured area was evidenced *via* the Damage Associated Molecular Patterns (DAMPs) that also activate TLRs. However, the extent and quality of the pro-inflammatory status of these pro-inflammatory macrophages have been poorly addressed *in vivo*. In this context, we recently showed that soon after skeletal muscle injury, monocyte-derived macrophages exhibit a pro-inflammatory status, which is different from that of classical M1 (IFN γ driven) activation and is likely related to tissue damage (Varga et al., 2016). Few days after the injury, the resolution of inflammation takes place and macrophages switch their phenotype towards an anti-inflammatory/recovery status to

exert repairing function in the tissue (Arnold et al., 2007; Mounier et al., 2013; Perdiguero et al., 2011; Varga et al., 2013) .

Whether pro-oxidant characteristics of macrophages that were described in the context of immune challenge does occur in the context of sterile inflammation is still uncertain. iNOS, which participates in the formation of Reactive Nitrogen Species (RNS) and ROS, is expressed by inflammatory macrophages during the first days of muscle regeneration, then its expression declines with time, notably when macrophages acquire the recovery inflammatory status (Mounier et al., 2013). Mice deficient for *inos* fail to regenerate correctly because of a defect of myogenesis (Rigamonti et al., 2013) suggesting, because *inos* is mainly expressed by inflammatory macrophages infiltrating the injured area, that macrophage-derived iNOS plays a role in skeletal muscle regeneration. However, despite the assumption that inflammatory macrophages generally produce ROS that are detrimental for the tissue, no study has characterized macrophage ROS production in the context of skeletal muscle regeneration or tissue repair.

The aim of the present study was to evaluate the production of ROS by macrophage populations during skeletal muscle regeneration. To address this question, we combined *in vitro* and *ex vivo* approaches of direct biochemical measurements of ROS entities, *ex vivo* analysis of the "oxidative" gene profile of macrophages isolated from regenerating muscle, and *in vivo* pharmacological antioxidant supplementation during skeletal muscle regeneration.

MATERIALS AND METHODS**Macrophage culture**

Macrophages were obtained from bone marrow (BM) precursor cells as previously described (Mounier et al., 2013). Briefly, total BM was cultured in DMEM (Gibco) containing 20% fetal bovine serum (FBS) and 30% conditioned medium of L929 cell line. Macrophages were seeded (200 000 cells/cm²) and activated in DMEM medium containing 10% FBS for 3 days as follows: IFN- γ (50 ng/ml), IL-4 (10 ng/ml), IL-10 (10 ng/ml), Muscle Damage Associated Molecular Patterns (DAMPS) (homogenate from a muscle injured by ischemia/reperfusion, 1 μ g/ml), lipopolysaccharide (LPS, 10 ng/ml) and Phorbol 12-myristate 13 acetate (PMA, 50 ng/ml). After several washes, conditioned medium was obtained after 24h of culture of activated macrophages in DMEM serum-free medium.

Superoxide ion production assessment

Superoxide ion production assessment procedure was previously fully detailed (Bernard et al., 2012). Ferricytochrome *c* reduction was used to assess the superoxide ion production by macrophages. Macrophages activated as described above were incubated at 37°C for 2 h with 1 ml of HEPES buffer (53 mM HEPES) pH 7.4 containing 14.6 mM glucose, 358 mM NaCl, 12.7 mM KCl, 3.1 mM KH₂PO₄, 6.1 mM MgSO₄, 3.1 mM CaCl₂, 13 mM NaHCO₃, 0.4 mM EDTA and 100 μ M ferricytochrome. Absorbance was read at 550 nm with a spectrophotometer (BioSpectrometer Basic Eppendorf). Contribution of ferricytochrome *c* at 100 μ M (absorbance at 800 nm) was subtracted. Similar measurements were realized on macrophages isolated by FACs (see below) and subsequently plated for 2 h in the absence or presence of PMA (50 ng/ml) to induce an oxidative burst (Abbas et al., 2014).

Superoxide dismutase expression

Protein concentration was determined in macrophage lysates using BCA protein assay (Uptima-Interchim). Western blot analysis was performed to examine the expression of MnSOD (rabbit polyclonal anti-human SOD2 200 µg/ml, Santa Cruz Biotechnology) and Cu-ZnSOD (rabbit polyclonal anti-human SOD1 200 µg/ml, Santa Cruz Biotechnology) *versus* that of actin (Goat polyclonal anti-human actin 200 µg/ml, Santa Cruz Biotechnology). Quantification of the band intensity was performed using ImageJ Software.

Macrophage H₂O₂ release

Extracellular H₂O₂ was detected using the Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen). Macrophage-conditioned medium was collected and incubated with the Amplex Red reagent (50 µl of 10 mM solution) and horseradish peroxidase (100 µl of 10 U/ml solution) in 0.25 M sodium phosphate buffer for 30 min. Absorbance intensity was measured on an Apollo LB 911 plate reader (Berthold Technologies) at 560 nm.

