

Macrophage Therapy Activates Endogenous Muscle Stem Cells and Accelerates Muscle Regeneration

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Research

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Abstract

Background: The effects of macrophage therapies on muscle regeneration and stem cell activation after injury remains unclear. This study aims to know the effect of macrophage therapies on muscle regeneration and endogenous stem cell activation towards new muscle

Methods: Adult male Swiss mice were subjected to an injury in the gastrocnemius, close to the myotendinous junction, using a 2-mm biopsy tube. Animals were assigned to the following groups: 1.- Injury; 2.- Injury+ clodronate. Macrophages were depleted 24 hours after injury by clodronate injection. 3. - Injury+ clodronate+ macrophage therapy. Macrophage depleted mice were treated with an intramuscular injection of 1 million peritoneal macrophages modified with intermittent anoxia reoxygenation. Animals were sacrificed at 4, and 15 days after the injury, (n = 8 per study time). Gene expression of proliferating cell nuclear antigen(PCNA) and Ki67 as cell proliferating markers, mannose receptor Ecotype 1(MRC1) and Interleukin10(IL-10) as anti-inflammatory markers, PAX7, MYOD as stem cell abundance and activation markers were evaluated by RT-PCR. Immunofluorescence analysis of PAX7, MYOD and histological scores of regeneration were performed.

Results: Macrophage depletion provoked an increase in anti-inflammation (IL-10, , MRC1), cell proliferation (Ki67, PCNA) and stem cell abundance(PAX7), indicating that during injury, endogenous macrophages are inducers of inflammation, anti-proliferation and inhibitors of stem cell abundance

Adoptive transfer of intermittent anoxia treated macrophages (M2 macrophages) to previously depleted animals increased cell proliferation (Ki67 and PCNA), stem cell activation (MyoD) and abundance (Pax7). Immunofluorescence revealed increases in positive PAX7 fluorescence, indicating that M2 are inducers of proliferation, anti-inflammation and stem cell proliferation and activation..

Conclusion: This study indicates that infusion of intermittent anoxia treated macrophages to injured muscle reduces inflammation, promotes cell proliferation and stem cell activation and proliferation, leading to muscle regeneration.

Intermittent hypoxia/reoxygenation induced macrophages, promote muscle regeneration without the addition of any external inducer agent, which simplifies the process and should allow to obtain a safer regenerative product from a clinical point of view.

Background

Muscle injuries are common disorders of the soft tissue and a challenge in traumatology and sports medicine practice. With severe injury, a total restitution is not achieved and scar tissue is formed, reducing the mechanical properties of the original tissue and making them more prone to re-injury.

Muscle tissue is formed by specialized cells called myofibers, multinucleated cells that contract and expand in order to produce movement. When a muscle is injured, the myofibers rupture and necrotize. In

order to regenerate the muscle, satellite cells, a specific subset of muscle stem cells located at the periphery of skeletal myofibers (1), are activated, proliferate and differentiate to myoblast which fuse with myofibers in order to restore the tissue. Muscle repair follows three overlapped stages: the inflammatory phase (1 to 3 days), the proliferative phase (3 to 4 weeks) and the remodeling phase (3 to 6 months). During the inflammatory phase, inflammatory cells (mostly polymorphonuclear leukocytes) migrate to the injured site, these are replaced by monocytes, which are transformed into macrophages. Macrophages not only phagocytose necrotic material, but also release growth factors, cytokines, and chemokines that can activate satellite cells and promote muscle regeneration (2). Termination of the inflammatory phase implies a switch from pro- to anti-inflammatory macrophages M2 which are detected in high number even 2 weeks after muscle damage and are critical for tissue restoration (3).

Previous studies of the group have demonstrated the regenerative capacity of macrophages in renal ischemia / reperfusion injury (4, 5). Macrophage therapy leads anti-inflammation and pro proliferation after kidney injury depending of macrophage phenotype. These studies indicated that macrophage M2 phenotype is influenced by the cellular environment (4). Other studies (6, 7, 8) have shown that macrophages, as well as drivers of proliferation and restorers of damaged tissue, they bring on the differentiation of endogenous stem cells towards mature functional differentiated cells that restore normal organ function.

In skeletal muscle, other authors have shown that macrophages seem to be essential for muscle regeneration (3) but few therapies with macrophages have been developed to regenerate a damaged muscle, being most of the approaches based on M1 macrophages (9, 10) and only few approaches are based on the use of M2 macrophages (11).

Once macrophages have been shown to be present in muscle injury (12) and knowing their regenerative role in other models, we have considered finding out their role in muscle regeneration.

On the other hand, the process of differentiation of muscle stem cells is defined and the markers of the different phases that lead to muscle regeneration are known. In this sense, MRC1 (mannose receptor Ecotype 1) mediates the endocytosis of glycoproteins by macrophages and is a marker of M2 macrophage phenotype (13). Interleukin 10 (IL-10) is a cytokine with potent and powerful anti-inflammatory properties that plays a central role in limiting host immune response to pathogens, inhibits the synthesis of a number of cytokines, including IFN-gamma, IL-2, IL-3, TNF and GM-CSF produced by activated macrophages and by helper T-cells (14).

The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G (1), S, G (2), and mitosis), but is absent from resting cells (G (0)), makes it an excellent marker for determining the so-called growth fraction of a given cell population (11). PCNA proliferating cell nuclear antigen (PCNA) (15) is also a marker of cell proliferation.

PAX 7 (13) is a transcription factor playing a role in myogenesis through regulation of muscle stem cells proliferation. MYOD regulates muscle cell differentiation by inducing cell cycle arrest, a prerequisite for myogenic initiation. The protein encoded by this gene is also involved in muscle regeneration. It activates its own transcription, which may stabilize commitment to myogenesis (16).

Previous studies explained that M2 macrophage polarization could be accomplished by subjecting cells to intermittent periods of anoxia-reoxygenation (17). In this work we will study the effect of injection of macrophages that exhibit a M2 phenotype, on a muscle injury.

The objective of the work is to know the role of a new macrophage therapy in muscle regeneration and its role as driver of the activation, and proliferation of muscle stem cells.

Methods

Experimental design

Mice were anesthetized with isoflurane by inhalation with an induction dose of 5%, an oxygen flow of 4–5 l / min and a maintenance dose of between 1-2.5%, before the surgical procedure. The muscle injury were performed in the gastrocnemius, close to the myotendinous junction, using a 2-mm biopsy tube. Muscle were transected, without removing.

