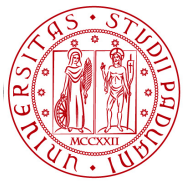

UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede amministrativa: Università degli Studi di Padova

Dipartimento di Biologia

Scuola di Dottorato in Bioscienze e Biotecnologie

Indirizzo Genetica e Biologia Molecolare dello Sviluppo

XXV Ciclo

ROLE OF MACROPHAGIC FACTORS IN MUSCLE REGENERATION: *IN VITRO* AND *IN VIVO* CHARACTERIZATION

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Abstract

Muscle regeneration is a complex process that involves many different types of cells, both myogenic and non-myogenic. In particular, macrophages play a fundamental role, since they exert many different functions: they phagocyte fiber debris, release cytokines to regulate the inflammatory response and stimulate satellite cells through the secretion of myogenic factors. Importantly, inflammatory processes mediated by macrophages are known to play a major role in the pathophysiology of many inherited muscular dystrophies, such as Duchenne Muscular Dystrophy (DMD).

There are two main classes of activated macrophages: M1 macrophages, which have a pro-inflammatory action, and M2 macrophages that are anti-inflammatory. The exact effect of these two types of macrophages on myogenic cells is still debated, although all Authors state that if given type has a pro-proliferation effect it does not exert a pro-differentiation effect, and *vice versa*.

In order to study the effects of macrophage-released factors onto myogenic cells we use the murine macrophage cell line J774 to obtain a serum-free, macrophage-conditioned medium (mMCM). We previously found that mMCM could enhance the proliferation rate and prevent the trans-differentiation of rat satellite cells, as well as human myoblasts from both normal and dystrophic muscles. Besides, it can greatly enhance the repair processes in muscles that underwent large surgical ablations. As these features could all be very useful in clinical terms to treat inherited and traumatic muscle diseases, we are now trying to clarify the mechanism(s) of action of mMCM. To do so, we have moved to murine *in vitro* and *in vivo* models, both *wild type* (*wt*) and dystrophic (*mdx*).

In this work, we confirmed the pro-proliferative effect of mMCM on murine satellite cells, both *wt* and *mdx*: mMCM significantly shortened their average duplication time from 90 to 48 hours and from 100 to 20 hours, respectively. The results were also confirmed with FACS-sorted *wt* satellite cells (duplication time reduced from 45 to 31 hours), suggesting that the effects were not limited to a specific subpopulation of satellite cells. At the same time, though, not only mMCM did not inhibit the differentiation process, but in some instances it seemed to enhance them.

We also tested the effect of mMCM on the proliferation of primary *wt* and *mdx* fibroblasts, finding that mMCM consistently had a clear anti-proliferative effect on *mdx* fibroblast, while it did not affect *wt* fibroblasts.

Experiments on the effect of mMCM pro-proliferative action on satellite cell transplantation in dystrophic muscle were carried out using GFP^+ satellite cells,

showing that when recipient muscles were treated with mMCM the number of GFP-positive fibers was higher that in controls. *In vivo* experiments on *wt* mice muscles showed that in intact tissue delivery of mMCM does not elicit an inflammatory response; on the other hand, in chemically pre-injured muscles mMCM injections lowered the expression levels of various macrophagic markers, both for M1 and M2, thus indicating that mMCM can interfere with the inflammatory process, apparently reducing the total number of macrophages.

In order to further elucidate the possible interactions between mMCM and activated macrophages we also investigated the effects of mMCM on macrophages polarization. To this aim, we used a model in which human monocytes obtained from blood that were differentiated into macrophages and then stimulated with cytokines to acquire either M1 or M2 phenotype. We then tested the effects on mMCM on non-stimulated, M1 and M2 macrophages. These analyses showed that mMCM alone cannot influence macrophages polarization but, when combined with polarization stimuli, it seems to enhance the M1 phenotype.

Finally, we began an analysis of mMCM composition, finding that it contains various cytokines, some pro- and other anti-inflammatory. We are also starting the mass spectrometry analysis, and the preliminary results identified some interesting candidates. mMCM also contain exosomes, whose content is however still under investigation.

Our data confirm the potential of mMCM as a therapeutic tool for muscular pathologies, but also underline the need of improving our understanding of the complex interplay between macrophages and muscle environment.

Riassunto

La rigenerazione muscolare è un processo complesso che coinvolge diversi tipi cellulari, sia miogenici sia non miogenici. In particolare, i macrofagi ricoprono un ruolo fondamentale, svolgendo diverse funzioni: fagocitano i detriti cellulari, rilasciano citochine che regolano la risposta infiammatoria e stimolano le cellule satelliti attraverso la secrezione di fattori miogenici.

I processi infiammatori mediati dai macrofagi hanno un ruolo fondamentale nella patofisiologia di molte distrofie muscolari ereditarie, come la Distrofia Muscolare di Duchenne (DMD).

Esistono due classi di macrofagi attivati: i macrofagi M1, che hanno un effetto pro-infiammatorio, e i macrofagi M2, che hanno un'azione anti-infiammatoria. L'esatto effetto di questi due tipi di macrofagi sulle cellule miogeniche è ancora oggetto di dibattito, sebbene tutti gli Autori concordino nell'affermare che se un certo tipo ha un'azione pro-proliferativa sulle cellule, non avrà anche un effetto pro-differenziativo sulle stesse e viceversa.