Skeletal muscle injury

Eight week-old C57/Bl6 and CX3CR1^{GFP/+} male mice were used for N-acetylcysteine (NAC, Sigma) supplementation protocol and extraction of macrophages from regenerating muscle, respectively. Mice were bred and used in compliance with French and European regulations. Principal investigators are licensed for animal experimentation and the ethical committee validated the protocol. Muscle injury was caused by injection of cardiotoxin (CTX) in the *Tibialis Anterior* muscle as previously described (Mounier et al., 2013). In some experiments, LPS (50 µg/ml final) was co-injected with CTX as previously described (Varga et al., 2016). In some experiments,

NAC was administered at 4% in drinking water for 8 days from the day of injury, as previously described (Terrill et al., 2012).

Isolation of immune cells from regenerating muscle

Immune cells were isolated as described previously (Mounier et al., 2013; Varga et al., 2016). Briefly, muscles were minced and digested with collagenase B 0.2%. CD45⁺ cells were isolated by magnetic sorting procedure (Miltenyi Biotec) and further labeled with APC-CD64 and PE-Ly6C antibodies for C57/Bl6 mice and with an APC-Ly6C (all from eBiosciences) antibody for CX3CR1^{GFP/+} mice. Cells were sorted using a FACS Aria II cell sorter (BD Biosciences).

Histological analysis

At different time-points after injury (day 4, 8 and 28) regenerating muscles were harvested, snap-frozen in nitrogen-chilled isopentane and kept at -80°C until analysis. 10 µm-thick muscle cryosections were prepared and used for H/E staining and immunofluorescent staining. Cryosections were treated with antibodies directed against F4/80 (#ab6640, Abcam), iNOS (#ab15323, Abcam), and CD206 (#sc-58987, Santa Cruz) revealed with FITC-or Cy3-conjugated antibodies (Jackson ImmunoResearch Inc). Pictures were recorded with Axioscop microscope (Zeiss) at 20X of magnification connected to AxioCam ICc5 (Zeiss). For each condition in each experiment, at least 8-10 randomly chosen fields were counted. The number of labeled macrophages was calculated using ImageJ software and was expressed as a percentage of total macrophages.

For Cross Section Area (CSA) analysis, cryosections were labeled with antibodies directed against Laminin (#L9393, Sigma-Aldrich) and revealed with FITC-conjugated secondary antibody. Pictures covering the whole cryosection were recorded with an Axio Observer.Z1 (Zeiss) at 10X of magnification connected to a CoolSNAP HQ2

CCD Camera (Photometrics). CSA was determined manually using ImageJ software.

A mean of 2345 +/- 542 fibers were analyzed *per* TA muscle.

For satellite cells analysis, fresh cryosections were labeled with antibodies directed against Pax7 (clone 4ea, DHSB) and Laminin (#L9393, Sigma-Aldrich) revealed with Cy3- and FITC-conjugated antibodies, respectively. Pictures were recorded with an Axio Imager.Z1 (Zeiss) at 20X of magnification connected to a CoolSNAP Myo CCD Camera (Photometrics). For each condition, 10 randomly chosen fields were counted. The number of satellite cells was determined manually using ImageJ software and expressed as the number of Pax7+ cells *per* 100 fibers.

Statistical analyses

Results are expressed as means \pm SEM. All experiments were carried out with at least 3 different primary cultures or 3 mice for *in vivo* analyses. Isolation of macrophages from regenerating muscle required 3 to 5 mice that were pooled. Mann-Whitney and Kruskal-Wallis tests were done with Prism software.

RESULTS

Pro- and anti-inflammatory macrophages release similar levels of ROS *in vitro*

To investigate ROS production by differentially activated macrophages, BM-derived macrophages were treated with a (IFN- γ), with IL-4 or with IL-10 to trigger pro-inflammatory, alternative and anti-inflammatory activation, respectively (Murray et al., 2014). Superoxide production in conditioned medium was assessed using ferricytochrome c reduction assay (Bernard et al., 2012). This method, based on the reduction of cytochrome c by superoxide to form detectable ferrocyanide, reflects the extracellular production of superoxide. No significant superoxide production was observed upon the 3 treatments, despite a slight increase by macrophages activated with IFN γ (**Figure 1A**). To investigate ROS production by pro-inflammatory macrophages *in vitro*, cells were treated with various effectors known to induce pro-inflammatory states including a ROS-inducer (PMA), a bacterial mimetic (LPS) and a lysate from injured skeletal muscle (DAMPS). Because the measurement of anion superoxide release has never been performed in these conditions, we added two positive controls known as superoxide generators: a strong chemical inducer (PMA) (Nathan and Root, 1977) and LPS. LPS activates superoxide production and hydrogen peroxide-derived superoxide. We also tested DAMPS as a putative superoxide activator. Hence, we validated our measurement setup and were able to underline the differences between classical models of activation based on cytokine treatment and the above-mentioned treatments. Among the 3 treatments, only PMA (+139 \pm 9%) (p<0.05) treatment induced superoxide detection as compared with untreated macrophages (NT) (**Figure 1A**). To further explore ROS metabolism in activated macrophages, the level of H₂O₂, which is the product of superoxide dismutation by superoxide dismutases (SODs) (Holmstrom and Finkel, 2014), was

measured in conditioned media. Surprisingly, hydrogen peroxide production was similar in all macrophage populations (**Figure 1B**). The important lability of H_2O_2 can explain these results. Overall, these data indicate that macrophages released similar levels of superoxide and H_2O_2 irrespective of their activation state.