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and followed the European Union guidelines for the handling and care of laboratory animals. Male Swiss mice (Charles River), weighing 25–30 g, were housed with 12:12-h light-dark cycle and were allowed free access to food and water. CD1 mice weighing approximately 30 g were used.

Postoperative analgesia (buprenorphine 0.1 mg / kg) was administered subcutaneously in all injured animals.

The following groups were performed:

1. 1.- Injury group. 24 hours after the lesion 100 ul of PBS were injected intramuscularly.
2. 2.- Injury plus clodronate.-Macrophages were depleted 24 hours after the injury.

4 and 15 days after the injury, animals were sacrificed (n = 8 per study time) n = 4 histology; n = 4 Biochemical determinations.

2.-Injury + clodronate + M2 therapy. -Mice that had been depleted endogenous macrophages 24 hours after the lesion were injected intramuscularly with 100 ul of PBS with 1 million peritoneal macrophages modified with intermittent anoxia reoxygenation (M2). 4 and 15 days after the lesion the animals were sacrificed (n = 8 per study time).

Proliferation, regeneration, inflammation and mesenchymal stem cell proliferation were measured by RT-PCR and histological analysis.

Depletion of resident macrophages with clodronate.

After 24 hours after the lesion, mice were anesthetized with isoflurane, placed in an immobilization tube and by means of a heat light the blood vessels of the lateral vein of the tail dilated. Next, 200 ul of clodronate liposomes were injected intravenously with a 30G needle through the lateral vein of the tail.

Cell culture and extraction of peritoneal macrophages.

Mice of strain CD1 weighing approximately 30 g were used to obtain peritoneal macrophages after provoking an inflammatory process by thioglycollate. 2.5 ml of thioglycollate were injected intraperitoneally with 2 ml syringes and 30G needles. 3 days after the injection, the animal were sacrificed by cervical dislocation and the peritoneal macrophages generated due to an inflammatory process caused by thioglycollate were extracted by means of cold PBS. The obtained cells were counted and cultured 2 million / well in 6-well plates with RPMI medium under standard cell culture conditions.

Modification of macrophage phenotype by intermittent anoxia/reoxygenation series.

A hypoxia chamber were used to perform intermittent series of anoxia and reoxygenation in the macrophage peritoneal cells previously obtained from mice (17). Then cells were cultured for 24 hours before its use as cell therapy.

Macrophage therapy.

48 hours after injury, 1 million resuspended macrophages were injected in a volume of 100 ul of PBS with a 30G needle in the gastrocnemius. The treatment has been done according to the treatment group, either with resting macrophages or macrophages modified with anoxia.

Sample collection.

Mice were anesthetized with isoflurane, placed prone and immobilized by all four extremities. The hair and the skin were removed and with silk of 5 zeros, the area of the injured muscle was cut and frozen at -80° or set in paraformaldehyde for histological analysis.

Real-time RT-PCR analysis.

The total RNA were extracted from the frozen muscles by the Trizol protocol. From this RNA, cDNA is synthesized with the iScript CDNA Synthesis kit. The expression of the genes GAPDH, NGAL, MRC1, IL-10, KI67, PCNA, MYOD, Pax7, is analyzed from primers designed in Mouse (Invitrogen).

Histological analysis.

Samples were embedded in paraffin, cut into 4 mm sections, and stained with hematoxylin and eosin.

Immunofluorescent staining of muscle sections.

Muscle sections were unmasked in sodium citrate buffer and blocked for 1 h. Staining of MYOD and Pax 7 was carried out. Briefly, slides were incubated with anti-MYOD and anti-PAX 7 antibody conjugated to Alexa fluor AF488 (SC-377460) and Alexa fluor AF647(SC-365843) respectively (Santa Cruz, Santa Cruz, CA), 90 minutes at room temperature. Sections were viewed using a Leica TCS NT laser microscope (Leica, Wetzlar, Germany) for histological evaluation.

Statistical Analysis.

Unpaired t-test was used to compare means among two groups. Differences in values were considered to be statistically significant if $P < 0.05$. Statistical analyses were performed with GraphPad Prism 8.0 software.

Results

Depletion of macrophages after skeletal muscular injury potentiates proliferation, anti-inflammation and endogenous stem cell activation. To study the impact of macrophage on proliferation, inflammation and stem cell activation, we used a macrophage depletion protocol, to eliminate endogenous macrophages. Mice received an intravenous bolus 100 μ l/g body weight of clodronate-encapsulated liposomes (lipo-CL2MBP) 1 day after muscle injury induction.

Previous results of the group confirmed the efficacy of lipo-CL2MBP treatment (5). The following markers were measured at 4 and 15 days after injury with or without lipo-CL2MBP treatment:

- -MRC1 and IL-10 expression to assess the anti-inflammatory state of skeletal muscle.
- -Ki 67, PCNA expression to assess proliferation.
- -MyoD and Pax 7 expression at tissue were measured to monitor stem cell activation (MyoD) and stem cell abundance (PAX7).

As shown in Fig. 1, at 4 days after injury, macrophage depletion leads to a significant increase in all the parameters measured. The expression of anti-inflammatory, proliferation and stem cell activation markers were significantly increased at 4 days' post injury in the lipo-CL2MBP treated group, and these expressions clearly decrease with the progression of injury. The results obtained indicate that macrophage depletion provoked an increase in anti-inflammation (IL-10 and MRC 1), proliferation (Ki67, PCNA), and stem cell abundance: (PAX7), thus indicating that macrophages, during the initial stages after the injury, are inducers of inflammation, anti-proliferation and inhibitors of stem cell proliferation.

At 15 days' post injury in the lipo-CL2MBP treated group, results revealed that, expression of anti-inflammatory (IL-10 and MRC1) and proliferation markers (Ki 67 and PCNA) were significantly reduced. markers of stem cell activation (MyoD) was increased while markers of stem cell abundance (PAX 7) was decreased with respect to injured groups without lipo-CL2MBP. This PAX 7 reduction is concomitant with an increase in stem cell proliferation and activation (MyoD).

Intermittent anoxia/reoxygenation changes macrophage phenotype towards anti-inflammatory phenotype (M2)

To study the impact of intermittent anoxia reoxygenation on macrophage phenotype, peritoneal macrophages underwent 4 series of 3 min of anoxia followed by 45 seconds of reperfusion.