Per studiare l'effetto dei fattori rilasciati dai macrofagi sulle cellule miogeniche, abbiamo utilizzato la linea cellulare di macrofagi murini J774, per ottenere un medium condizionato da macrofagi privo di siero, chiamato murine Macrophage Conditioned Medium (mMCM). In lavori precedenti abbiamo dimostrato che mMCM può aumentare la proliferazione e impedire il trans-differenziamento di cellule satelliti di ratto, nonché di mioblasti umani, sia di individui sani che di pazienti distrofici. Può inoltre migliorare grandemente la rigenerazione in muscoli che hanno subito grosse rimozioni chirurgiche di massa.

Tutte queste caratteristiche potrebbero essere molto utili nel trattamento clinico di patologie muscolari sia traumatiche sia ereditarie e stiamo quindi cercando di chiarire quale sia il meccanismo (o i meccanismi) di azione del mMCM. Per far ciò, abbiamo deciso di utilizzare il modello murino *in vitro* e *in vivo*, sia selvatico (*wild type*, *wt*), sia distrofico (*mdx*).

In questo lavoro, abbiamo confermato l'effetto pro-proliferativo del mMCM sulle cellule satelliti di topo, sia *wt* che *mdx*: mMCM ha ridotto significativamente il loro tempo di duplicazione medio rispettivamente da 90 a 48 ore e da 100 a 20 ore. Tali risultati sono stati anche confermati con cellule satelliti *wt* isolate tramite FACS (tempo di duplicazione ridotto da 45 a 31 ore), indicando che gli effetti visti sulle cellule non sono limitati ad una specifica subpopolazione di cellule satelliti. Allo stesso tempo, mMCM non solo non ha inibito il processo di

differenziamento delle cellule, ma in alcuni casi è anche sembrato che lo migliorasse.
Abbiamo anche studiato l'effetto del mMCM sulla proliferazione di fibroblasti primari, sia *wt* che *mdx*, trovando che mMCM ha un significativo effetto anti-proliferativo sui fibroblasti distrofici, mentre non ha effetto sui *wt*.
Abbiamo poi condotto alcuni esperimenti *in vivo* per capire l'effetto del mMCM sul trapianto di cellule satelliti in muscolo distrofico, usando satelliti GFP^+, e abbiamo dimostrato che se il muscolo ricevente viene trattato con mMCM, il numero di fibre GFP-positive è più alto dei controlli. Gli esperimenti condotti *in vivo* su muscoli *wt* intatti hanno mostrato che mMCM da solo non stimola una risposta infiammatoria, mentre la somministrazione di mMCM in muscoli danneggiati chimicamente ha diminuito l'espressione di vari marcatori macrofagici, sia M1 che M2, indicando che mMCM può interferire con il processo infiammatorio, apparentemente riducendo il numero totale di macrofagi.
Per indagare più approfonditamente sulle possibili interazioni fra mMCM e i macrofagi attivati, abbiamo poi investigato gli effetti del mMCM sulla polarizzazione di macrofagi. Abbiamo dunque usato un modello in cui monociti umani ottenuti dal sangue sono differenziati in macrofagi e poi stimolati con citochime per acquisire un fenotipo M1 o M2. Abbiamo poi studiato gli effetti del mMCM sui macrofagi già polarizzati o ancora vergini. Queste analisi hanno mostrato che mMCM da solo non può influenzare la polarizzazione dei macrofagi, ma che quando è in combinazione con stimoli polarizzanti, sembra spingere verso il fenotipo M1.
Infine, abbiamo iniziato l'analisi della composizione del mMCM. mMCM contiene diverse citochine, alcune pro- e altre anti-infiammatorie. Stiamo inoltre iniziando l'analisi con la spettrometria di massa sul mMCM e i risultati preliminari hanno identificato alcune proteine interessanti. mMCM contiene inoltre anche exosomi, il cui contenuto è ancora oggetto di analisi.
I nostri dati confermano il potenziale del mMCM come strumento terapeutico per le patologie muscolari, ma evidenziano anche il bisogno di migliorare le nostre conoscenze sulle complesse interazioni fra macrofagi e ambiente muscolare.

Introduction

Skeletal muscle regeneration

Skeletal muscle is one of the most abundant tissues in the body, accounting for about 30-40% of the total human body mass. Single muscles vary greatly in size and shape, from very tiny –such as those present in the ear– to very large, like those of the leg.
Its functional unit is the myofiber, a long cylindrical syncitial multinucleated cell that contains many myofibrils, formed by repeating contractile units called sarcomeres. During development, myofibers are originated by the fusion of many mononucleated cells, called myoblasts, which arise from myogenic precursor cells present in the somites. Myoblasts fusion initially leads to the formation of thin syncitia called myotubes, which progressively increases in size due to the fusion of more myoblasts. As differentiation proceeds, nuclei are pushed near the cell membrane, called sarcolemma, while the cytoplasm is entirely filled with sarcomeres. Once formed, the muscle fiber is mitotically inactive (*Yusuf and Brand-Saberi, 2012*). Importantly, differentiated muscles also contain a population of myogenic adult stem cells, termed "satellite cells", positioned between the sarcolemma and the basal lamina. It is their presence that confers to skeletal muscle its remarkable self-repair properties.