We further examined the expression of superoxide dismutase (SOD) enzymes by activated macrophages. MnSOD (or SOD2) is a mitochondrial enzyme responsible of the dismutation of primary formed superoxide into hydrogen peroxide, the most common reaction of ROS metabolism cascade (Holmstrom and Finkel, 2014). MnSOD expression was higher in $IFN\gamma$ macrophages as compared with (IL-10) macrophages ($p < 0.05$) (**Figure 1C**). Additionally, cytosolic SOD1 (or CuZn-SOD) expression was not significantly different in the various activated macrophages (**Figure 1D**). These data indicate that mitochondrial SOD2 was overexpressed only in pro-inflammatory macrophages, leading to an improvement of the dismutation capacity of pro-inflammatory macrophages that may result in a similar ROS release as compared with other activated macrophages.

Macrophages produce low levels of superoxide during post-injury skeletal muscle regeneration *in vivo*

To investigate ROS production by macrophages during muscle regeneration, macrophages subsets and neutrophils were sorted from regenerating muscle at day 2 after injury and assayed for the evaluation of superoxide production (**Figure 2A**). At day 2, both $Ly6C^{pos}$ and $Ly6C^{neg}$ populations are equally present (Mounier et al., 2013; Varga et al., 2013). No significant superoxide production was detected in both $Ly6C^{pos}$ and $Ly6C^{neg}$ macrophage subsets and neutrophils, as compared with the control solution (**Figure 2A, right panel**). To test the experimental conditions and the

ability of myeloid cells isolated from regenerating muscles to produce superoxide, sorted macrophages and neutrophils were immediately treated with PMA, which induces an oxidative burst and is widely used to study ROS production in myeloid cells. In this condition, PMA-stimulated neutrophils significantly produced ROS, with an upregulation of superoxide release of $162.6 \pm 9.4\%$ ($p < 0.001$) (**Figure 2A, right panel**).

Additionally, a skeletal muscle injury model with concomitant bacterial contamination with LPS was used to induce a stronger pro-inflammatory profile of Ly6C^{pos} macrophages (Varga et al., 2016) (**Figure 2B, left panel**). No significant superoxide production was detected in both macrophage subsets and neutrophils, as compared with the control solution (**Figure 2B, right panel**). Again, treatment with PMA stimulated only neutrophils to produce superoxide ($144.1 \pm 13.2\%$, $p < 0.05$) (**Figure 2B, right panel**). Taken together, these results indicate that after an injury, macrophages invading skeletal muscle release, if any, very low levels of superoxide.

Gene expression profiling does not reveal antioxidant-related response in macrophages after skeletal muscle injury

The oxidative stress signature of macrophages present in regenerating muscles was investigated, using a microarray analysis published in (Varga et al., 2016). As expected, Ly6C^{pos} macrophages expressed higher levels of a series of genes known to be associated with pro-inflammatory macrophages in skeletal muscle regeneration, including selectin L, CSF1, IL6 and CCR1 (**Figure 3, upper panel**). A set of antioxidant genes that would identify an antioxidant-related transcriptional signature was analyzed in both macrophage subsets. There was no significant differential expression of 30 genes associated with response to oxidative stress

between inflammatory Ly6C^{pos} and restorative Ly6C^{neg} macrophage subsets (**Figure 3, lower panel**).

NAC treatment impairs skeletal muscle regeneration after injury

To further assess a potential functional role of ROS in macrophages during skeletal muscle regeneration, we used a pharmacological approach using antioxidant NAC oral treatment, given from the time of muscle injury. Indeed, NAC should counteract the ROS released at early time points by pro-inflammatory macrophages. Inversely, if no ROS are present in the injured muscle, NAC should not affect skeletal muscle regeneration.

First, macrophage inflammatory status was investigated by performing double immunofluorescent staining for F4/80 and iNOS or CD206, to identify pro- and anti-inflammatory macrophages, respectively (Mounier et al., 2013). At day 4 after injury, there was no difference in the percentage of macrophages expressing iNOS or CD206 between control and NAC-treated muscles (**Figure 4A**). The number of necrotic and phagocytosed myofibers was calculated 8 days after injury, a time point when most of necrosis and phagocytosis of injured myofibers is over (Mounier et al., 2013). Although exhibiting high variations between individuals, NAC-treated group did not significantly differ from the control group (**Figure 4B**), indicating no significant difference in the time course of the regeneration process. Four weeks after injury, NAC treatment was associated with a reduction of body weight (-9%, $p < 0.01$), *Tibialis Anterior* muscle mass (-15%, $p < 0.001$) and *Tibialis Anterior* mass/body mass ratio (-8%, $p < 0.05$) (**Figure 5B**). This was correlated with a decrease in mean Cross Section Area (-15%, $p < 0.05$) and a shift of the myofiber size towards the smaller ones (**Figure 5C**). Additionally, muscles from NAC-treated group displayed a

significant lower number of nuclei *per fiber* as compared with the control group (-22%, $p < 0.001$) (**Figure 5D**). Finally, NAC-treated muscles did not show a significant difference in the number of satellite cells (**Figure 5E**). Altogether, these results suggest that NAC treatment primarily affected myofiber growth than early events related to inflammation during skeletal muscle regeneration.