TGF and IL-10 expression were measured to assess the anti-inflammatory state of macrophages.

As shown in Fig. 2 the intermittent anoxia protocol leads to an anti-inflammatory phenotype (IL-10 expression is increased with respect to control cells) and expression of the pro-fibrotic marker TGF is reduced, thus indicating that this protocol were suitable to be used as macrophage switch inducer in our experimental protocol.

Adoptive transfer of M2 macrophages to previously depleted animals, potentiates the anti-inflammation, proliferation and activation of muscular stem cells to regenerate muscle.on new muscle

Modified M2 macrophages were administered to previously macrophage depleted animals with clodronate administration. We injected control macrophages and M2 induced macrophages (1 million cells per mouse iv) 24 h after clodronate administration.

At 4 days of injury, as shown in Fig. 3, adoptive transfer of control macrophages to previously depleted animals does not modify the effects of macrophage depletion with clodronate in any of the measured markers, except in stem cell activation (MyoD), On the other hand, M2 macrophages injection potentiates cell proliferation (Ki67 and PCNA), stem cell activation (MyoD) and stem cell abundance (Pax 7).

At 15 days post-injury, adoptive transfer of control macrophages to previously depleted animals leads to a significant decrease in all the measured parameters with respect to the clodronate treated group.

The injection of M2 macrophages potentiates proliferation (PCNA and Ki67 are increased vs clodronate treated animals) anti-inflammation (IL-10 and MRC1 expression is increased with respect to clodronate treated animals,) and stem cell abundance (PAX 7 is increased) while stem cell activation (MyoD) is decreased with respect to clodronate treated group.

Altogether, showing that M2 are mainly inducers of proliferation, anti-inflammation, stem cell proliferation and activation

Immunofluorescence result on PAX 7 (as shown is Fig. 4) revealed increases in positive PAX7 fluorescence in the Cl2 MBP + injury + M2 treatment group at 4 and 15 days after injury with respect to the Cl2MBP + injury treated groups, indicating the ability of M2 treatment to enhance mesenchymal stem cell abundance.

MYOD fluorescence increases at day 4 in the Cl2 MBP + injury + M2 treatment group with respect to the Cl2MBP + injury treated group, while a slight decrease was observed at day 15 in the Cl2 MBP + injury +

M2 treatment group with respect to the Cl2MBP + injury treated group.

As shown in Fig. 5a, histological analyses revealed that injection of macrophages M2 to previously injured and clodronate treated animals, provoked a significant decrease in the area of mesenchymal phenotype extension that continues at day 15.

The decrease in mesenchymal phenotype extension was concomitant with a significant increase in muscle phenotype extension at days 4 and 15 post injury. This data could be indicative that M2 therapy provokes mesenchymal activation towards new muscle formation.

The increase in muscle fiber diameter observed after M2 treatment at 4 and 15 days is indicative of a more advanced muscle formation. Larger fiber size is observed in M2 treated mice, suggesting the positive effect of M2 in myotube formation.

Macrophage therapy to injured muscle potentiates antiinflammation and proliferation.

To study the impact of adoptive transfer of different types of macrophages to restore the effects of muscle injury, on proliferation and anti-inflammation, we injected resting peritoneal macrophages and M2 induced peritoneal macrophages (1 million cells per mouse iv) 48 hours' after injury induction.

As shown in Fig. 6, at 4 days of injury, adoptive transfer of resting macrophages

provoked an increase in the anti-inflammation (MRC1 and IL-10), proliferation (Ki 67 and PCNA).

Injection of M2 macrophages to injured animals provoked an increase in antinflammatory parameters (MRC1 and IL-10) and proliferation (Ki 67 and PCNA) that was maintained at day 15. Indicating the ability of macrophage therapy to induce muscle repair and antiinflammation.

Discussion

This study shows the effect of macrophage depletion on inflammation, cell proliferation and stem cell activation/proliferation after skeletal muscle injury and the impact of adoptive transfer of macrophages on muscle regeneration.

It is commonly accepted that in the initial stages after injury, macrophages with a pro-inflammatory profile predominate, playing a key role in muscle repair, removing cell debris, and releasing several pro-inflammatory factors, such as TNF or IFN γ , that can regulate the initial stages of myogenesis (18). Both TNF and IFN γ can mediate the inhibition of stem cell differentiation (19, 20), but regarding their role in cell proliferation different results have been previously obtained. Although various in vitro studies suggest that M1 macrophages can promote myocyte proliferation (21, 22), other studies show that TNF can silence Pax7 expression, negatively regulating Satellite cell number (23) and, depending on their concentration, IFN can also reduce myocyte proliferation (24).

Regarding previous *in vivo* studies, the role of M1 macrophages on cell proliferation also differs depending on the experimental conditions. Although it has been observed that M1 macrophages, co-injected with human myoblast, promote stem cell proliferation (22), other previous *in vivo* studies (25) showed that monocyte/macrophage depletion on animal models before the injury, delays the clearance of necrotic fibers impairing skeletal muscle regeneration, but they had no effect on myocyte proliferation and differentiation (25).

By contrast, our experiments showed that the absence of macrophages in the injured muscle is beneficial for regeneration, since macrophage depletion with clodronate liposomes 24 hours after the injury, increases cell proliferation. In concordance with our results, other studies have reported an inhibitory effect of TNF and IFN γ on cell proliferation (23, 24).

Eliminating inflammatory macrophages dramatically shifts the pro-inflammatory microenvironment towards an anti-inflammatory profile, as it can be show by the elevated levels of IL10 and MRC1 at 4 days' post injury. This is associated with an increase in proliferation markers (ki 67 and PCNA) and stem cell abundance (PAX 7).

Thus indicating that elimination of macrophages during the inflammatory phase has a beneficial effect on muscle proliferation and anti-inflammation. The increase of Pax7 in the clodronate treated mice indicates the elevated abundance of muscle stem cells when inflammatory macrophages are not present, suggesting and inhibitory effect of inflammatory macrophages on stem cell proliferation. This result contrast with previous *in vitro* and *in vivo* studies (21, 22) that indicate that M1 macrophages promote stem cell proliferation. It is important to point out that these previous studies were performed *in vitro* and in the case of *in vivo* studies, macrophages were coinjected with human myoblasts. Our results indicate that the proliferative effect of M1 macrophages is very context dependent, not depending only on macrophage phenotype and being the inflammatory microenvironment also a key driver on stem cell abundance and proliferation. In addition, our results indicate that macrophage depletion increases stem cell activation, as it can be show by the elevated levels of MYOD in the clodronate treated group at 15 days' post injury, indicating that M1 macrophages avoids stem cell activation, as it has been previously reported (22).