Despite being formed by non-mitotic fibers, skeletal muscle is able to regenerate very efficiently, even after severe damage. Its regeneration is a complex process that involves the interplay of satellite cells and immune system cells.
Regardless of the type of injury, skeletal muscle regeneration always follows the same pattern of events. The process can be divided into three partially overlapping phases (Figure 1a), whose extent and duration can vary in respect to the type and the extent of the injury:

- Destruction: the length of this phase depends on the type of the injury; myofibers that are damaged beyond repair are disrupted and die, mostly by necrosis. There could be also damage to the vasculature. Neutrophils invade the within an hour from the damage; tissue resident macrophages release cytokines that regulate the inflammatory process and recruit other macrophages from the blood, that start to phagocyte cellular debris.

- Repair: During this phase satellite cells are activated, exiting from their state of quiescence forming high number of proliferating myoblasts; these latter then can either fuse to generate new fibers or repair the less damaged ones. Macrophages play a main role in the progression of healing, as they can stimulate the proliferation and the differentiation of satellite cells to generate new fibers. There can also be scar tissue formation.
- Remodelling: in this last phase, the formation of the new fibers reaches completion and the muscle structure is reorganized with revascularization and reinnervation to achieve complete recovery of its function (*Smith et al., 2008*).

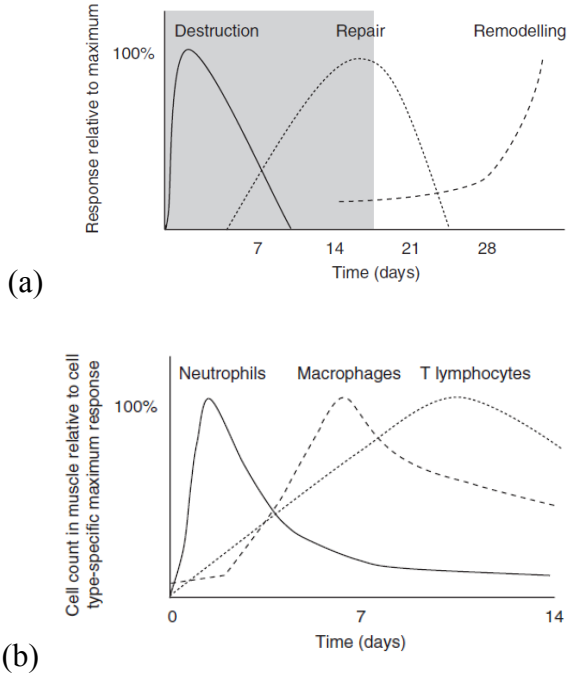


Figure 1: (a): The three phases of skeletal muscle regeneration (b) Schematic representation of the different immune system cells types that invade the site of the injury. (Reprinted with permission from *Smith et al., 2008*)

Immune system cells play a fundamental role during muscle regeneration (Figure 1b). After damage, damage of the plasma membrane of the fibers causes calcium entry and activates calcium dependent proteases such as calpains that can rapidly destroy cell components, leading to plasma protein influx and activation of the complement. Resident macrophages present in the connective *fasciae* that envelop the fibers release chemoattractants for both neutrophils and macrophages. These events start the recruitment of leukocytes at the site of injury (*Ciciliot and Schiaffino, 2010; Moyer & Wagner, 2011*).

The first immune cells that can be found at the site of the injury in just 1 hour after the damage are neutrophils. Neutrophils reach their peak of concentration about 24-48 hours after the damage, but can persist for about 5 days. Invading neutrophils are mainly phagocytic and they also can release proteases to help the degradation of the necrotic debris. Interestingly, they can also release cytotoxic molecules that can damage the surrounding healthy tissue and produce free radicals, leading to a secondary damage of the injured muscle (*Tidball, 2005*). Besides, neutrophils can release pro-inflammatory cytokines, such as IL6 and TNFα, that enhance the inflammatory process (*Smith et al., 2008*).
Macrophages start to invade the injured site around 1 day after injury, when neutrophil number start to decline, and they are present until the regeneration process is complete. The first macrophages that invade the site of the injury are pro-inflammatory macrophages. They can phagocytize tissue debris as well as recruit other macrophages from the blood flow. After 48 hours, these macrophages begin to switch to an anti-inflammatory profile, stimulating regeneration and regulating immune responses. Macrophages also stimulate satellite cells, helping them in the formation of new fibers (*Smith et al., 2008*).