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DISCUSSION

We investigated ROS release from macrophages during regenerating skeletal muscle and provided evidence that after a sterile injury, infiltrated macrophages did not release ROS and that antioxidant supplementation impaired long term skeletal muscle regeneration. To our knowledge, only one study investigated whole skeletal muscle redox changes following skeletal muscle injury induced by venom injection. It was described that skeletal muscle injury is accompanied by an increase of ROS production within the whole tissue from 1 to 7 days after injury (Vezzoli et al., 2011). However, the pro/antioxidant-related measurements were not specific to macrophage populations. These results, combined to those of the present study, suggest that ROS present in the early injured muscle are mainly derived from the myofiber than from the inflammatory compartment.

ROS are short lived and direct detection is difficult, notably *in vivo*, despite recent technological advances (Murphy et al., 2011; Winterbourn, 2008). Here, we used biochemical assays such as ferricytochrome c reduction and Amplex Red technology, which are robust, specific and adequate methods to detect the major ROS that are superoxide and hydrogen peroxide produced by the cells and released in their microenvironment (Bernard et al., 2012; Wang et al., 2012).

We report that among a set of *in vitro* activation, only macrophages treated with PMA, which induces the oxidative burst in macrophages (Abbas et al., 2014), present a significant ROS production. Additionally, no difference was observed in the release of H₂O₂, which is the product of superoxide dismutation, between the different activated macrophage populations. Protein expression of MnSOD, responsible of the dismutation of superoxide into hydrogen peroxide, the most common reaction of ROS metabolism cascade at this step (Holmstrom and Finkel, 2014), is higher in IFN γ

treated macrophages as compared with other activated macrophages. This distinct pattern of MnSOD is a hallmark of IFN γ -activated macrophages (Lawrence and Natoli, 2011). This result indicates that a higher MnSOD protein expression may counteract a higher production of superoxide (not detectable in our experimental conditions) as a stress response to restore cellular homeostasis.

Ex vivo isolation of pure populations of pro-inflammatory and anti-inflammatory macrophages (Ly6C^{pos} and Ly6C^{neg}, respectively) two days after muscle injury revealed no significant superoxide release by these cells. Technically, sorted cells, here neutrophils, were shown to secrete superoxide after PMA treatment. Similar results were observed after muscle injury upon LPS co-injection, that induces a stronger pro-inflammatory profile in Ly6C^{pos} macrophages (Varga et al., 2016). ROS production is an important feature of macrophages, notably following activation with pathogens (Fang, 2004; Laskin et al., 2011; West et al., 2011), that constitutes a different context from sterile tissue injury. The absence of a high oxidative response in macrophages upon muscle injury, notably in Ly6C^{pos} macrophages at early time points after injury, was confirmed by gene expression analysis. The mRNA expression of about 30 genes related to oxidative stress did not differ between inflammatory Ly6C^{pos} and restorative/Ly6C^{neg} macrophage from injured muscle. More broadly, gene profiling analysis of these two populations indicated that following a sterile muscle injury, inflammatory Ly6C^{pos} macrophages exhibit a specific inflammatory profile which is different from the classical M1 (IFN γ driven) status and which is strongly different from that of pathogen-challenged macrophages (Varga et al., 2016).

Finally, our *in vivo* pharmacological approach aimed at targeting ROS production early after injury, with the antioxidant NAC, a molecule commonly used as

therapeutics in a large number of pathophysiological contexts including skeletal muscle diseases and in exercise and sport performance context (Le Moal et al., 2017; Michailidis et al., 2013). We showed that NAC treatment did not alter macrophage phenotype at early time points after injury, in accordance with the above results indicating the absence of ROS release from macrophages in this context. While the number of necrotic myofibers was not significantly different from that observed in the control muscle, NAC-treated muscles showed a highly heterogeneous, somewhat increased level of necrosis 8 days after the injury, deserving further investigation on the phagocytic capacities of macrophages in these conditions. Finally, parameters of myofiber growth and fusion were altered in NAC-treated muscle 28 days after injury, suggesting an impact of anti-oxidant treatment on ROS-unrelated properties of restorative macrophages or directly on the myogenesis process. However, the number of satellite cells was unchanged after NAC treatment, suggestive of an impact of NAC on the late stage of myogenesis, that deserves further exploration. Indeed, several pathways involved in myogenesis processes may be regulated by oxidative stress, for instance IGF-I signaling or Akt-mTOR pathways (Le Moal et al., 2017). This is also suggested by a study showing that NAC supplementation after an exercise-induced muscle damage (EIMD) in human blunts the circulating inflammatory response (assessed by indirect measurements in blood) and decreases the EIMD-induced increase in the phosphorylation of a series of proteins involved in myofiber growth (PKB, mTOR, p70S6K, S6, MAPK P38). Consequently, performance of the NAC-treated group was decreased (Michailidis et al., 2013).