Late steps of muscle regeneration are associated to anti-inflammatory macrophages M2, the switch from M1 to M2 macrophages is essential in order to follow the regenerative process. Phagocytosis of cell debris by M1 macrophages can promote the phenotype switch (21) and the inflammatory cytokine IL-10 can play a key role regulating this process (11). Various subsets of M2 macrophages have been described depending on their cytokine expression profile, playing different roles in tissue repair, noticeably M2a and M2c seems to be especially relevant in muscle repair.

In vitro studies show how both M2a and M2c macrophages promote stem cell differentiation to myocyte (21, 26) and myotube fusion (21, 26 and 22). On the other hand, co-culture of myoblasts with M2 macrophages activated with IL10 caused significant increases in myoblast proliferation (11) suggesting that M2 macrophages can also promote the initial stages of cell proliferation.

Our approach is based on the use of intermittent hypoxia/reoxygenation induced macrophages, following a process previously described (17). This process allows for an important increase of IL-10 expression, indicating an anti-inflammatory profile, without the use of any external agent (such addition of IL-10), which simplifies the process and should allow to obtain a safer product from a clinical point of view.

When, after macrophage depletion with clodronate, intermittent hypoxia induced M2 macrophages were injected, Pax7 levels were elevated at 4 and 15 days after injury, suggesting a positive role of M2 macrophages on stem cell abundance. PCR gene expression revealed also increased levels of the stem cell activation marker, MYOD. Immunofluorescence analysis are in line with these results, showing that both PAX7 an MYOD expression are promoted by M2 macrophages, these results are also supported also by our histological analysis, were larger muscle phenotype extension is observed in the group treated with M2 macrophages. This is in accordance with previous in vitro studies, showing the positive effect of anti-inflammatory macrophages on cell proliferation (11) .

Moreover, histological results indicate an increase in muscle phenotype extension and in muscle fiber size after M2 treatment, both indicative of a more mature muscle formation / regeneration., while mesenchymal phenotype extension is reduced. This fact could be attributed to the transformation of mesenchymal stem cells in muscular mature fibers. These results are in accordance with previous in vitro studies, showing the effect of anti-inflammatory macrophages on myocyte differentiation (21, 26).

Conclusions

This study indicates that M2 macrophage therapy reduces inflammation, promotes cell proliferation and stem cell activation.

Conveniently modulated, macrophages can be powerful therapeutic agents for the treatment of musculoskeletal injuries. Inflammatory macrophages, at the early stages of injury, seems to inhibit not only differentiation but also proliferation, and M2 macrophages could promote not only the differentiation but also de proliferation of satellite cells. Proliferation is special favored by M2 macrophages even 15 days after the injury and could be very useful for severe injuries with an elevated loss of muscle tissue.

Abbreviations

MRC1

mannose receptor Ecotype 1, IL-10:Interleukin10, PCNA proliferating cell nuclear antigen,

Declarations

Ethics approval

All animal procedure were approved by the Institutional Animal Care and use Committee(IACUC) and followed the European Union guidelines for the handling and care of laboratory animals.

Consent for publication

Not applicable

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interest

The authors declare that they have no competing interest.

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Author's contributions

G.H, A.S and J.M. developed the concept and designed this study, A.C., A.R., J.M performed experiments, A.C., A.R., J.M., A.S. and G.H analyzed and interpreted the data. G. H and X.G. wrote the manuscript, A.C and J.M. generated all figures and G.H supervised the project. All authors discussed the results and implications and commented on the manuscript at all stages. Georgina Hotter is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity and accuracy of the data analysis.

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Tables

Table 1.-Primers sequences used for qPCR.

Gen		Sequence
Mn MRC 1	Invitrogen	5'-CAG-GTG-GCT-TAT-GGG-ATG-TT-3'
		Revers 5'-GAG-TTG-TTG-TGG-GCT-CTG-GT-3'
Mn TGF B1	Invitrogen	5'-ATT-CCT-GGC-GTT-ACC-TTG-G-3'
		Revers : 5'-CCT-GTA-TTC-CGT-CTC-CTT-GG-3'
Mn_IL-10	Invitrogen	5'-CAT-GGG-TCT-TGG-GAA-GAG-AA-3'
		Revers : 5'-AAC-TGG-CCA-CAG-TTT-TCA-GG-3'
Mn_MYOD	Invitrogen	5'-TCC-TCA-TAG-CAC-AGG-GGT-GA-3'
		Revers : 5'-GCA-AGC-TGT-GGG-GAA-AAG-TG-3'
Mn_KI67	Invitrogen	5'-CAG-TAC-TCG-GAA-TGC-AGC-AA-3'
		Revers : 5'-CAG-TCT-TCA-GGG-GCT-CTG-TC-3'
Mn_PCNA	Invitrogen	5'-AAT-GGG-GTG-AAG-TTT-TCT-GC-3'
		Revers : 5'-CAG-TGG-AGT-GGC-TTT-TGT-GA-3'
Mn_PAX 7	Invitrogen	5'-CAA-GCC-CTG-AGT-CTC-CTC-AC-3'
		Revers : 5'-CAT-GGG-TAG-ATG-GCA-CAC-TG-3'