Satellite cells

Satellite cells were identified for the first time by Alexander Mauro, in 1961 *via* electron microscopy (*Mauro, 1961*). Mauro found a cell that was comprised between the sarcolemma and the basal lamina of the fiber in the *tibialis anterior* (TA) muscle of a frog, having a little cytoplasm and a large nucleus. The name "satellite cell" was due to its location. Mauro hypothesized that these cells were myoblasts that failed to fuse with the fiber.
Satellite cells comprise about 2-7% of the total myonuclei and their number varies greatly between different muscles (*Hawke and Garry, 2001*). They are the main players in muscle regeneration, as demonstrated by many different studies (as recently reviewed by *Relaix and Zammit, 2012*). As mentioned above, they are normally quiescent in an uninjured muscle but can quickly be activated when the muscle is damaged to proliferate again and originate new myoblasts.
An important experiment that confirmed the “stem cell” status to satellite cells was performed by Morgan and colleagues. They grafted single myofibers from *Extensor Digitorum Longus* (EDL) (that contains about 7 satellite cells) of *lacZ*-transgenic mice in the irradiated TA muscles of recipient mice, and found that just

the few satellite cells carried by one single fiber were able to not only to generate more than 100 new myofibers, but also of repopulating the satellite niche (*Collins et al., 2005*).

Quiescent satellite cells seem to form a quite heterogeneous population. For example, CD34 or M-cadherin are widely used markers of satellite cells, but not all satellite cells express it. Some satellite cells express Myf5, while other do not (*Biressi et al., 2010*). Satellite cells of some muscles, such as the diaphragm and the *gracilis*, also express Pax3. (*Relaix et al., 2006*) Satellite cells markers also differ within different species: CD34 is a marker of murine satellite cells, but not human, while M-cadherin is considered to be not a reliable marker of human satellite. CD56 is considered a marker of human satellite cells, but it is also expressed by other cell types. (*Tedesco et al., 2010*)

Almost all quiescent satellite cells express Pax7, a member of the Pax gene family that is involved in the formation of tissues and organs during embryonic development. They are transcription factors that contains a 128 amino acids paired domain composed by two sub-domains, the PAI domain at the N-terminus and the RED domain near the C-terminus, that can bind a specific DNA sequence of about 17 nucleotides. They may also contain an octapeptide motif and part or all of a homeobox DNA binding domain (*Buckingham and Relaix, 2007; Chi and Epstein, 2002*). Along with another member of this family, Pax3, Pax7 is present during myogenesis in the embryo, although Pax3 is expressed earlier in a wider range of cells. Pax3 is later down-regulated in the fetal period, while Pax7 expression remains (*Relaix and Zammit, 2012*). During adult life, satellite cells express Pax7 during quiescence and after activation, down-regulating it after myogenic differentiation (*Seale et al., 2000*). Knock-out mice for Pax7 are born without any particular problem, but they are smaller and usually die before the third week after birth (*Mansouri et al., 1996*). Satellite cells are present at birth and they can proliferate and differentiate to generate myoblasts, but they disappear quickly, probably because of apoptosis, and this lead to impaired muscle maintenance and in the end, death (*Mansouri et al., 1996; Seale et al., 2000; Oustanina et al., 2004*). Interestingly, though, Pax7 does not seem necessary for the survival of adult satellite cells itself. Tamoxifen inactivation of Pax7 in adult transgenic animals showed that muscle regeneration occured normally, even for severe injuries. Satellite cells could be activated, proliferate and fuse. This was found also when Pax3 and Pax7 were both inactivated in the adult (*Lepper et al., 2009*). The meaning of this surprising finding is still debated, considering that Pax7 controls the expression of genes involved in the myogenic program, such as MyoD and Mrf4. In Pax7-null mice, there is still expression of

MyoD and other myogenic markers, probably because of redundancy between Pax7 and Pax3. In fact, Pax3/Pax7 null mice show major defects in muscle development, as muscle progenitors fail to enter in the myogenic program (*Relaix et al., 2005, Buckingham, 2007*).

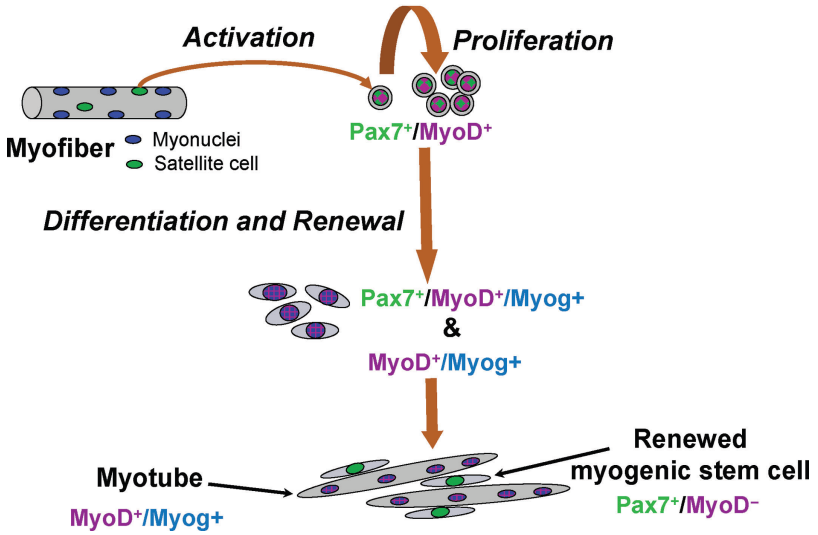


Figure 2: Schematic illustration of satellite cells activation after injury. (Reprinted with permission from *Yablonka-Reuveni et al., 2008*)

Activation of satellite cells after injury (Figure 2) is a process that requires multiple signals. Sphingolipid signaling, especially the generation of sphingosine-1-phosphate in the inner part of the plasma membrane of the satellite cells is necessary, and when its synthesis is blocked, satellite cells remain quiescent and the muscle regeneration process is defective (*Nagata et al., 2006*). Nitric oxide production (NO) is also important, via the stimulus of hepatocyte growth factor (HGF). HGF can bind to c-met and stimulate satellite cells activation (*Tatsumi et al., 2006*), but it also inhibits its differentiation (*Miller et al., 2012*).