Our results appear to be in sharp contrast with what is observed during muscular dystrophies. Indeed, the increase in oxidative stress in muscular dystrophies is a

well-known process and correlates with the severity of the pathology (Le Moal et al., 2017). Accordingly, anti-oxidant treatments ameliorate the morphological, biochemical, and functional phenotype of the diseased muscle, including a decrease in inflammatory cells (Le Moal et al., 2017). However, the redox status of inflammatory cells, including macrophages, has not been addressed in this pathological context yet. Nevertheless, as the inflammatory status of macrophages is different during skeletal muscle regeneration and during dystrophies (Lemos et al., 2015; Vidal et al., 2008), it is likely that their redox status will also differ significantly between acute and chronic injuries.

In conclusion, this study indicates that, contrary to an immune challenge implicating bacteria or virus, macrophage-derived ROS release is absent or weak after a sterile tissue damage, and does not impact the skeletal muscle regeneration process. Additionally, we report that antioxidant supplementation from the time of injury does not impact macrophage inflammatory status but may conversely be deleterious in the later restorative phase of skeletal muscle regeneration.

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DISCLOSURE STATEMENT

Authors declare no conflict of interest.

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FIGURES LEGENDS**Figure 1. ROS and antioxidant measurements in activated macrophages *in vitro*.**

BM-derived macrophages were activated *in vitro* using different effectors. **(A)** Detection of superoxide in conditioned medium was performed after 1h of incubation with ferricytochrome *c* solution using spectrophotometry (Optical Density, O.D.). **(B)** Detection of hydrogen peroxide was performed on conditioned medium with the Amplex Red hydrogen peroxide/peroxidase assay kit. **(C)** Mitochondrial MnSOD (SOD2) expression by western-blotting. Panel illustrates a representative gel. **(D)** Cytosolic Cu-ZnSOD (SOD1) expression by western-blotting. Panel illustrates a representative gel. Results are mean \pm SEM. A minimum of 3 independent experiments were performed. * $p < 0.05$ different from untreated (NT) macrophages (A) or IL-10 activated macrophages (C).

Figure 2. Superoxide detection in conditioned medium of macrophages sorted from injured skeletal muscle.

Ly6C^{pos} and Ly6C^{neg} macrophages were sorted from regenerating *Tibialis Anterior* muscles 2 days after injury with cardiotoxin (A) and with cardiotoxin + LPS (B). On the left, flow cytometry dot plots illustrate a representative experiment showing the 3 sorted populations as neutrophils (black dotted line circle), Ly6C^{pos} inflammatory macrophages (black plain circle) and Ly6C^{neg} restorative macrophages (plain gray circle). On the right, detection of superoxide in conditioned medium was performed after 1h of incubation with ferricytochrome *c* solution using spectrophotometry. When indicated PMA treatment (50 ng/ml) was applied for 60 min. Results are expressed as a percentage of the quantity of superoxide detected in the condition medium of

control solution (ferricytochrome c solution, without cells). A minimum of 3 independent experiments were performed. ** $p < 0.01$, *** $p < 0.001$ different from control.

Figure 3. Gene expression profiling of oxidative stress-associated network in macrophages sorted from injured skeletal muscle.

Two days after injury, Ly6C^{pos} and Ly6C^{neg} macrophages were sorted from regenerating muscle and processed for microarray as in (Varga et al., 2016). Upper panel: heatmap of genes referred as highly expressed in Ly6C^{pos} macrophage subset. Lower panel: heatmap of 30 genes associated with oxidative stress. Results represent the mean of 3 independent experiments.

Figure 4: NAC supplementation effect on early steps of muscle regeneration: impact on macrophages.

Tibialis Anterior muscle was injured and mice were treated or not with NAC for 8 days. **(A)** Upper panel: Immunofluorescent staining for F4/80 and iNOS or CD206 on regenerating muscle 4 days after injury in control and NAC-treated mice. Arrow represents double-positive cells and arrowheads represent macrophages negative for the inflammatory marker. Bar=50 μm . Lower panel: quantification of the number of iNOS^{pos} and CD206^{pos} macrophages. **(B)** Necrotic/phagocytosed fibers were counted on muscle sections stained with H/E at day 8 after injury. Results are mean \pm SEM of a minimum 3 mice.

Figure 5. NAC supplementation effect on late steps of muscle regeneration: impact on myogenesis.

Tibialis Anterior muscle was injured and mice were treated or not with NAC for 8 days. Muscles were analyzed at day 28. **(A)** Hematoxylin/Eosin staining of *Tibialis Anterior* muscle sections of control mice. Bar=50 μm . **(B)** Total body weight, *Tibialis Anterior* mass and body mass/ *Tibialis Anterior* muscle/body mass ratio. **(C)** Mean and distribution of Cross Section Areas. **(D)** Number of nuclei *per* myofiber in regenerating muscle. **(E)** Number of Pax7+ cells *per* 100 fibers in regenerating muscle. Results are mean +/- SEM of a minimum 3 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ different from control.