Figures

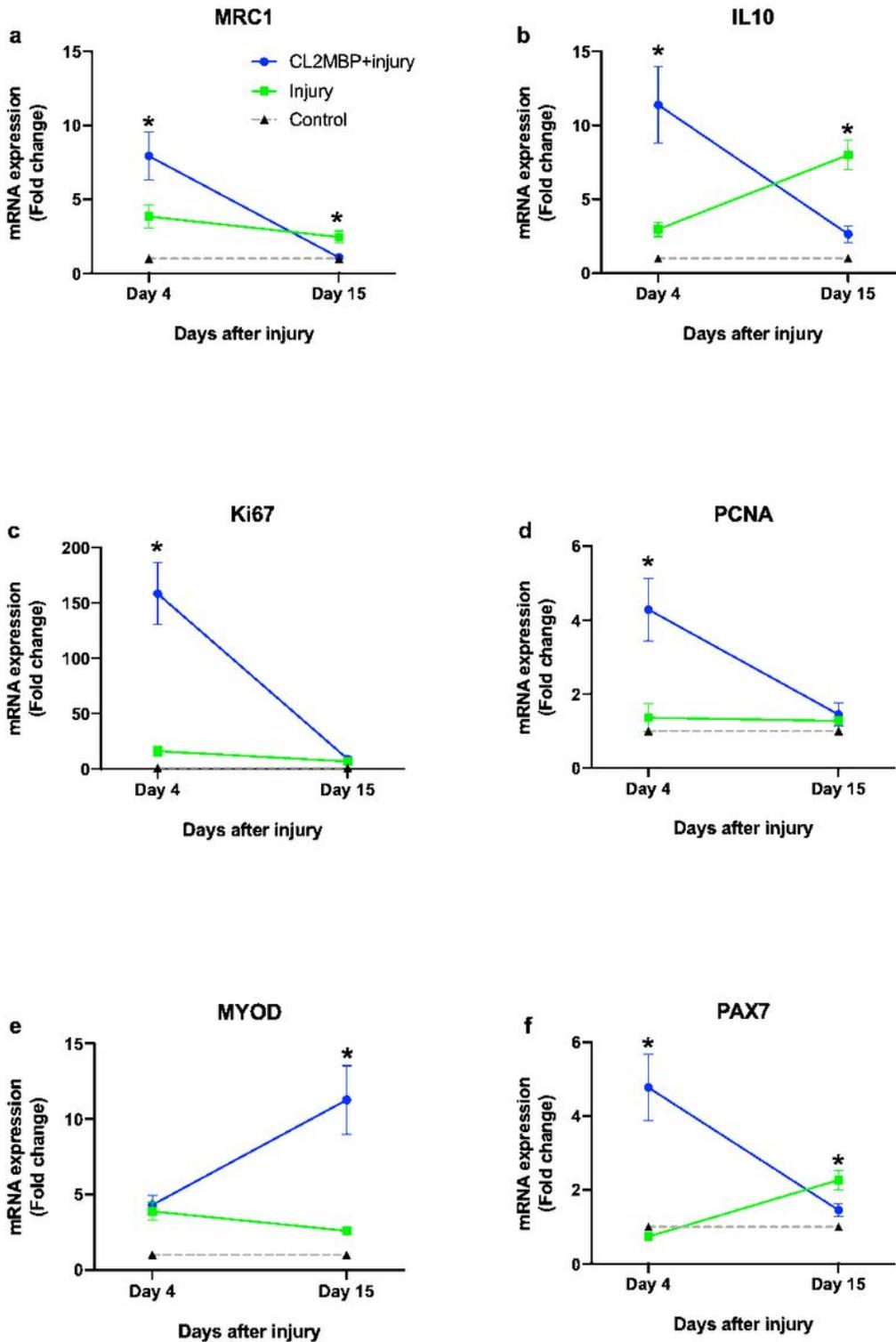


Figure 1

Patterns of normalized MRC1 (a), IL10 (b), Ki67 (c), PCNA (d), MYOD (e), Pax7 (f) fold mRNA expression referred to control, assessed by quantitative RT-PCR in skeletal muscle; muscle tissue was subjected to injury with and without macrophage depletion (24 hours after injury, lipo- CL2MBP were injected to provoke macrophage depletion) and at 4 and 15 days tissue was obtained. Fold mRNA expression normalized to GAPDH is represented as mean \pm SEM. * $p < 0.05$ versus injury group. N=4.