A group of bHLH DNA-binding proteins known as myogenic regulatory factors – MRFs: namely MyoD, Myf5, Mrf4 and Myogenin– play crucial roles during satellite cells activation and differentiation.

The first one to be discovered was MyoD (*Lassar et al., 1986*), thanks to the fact that its forced expression was sufficient to convert 10T1/2 fibroblasts into myoblasts. MyoD can bind DNA at an E-box sequence (CANNTG) that is present in the promoter sequences of muscle-specific target genes, in collaboration with myocyte enhancer factor 2 (Mef2C). These two factors dimerize with E-proteins to activate gene expression, but are inhibited by the presence of particular inhibitor of DNA binding (Id) proteins. MyoD complex interaction with Id proteins and other transcriptional repressors regulates the activation of the

myogenic program (*Berkes and Tapscott, 2005; Yokoyama and Asahara, 2011*). MyoD targets seem to be various and not all the targets have been identified yet). MyoD also seems to interact with hystone deacetylase complexes, allowing chromatin remodelling at specific loci (*Tapscott, 2005*).

Upon activation, satellite cells upregulate both MyoD and Myf5, but this second factor is expressed even by a subset of the quiescent satellite cells in the body. Given these observations, Rudnicki and colleagues proposed that those satellite cells which show Myf5 expression may be more committed progenitors, while the $Myf5^{-}$ population may be the real stem cell compartment, that could produce both satellite cells and committed progenitors via asymmetric division (*Kuang et al., 2007*). Single knock-out mice for either MyoD or Myf5 showed normal myogenesis, even if Myf5 expression was increased in $MyoD^{-/-}$ mice, suggesting a probable compensation by that factor. To further investigate the role of those two factors, a double knock-out for both MyoD and Myf5 was generated, and these mice showed no formation of muscle, as well as complete absence of myogenic stem cells (*Rudnicki et al., 1993, Berkes and Tapscott, 2005*).

The role of the third MRF, Mrf4 (previously known as myf6 or herculin) is more complex. It appears to be involved both in activation and differentiation of satellite cells into myoblasts, since it can target the expression of other MRFs targets (*Berkes and Tapscott, 2005*). Mutations in Mrf4 can lead to myopathies, even when present in heterozygosis (*Kerst et al., 2000*).

Myogenin is the last activated gene, marking the differentiation of the activated myoblasts into myotubes. A recent work showed that down-regulation of myogenin by RNA interference in C2C12 myotubes disrupted their structure, generating mononucleated cells that re-entered the cell cycle (*Mastroyiannopoulos et al., 2012*). Knock-out mice for myogenin die immediately after birth and show heavily reduced muscle mass, accumulation of fat tissue, abnormal curvature of the vertebral column, deformed rib cage and an overall more fragile skeleton, probably due to the absence of muscle mass (*Hasty et al. 1993*).

After activation, satellite cells start proliferating, and the vast majority of them down-regulate Pax7, up-regulate MyoD and Myf5, committing to the myogenic fate. After a while, these activated myoblasts up-regulate myogenin and Mrf4 and down regulate MyoD and Myf5, leading to terminal differentiation and fusion to generate new fibers or repair injured ones.

The switch from proliferation to differentiation appears to be controlled by the Wnt and Notch signaling pathways. Notch signaling is required during the first days of muscle regeneration, then the Wnt signaling pathway takes the lead and

participate in the process of muscle differentiation. Forced activation of the Wnt pathway led to premature differentiation *in vivo*, while Notch forced activation inhibited myogenesis (*Brack et al., 2008*).

Not all the satellite cells become myoblasts and then fuse to generate new fibers, since at the end of this process, the satellite cells pool remains intact. To explain this, two models were proposed by Rando and colleagues in 2005 (*Dhawan and Rando, 2005*): stochastic cell fate and asymmetric cell division (Figure 3).

In the former, some activated satellite cells that are proliferating to generate myoblasts down-regulate MyoD and return to quiescence, becoming satellite cells again for the newly formed fibers, while the majority of the cells become myoblasts and then fuse. In the latter, activated satellite cells can divide asymmetrically, generating one daughter cell that returns to quiescence and another committed daughter cell that continues to proliferate to generate new myoblasts. Indeed satellite cells seem to be able to return to quiescence (*Zammit et al., 2004*) and asymmetric division has been reported by Rudnicki's group as mentioned above (*Kuang et al., 2007*), but the exact mechanism of the preservation of the satellite cells pool is still unknown.