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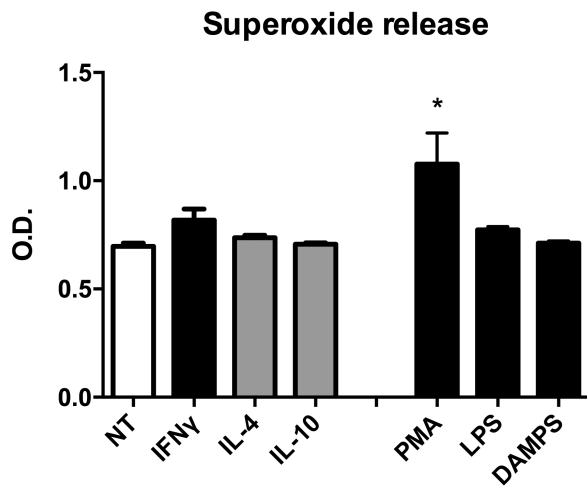
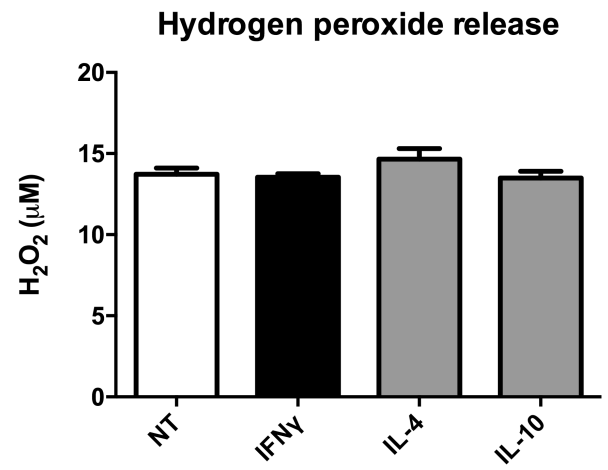
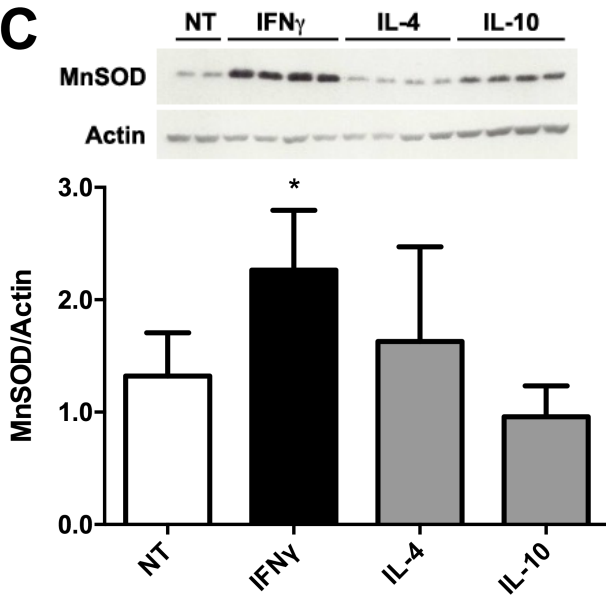
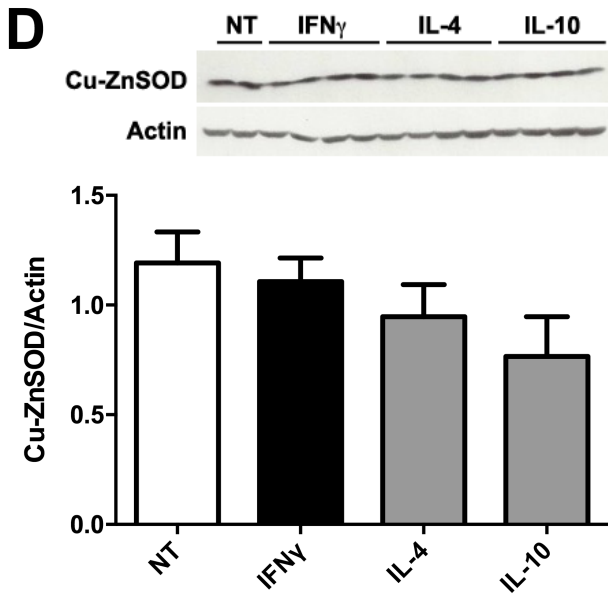
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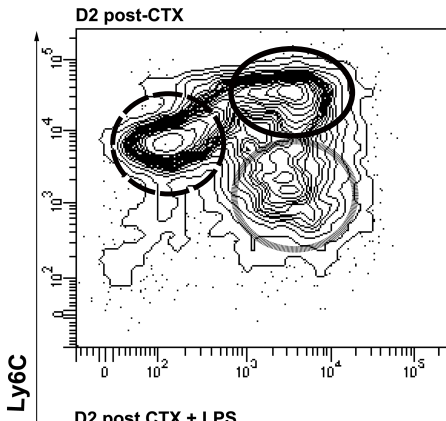
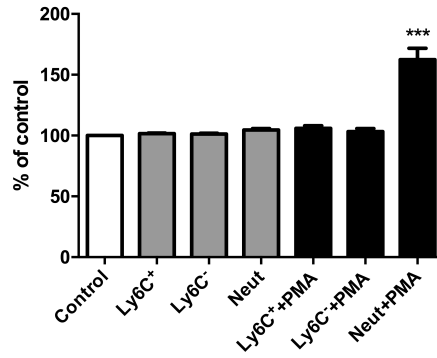
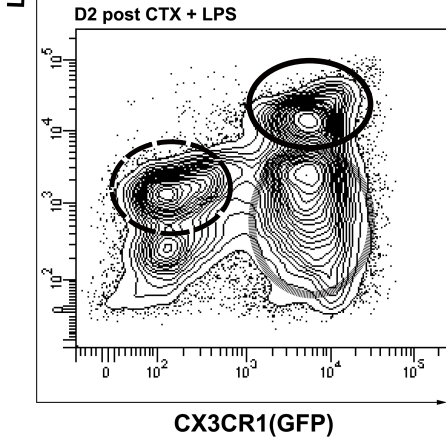
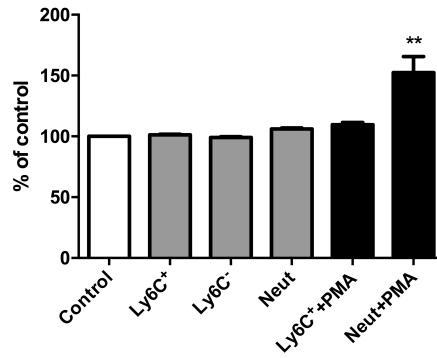
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A**B****C****D**