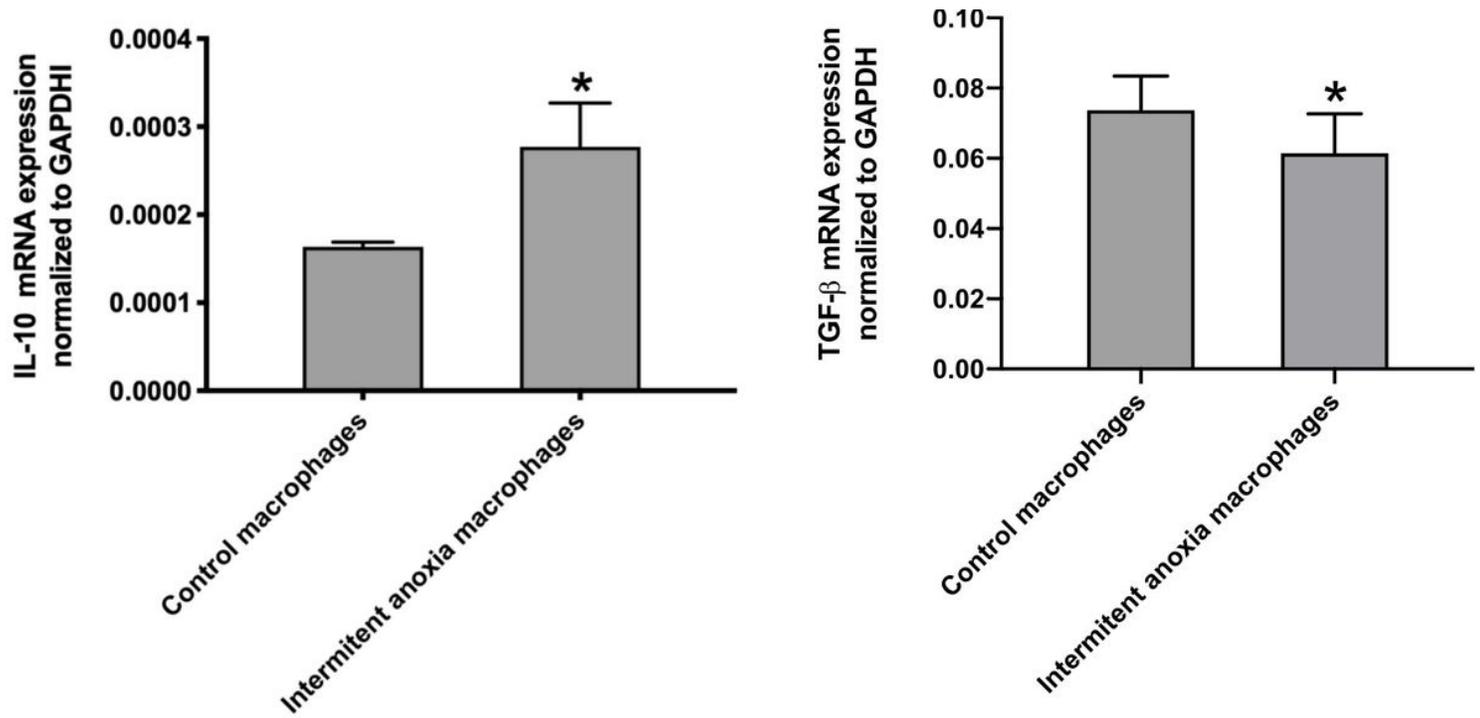


Figure 2

Quantitative real-time PCR gene expression for IL10 and TGFb in mouse peritoneal macrophages (resting macrophages) and peritoneal macrophages subjected to 4 series of intermittent anoxia re-oxygenation (3 min of anoxia followed to 45 seconds of reperfusion) (M2 macrophages) .Data are mean \pm s.e.m.; N = 5 mice were analyzed. t -test; * $P \leq 0.05$ vs. control.

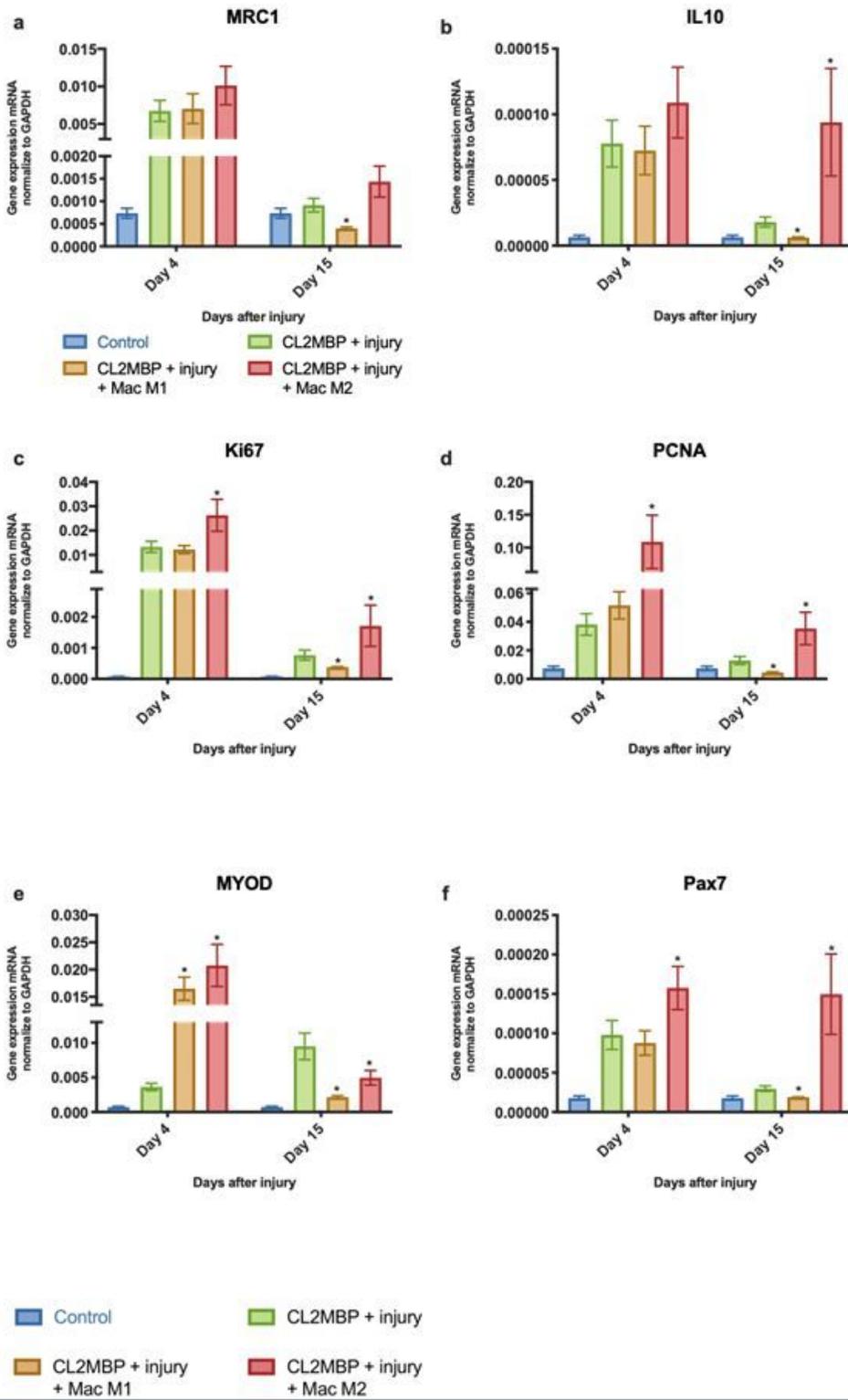


Figure 3

Evolution of quantitative real-time PCR gene expression for MRC1 (a), IL10 (b), Ki67 (c), PCNA (d), MYOD (e), Pax7 (f) assessed by quantitative RT-PCR in skeletal muscle; muscle tissue was subjected to injury. At 24 hours lipo- CL2MBP were injected to provoke macrophage depletion and 24 hours later Macrophages resting or M2 were injected. Skeletal muscle was obtained at 4 and 15 days post- injury. mRNA

expression normalized to GAPDH is represented as mean \pm SEM. * $p < 0.05$ versus Chlodronate treated group. N=4.