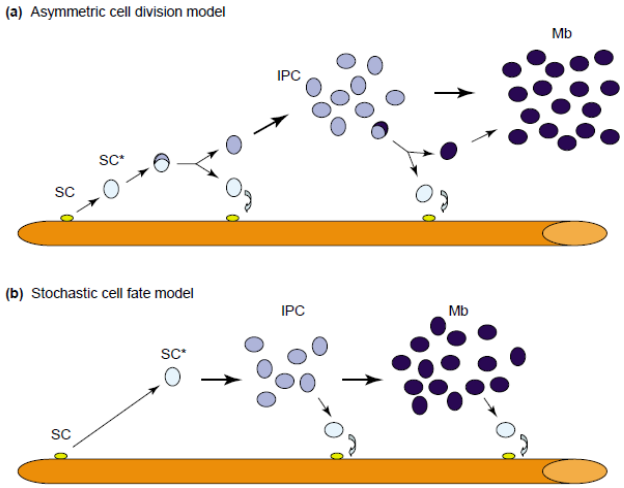


Figure 3: Rando and colleagues proposed two different models to explain the maintenance of the satellite cells pool. The first model (a) shows the capability of satellite cells to asymmetrically divide, generating another stem cell and a committed myogenic progenitor; the stochastic cell fate mechanism (b) hypothesizes that some of the proliferating, committed cells revert to a 'stem-state' and return to quiescence. SC = satellite cells; SC* = activated satellite cells, IPC = intermediate progenitor cells; Mb = myoblasts. (Reprinted with permission from *Dhawan and Rando, 2005*)

Satellite cells heterogeneity

Skeletal muscles are derived mainly from the somites, with the exception of the vast majority of the head muscles, which are derived from the mesodermal core of

the branchial arches and from the prechordal mesoderm. These muscles follow different genetic programs during development and present differences in gene expression, fiber composition and signaling cascades. Differences in the development and the innervation are present even between muscles derived from the somites, such as limb and back muscles (*Biressi et al., 2007*, *Biressi and Rando, 2010*).

Similarly, satellite cells from different muscles also show dissimilarities, such as the expression of different combination of markers (*Day et al., 2007, Harel et al., 2009*). For example, Zammit and colleagues examined satellite cells isolated from a muscle of somitic origin (EDL) and a muscle from the cranial mesoderm (masseter), finding differences both in their proliferation rate and differentiation capacity (*Ono et al., 2010*).

There are differences also between cells derived from fast and slow muscles, since they are able to generate physiologically different cells after *in vitro* cultivation (*Huang et al., 2006*). At the same time, there are also evidences that upon transplantation newly formed fibers can override their "signature" and generate myoblasts with the same characteristics of the host muscle (*Kalhovde et al., 2005*).

Satellite cell niche

Satellite cells, as other stem cells in the body, are located in their specific “niche”, a microenvironment that plays a fundamental role in regulating their behavior in response to different stimuli, both via soluble factors present in the surrounding area of the cell and by biophysical and mechanical factors. Many different stem cells niches have been identified in the human body, for example in bone marrow, skin, intestine, brain and skeletal muscle. It has been proved that signals regulated by the niche environment can influence self-renewal and generation of more committed cells (*Jones and Wagers, 2008*).

Jones and Wagers made a list of elements that should be present in a stem cell niche, although not necessarily all at once in each specific instance. The list comprises stromal support cells that interact with stem cells, through receptors, soluble factors and gap junctions, ECM proteins that organize the structure of the niche, blood vessels to carry the signal molecules to the cells and neural inputs to transmit physiological cues (*Jones and Wagers, 2008*).

At present, satellite cell niche is still poorly characterized, even if some interesting data have been published. As already mentioned, satellite cells are located between the sarcolemma and the basal lamina that surrounds the fiber itself; such spatial organization gives to the niche an asymmetrical distribution of its

component, since the fiber-derived cues would arrive from one side of the satellite cells, while stimuli from other tissue would arrive from the basal lamina (Figure 4). This asymmetry is present also in other stem cell niches, and it is thought to be very important for stem cells self-renewal (*Kuang and Rudnicki, 2008*).

On the basal lamina side there are many extracellular matrix components –like collagen IV, laminin, fibronectin, entactin and other glycoproteins– that interact with the satellite cells through the α7/β1-integrin expressed on its cell surface. These components can also bind various secreted factors, like some ligands involved in the Wnt signalling pathway, basic fibroblast growth factor (FGFb), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and epidermal growth factor (EGF) (*Cosgrove et al., 2009*).

Myofibers also express and secrete various factors that can interact and influence satellite cells, like SDF-1 (that can stimulate cell migration) or M-cadherin (that facilitates cell adhesion and may be involved in cell fusion) (*Cosgrove et al., 2009*).

To complete the picture, satellite cells express many different factors that interact with the niche, like ligands and receptors for the Notch pathway that could regulate satellite cell behavior (*Cosgrove et al., 2009*).

In addition to all these elements, elastic stiffness of the surrounding environment could also influence satellite cell behavior, especially in the aging process (*Cosgrove et al., 2009*).