Accet

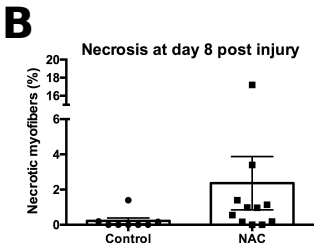
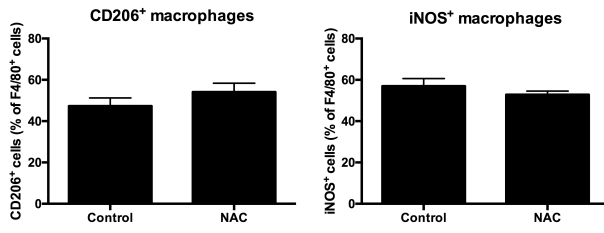
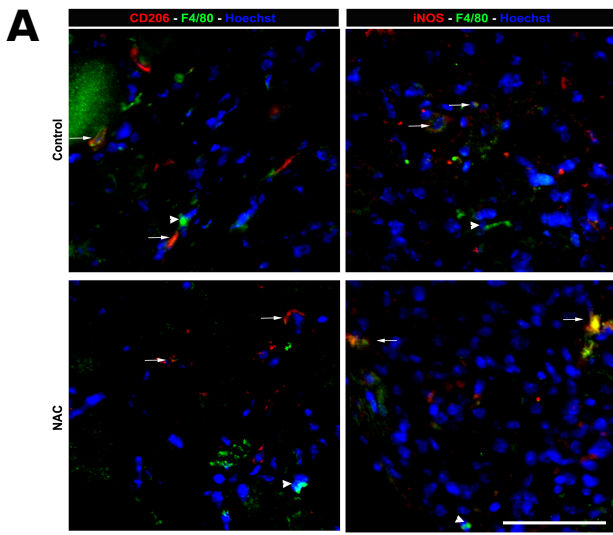
A**Superoxide release - D2 post-CTX****B****Superoxide release - D2 post-CTX+LPS**

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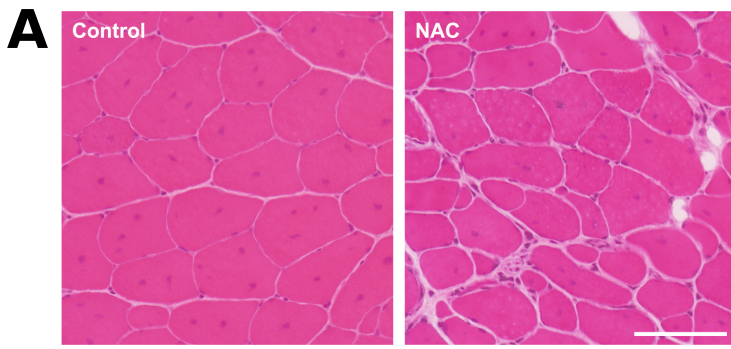
Gene	Ly6C ⁺	Ly6C ⁻
<i>Sell</i>	1.30	0.22
<i>Csf1</i>	1.12	0.07
<i>Il6</i>	1.28	0.40
<i>Ccr2</i>	0.00	-0.25
<i>Ccr1</i>	1.16	0.60
<i>Ccr5 Ccr2</i>	0.18	0.06
<i>Acox1</i>	-0.19	0.23
<i>Cat</i>	-0.24	0.18
<i>Cybs</i>	0.10	0.11
<i>Glrx</i>	0.42	0.02
<i>Glrx2</i>	0.30	0.45
<i>Glrx3</i>	0.06	0.46
<i>Glrx5</i>	0.34	0.57
<i>Gpx1</i>	-0.05	0.06
<i>Gpx3</i>	-0.46	-0.06
<i>Gpx4</i>	-0.02	0.32
<i>Grxcr1</i>	0.25	0.06
<i>Gss</i>	0.06	0.14
<i>Gstp1</i>	0.29	0.05
<i>Gstp2</i>	0.17	-0.01
<i>Mgst3</i>	0.01	0.60
<i>Mpv17</i>	-0.27	0.27
<i>Msrb2</i>	-0.20	0.07
<i>Msrb3</i>	0.00	-0.04
<i>ND1</i>	0.00	-0.07
<i>Ndor1</i>	-0.13	-0.04
<i>Prdx1</i>	0.11	0.11
<i>Prdx2</i>	0.18	0.17
<i>Prdx3</i>	0.13	0.41
<i>Prdx4</i>	-0.12	0.44
<i>Prdx5</i>	0.27	-0.05
<i>Prdx6</i>	0.34	0.04
<i>Sod1</i>	-0.01	0.11
<i>Sod2</i>	0.27	0.10
<i>Txnrd1</i>	-0.01	0.07
<i>Txnrd2</i>	0.05	0.18