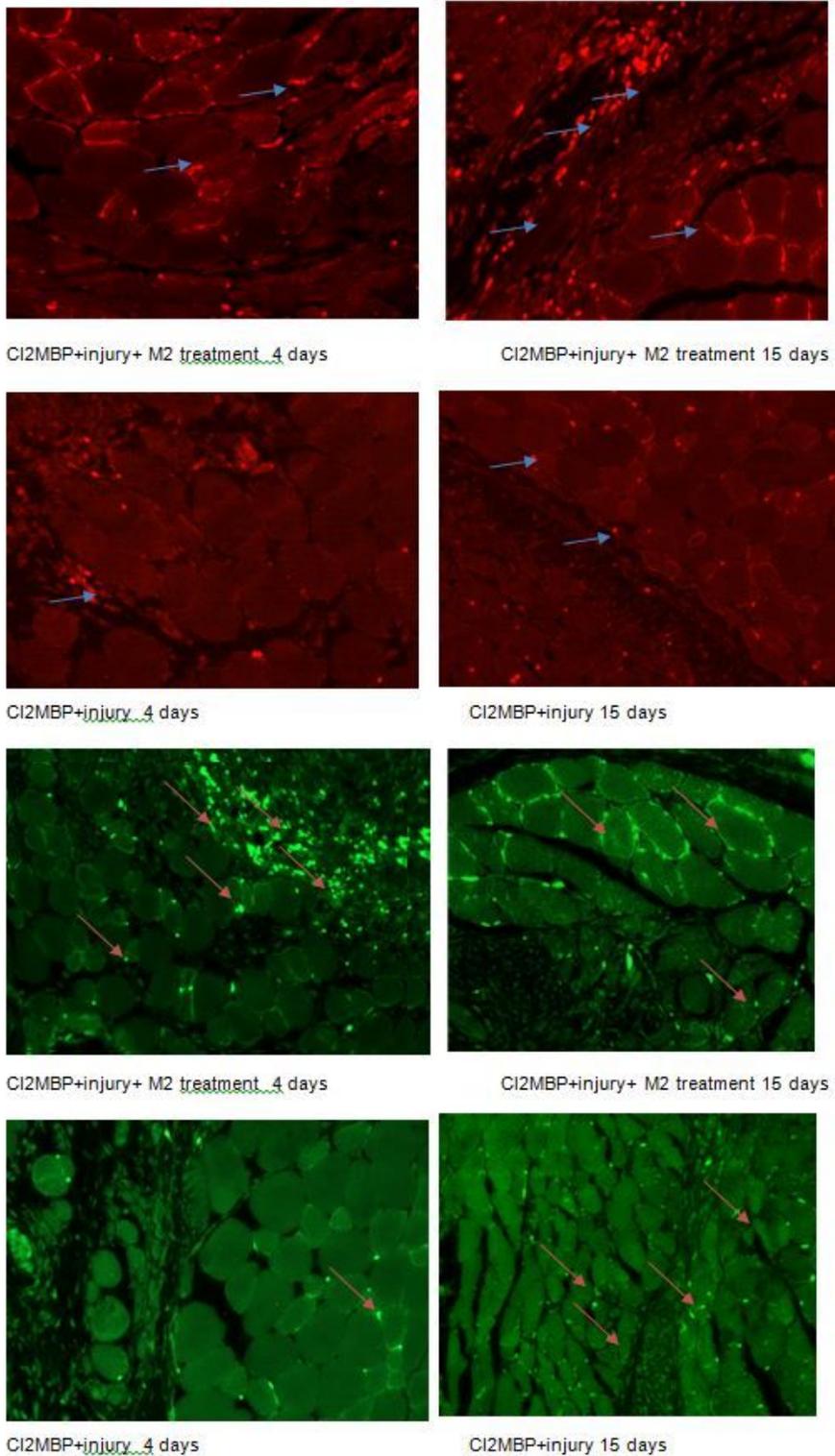
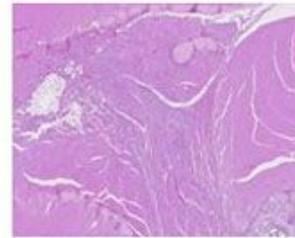
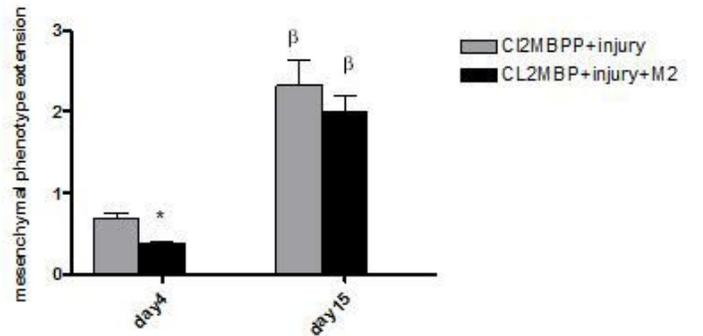


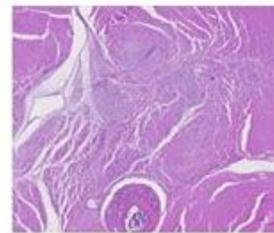
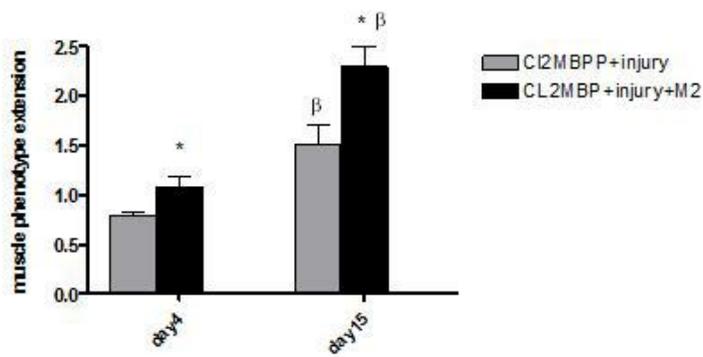
Figure 4

Representative images of immunofluorescence analysis of PAX 7(red) and MyoD (green) (original magnification _400) of the gastrocnemius region. After 24 hours of muscle injury, lipo-CI2MBP were injected to provoke macrophage depletion and 24 hours M2 were injected. Skeletal muscle was obtained

at 4 and 15 days post- injury to evaluate histological damage. (n=4 per group) Results revealed increases in positive PAX7 fluorescence in the Cl2MBP+injury+M2 treatment group at 4 and 15 days after injury with respect to the Cl2MBP+injury treated group. MYOD fluorescence increases at day 4 in the Cl2MBP+injury+M2 treated group with respect to the Cl2MBP+injury treated group., while decreased at day 15 in the Cl2MBP+injury+M2 treated group.