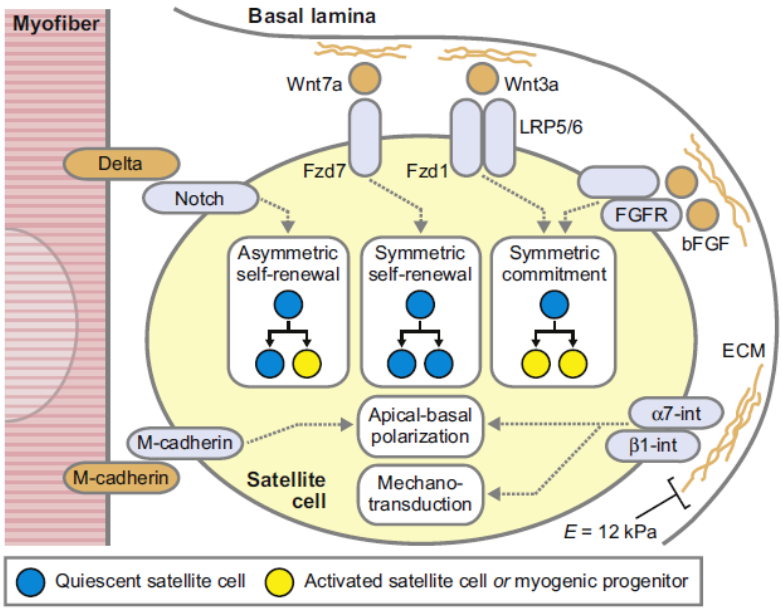


Figure 4: Schematic representation of the satellite cell niche: major components are shown, highlighting their influence on satellite cell division. (Reprinted with permission from *Cosgrove et al., 2009*)

Satellite cells are not the only source of myogenic cells

Satellite cells are by far the main compartment of myogenic stem cells, both numerically and functionally, but are not the only one. A multipotent stem cell population, named muscle-derived stem cells (MDSCs), has been isolated more than 10 years ago. These cells appear to be a possible progenitor of satellite cells, being able to differentiate into other lineages (for example osteogenic, adipogenic and endothelial). They can proliferate for a long period of time, can self-renew and are less attacked by the immune system when transplanted into a host muscle (*Qu-petersen et al., 2002*).

Even in tissues different from the muscle, various cell types were found to be able to commit to the myogenic lineage.

Almost 20 years ago, Tajbakhsh and colleagues found Myf5 expressing cells in the murine brain and spinal cord (*Tajbakhsh et al., 1994*). Later, it was found that neural stem cells are capable of differentiating into skeletal muscle cells, when transplanted into a regenerating muscle (*Galli et al., 2000*).

Cells derived from the hematopoietic system can also generate myoblasts: the first evidences were found in a study in 1998, where the investigators found β-gal positive muscle fibers in mice transplanted with the BM of a β-gal transgenic mouse (F*errari et al., 1998*). This was also found in a dystrophic patient that received bone marrow transplantation and subsequently showed the presence in his skeletal muscles of a very low percentage of donor-derived cells, which persisted even after 13 years (*Gussoni et al., 2002*).

Subsequent studies identified a fraction of the BM, expressing CD45, that possess myogenic potential and that was able to generate muscle fibers when transplanted into irradiated murine muscle (*Mckinney-freeman et al., 2002*).

Another subpopulation of circulating cells, called Ac133 as they express CD133 (a marker of hematopoietic stem cells) also express satellite cells markers such as m-cadherin and Myf5. These cells are not able to fuse in culture, but when injected in the TA of dystrophic mice they were able to generate dystrophin positive fibers, ameliorating also their maximum specific force (*Torrente et al., 2004*).

Mesenchimal stem cells can also be committed to the myogenic lineage by overexpressing Pax3 (*Gang et al., 2008*), but injection of these cells of murine origin in mice led to formations of rhabdomyosarcomas. The same experiment conducted with human mesenchimal stem cells reprogrammed with overexpression of Pax3 did not lead to tumor formation, and they were able to

successfully fuse and generate dystrophin positive fibers in dystrophic mice, but there was no significant improvement of muscle function (*Gang et al., 2009*).
Mesangioblasts, multipotent progenitor cells associated to the vessels (*Minasi et al., 2002*), can also commit the myogenic lineage. These cells were also isolated from human adult skeletal muscle, and were able to form myotubes that express myogenic markers and to form dystrophin-expressing fibers in a dystrophic immunodeficient mouse (*Dellavalle et al, 2007*).
Péault and colleagues identified a population derived from the vascular endothelium of human adult skeletal muscle that is able to differentiate into myoblasts, and could regenerate fibers in an injured immunodeficient mouse muscle (*Zheng et al., 2007*).
Finally, human multipotent adipose-derived stem cells, isolated from the adipose tissue, can differentiate into different lineages, including the myogenic lineage. These cells were found to be able to engraft and generate fibers in dystrophic mice, and when MyoD expression was forced the myogenic program was enhanced, while they were not able to generate adipocytes (*Goudenege et al., 2009*).

Macrophages and muscle regeneration

Macrophages are phagocytic cells that differentiate from circulating monocytes in the blood. These in turn originate in the bone marrow from a common myeloid progenitor that also generates neutrophils, eosinophils, basophils, dendritic cells and mast cells. They constitute about 5-10% of the peripheral blood leukocytes in the human body. Monocytes migrate from the blood to the various tissues and differentiate into macrophages to maintain the resident populations that are present in every tissue (*Mosser and Edwards, 2008*).
Macrophages are not a homogeneous population, reflecting the many different roles that these cells have in the various physiological processes of the body. The different macrophagic populations are commonly divided into two main classes: M1 and M2, with the M2 type divided again into 3 different subtypes. These three different subtypes are stimulated by different factors, express different marker combinations, have distinct cytokine secretion patterns and play different roles during the inflammatory response (Figure 5).