-1.5

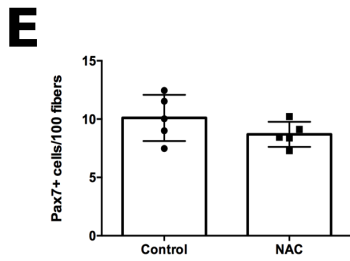
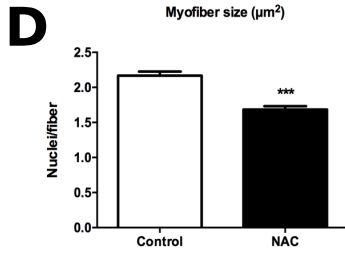
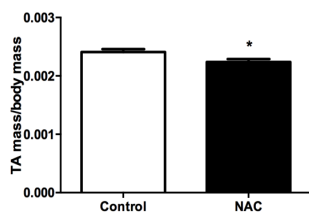
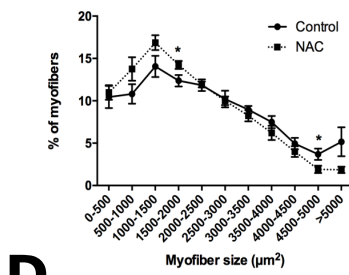
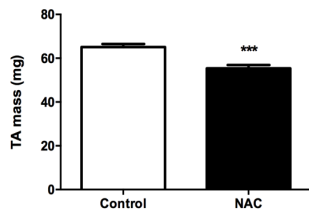
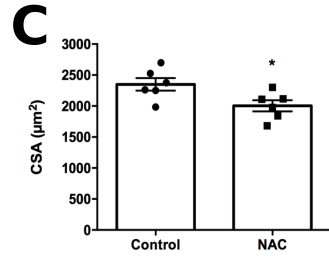
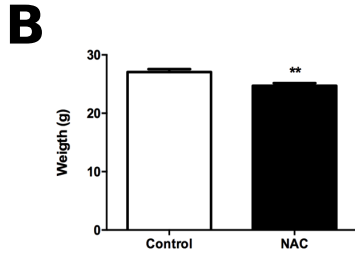
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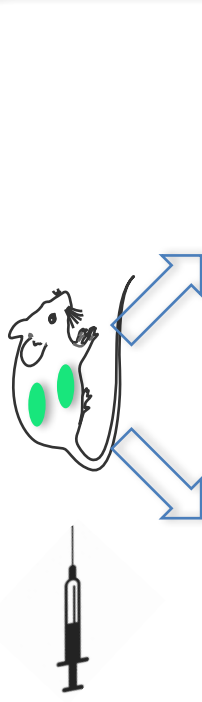


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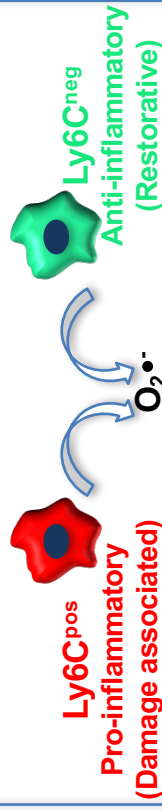
Manuscript

Graphical abstract

Skeletal muscle injury in CX3CR1:GFP/+ mice



Isolation of macrophage subsets 2 days P.I

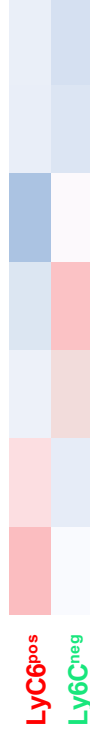


release detection

Weak O₂^{•-} release from Ly6C^{pos} cells

No trace of O₂^{•-} released from Ly6C^{neg} cells

Gene expression profiling
Oxidative stress response / Antioxidant pathway



NO O₂^{•-} RELEASE BY MACROPHAGES
FOLLOWING SKELETAL MUSCLE INJURY

Skeletal muscle injury in wild type mice

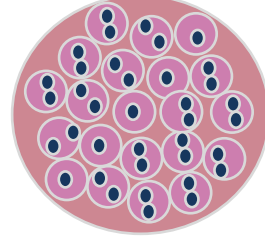


CONTROL

NAC
TREATMENT

J8
P.I

J28
P.I



NAC TREATMENT IMPAIRS SKELETAL
MUSCLE REGENERATION