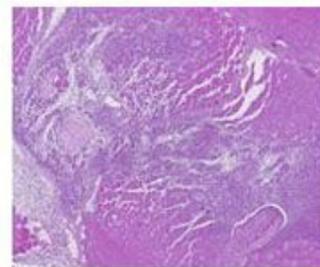
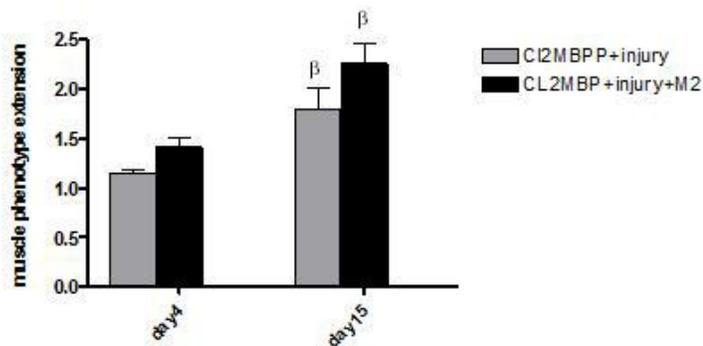
Cl2MBP+injury+ M2 treatment (15 days)



Cl2MBP+injury (15 days)



Cl2MBP+injury+ M2 treatment (4 days)



Cl2MBP+injury (4 days)

Figure 5a

Figure 5b

Figure 5

Conventional histological analysis of hematoxylin and eosin-stained skeletal muscle sections (original magnification $\times 400$) of the gastrocnemius region. After 24 hours of muscle injury, lipo-Cl2MBP were injected to provoke macrophage depletion and 24 hours M2 were injected. Skeletal muscle was obtained at 4 and 15 days post- injury to evaluate histological damage. Conventional histological analysis of hematoxylin and eosin-stained muscle sections confirmed protective effects of M2 therapy compared to Cl2MBP treated rats without M2 treatment. a) Representative images are shown. At 4 days we observed a reduction of mesenchymal area and an increase in muscle phenotype extension concomitant with an increase in muscle fiber size in the Cl2MBP+injury+ M2 treatment , indicating the potential of M2 therapy to accelerate new muscle formation. At 15 days of injury results revealed the same tendency. b). Histological damage was quantified in a blinded manner by two different skilled experts in the field (n=4 per group) . *P<0.005 vs. injury+ lipo- Cl2MBP; β p< 0.005 vs 4 days.

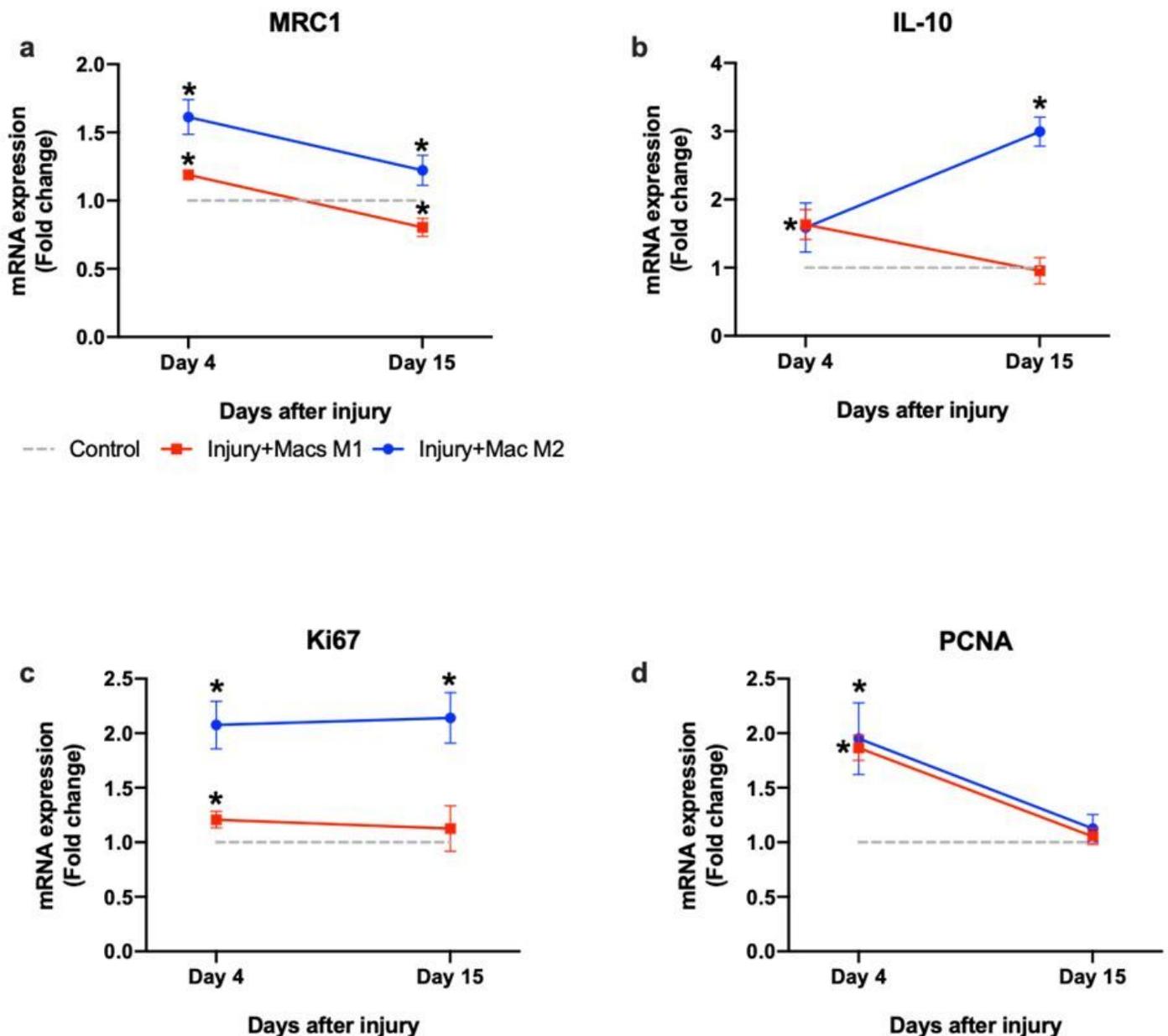


Figure 6

Patterns of normalized MRC1 (a), IL10 (b), KI67 (c), PCNA (d), fold mRNA expression referred to control, assessed by quantitative RT-PCR in skeletal muscle; muscle tissue was subjected to injury . 24 hours after injury, Macrophages resting or M2 were injected. Skeletal muscle was obtained at 4 and 15 days post-injury. mRNA expression normalized to GAPDH is represented as mean \pm SEM. *p < 0.05 versus injury.