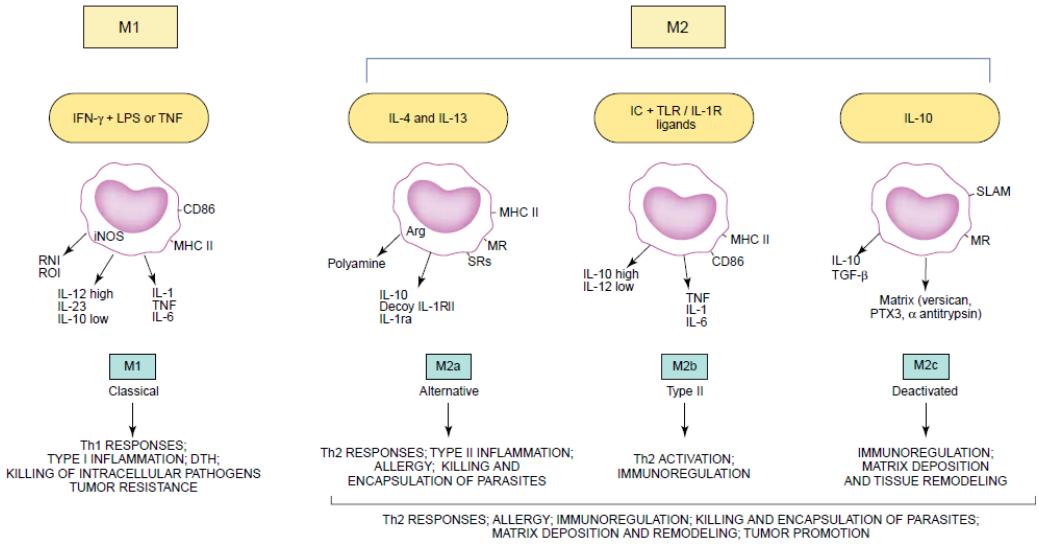


Figure 5: Macrophages can be divided into two main categories: M1 macrophages, that elicit a Th1 immune response, and M2 macrophages, that stimulate a Th2 immune response. M2 macrophages can be divided again into three subtypes: M2a, M2b and M2c. (Reprinted with permission from *Mantovani et al., 2004*)

M1 macrophages, also called classically activated macrophages, show a pro-inflammatory behavior: they have a high capacity of presenting antigens, produce pro-inflammatory cytokines, nitric oxide (NO) and reactive oxygen intermediates. *In vitro*, monocytes can be differentiated into M1 macrophages upon stimulation by interferon γ (INFγ) together with either lipopolysaccharide (LPS) or tumor necrosis factor (TNFα) (*Tidball and Villalta, 2010*).

The so-called "alternative" activation of macrophages, obtained *in vitro* through stimulation with IL-4 and IL-13, generates M2a macrophages. They can elicit anti-inflammatory responses, are involved in allergies and can encapsulate and kill parasites. They are also involved in wound healing and tissue repair.

Treating macrophages with IL-10 generates M2c macrophages, which are involved in tissue regeneration and remodelling and can release cytokines that deactivate the M1 phenotype.

The last M2 subtype is M2b macrophages, which are induced via exposure to immune complexes and Toll-like receptors or IL-1R. They can release anti-inflammatory cytokines (*Mantovani et al., 2004*; *Tidball and Villalta, 2010*).

Macrophage polarization is a very complex process, which more recent literature suggest should be considered more like a continuum, with M1 and M2 fully polarized cells at the two extremes (*Mantovani et al., 2009*). Consistently with this observation, it is not easy to identify 100% specific markers for M1 and M2 cells, and even for total macrophages. For example, F4/80, a member of the Adhesion-GPCRs receptors, is expressed in most macrophages in the mouse; in

human, F4/80 homolog EMR1 is predominantly expressed in eosinophils (*Murray and Wynn, 2011*).

Many groups use CD68 as a marker for M1 macrophages but M2 macrophages present in secretory organs can express it too (*Linehan, 2005*). CD68, also called macrosialin, is a type I integral membrane protein with a heavily glycosylated extracellular domain, member of the scavenger receptor family that is also able to bind to oxidized low-density lipoproteins (*Rabinowitz and Gordon, 1991; Ramprasad et al., 1995*). Binding to oxidized LDLs is supposed to activate phagocytosis by macrophages (*Ottnad et al., 1995*). A recent study found that CD80 could be a good marker for M1 human macrophages, but it still has to be validated in mouse (*Ambarus et al., 2012*). Another commonly used marker for M1 macrophages is CCR2, the receptor for the MCP-1 chemokine, involved in the recruitment of M1 macrophages at the site of the injury. Recently, Leuschner and colleagues demonstrated that CCR2-silencing by siRNA administered systemically in mice prevented macrophages accumulation in inflammation sites, proving itself beneficial for various pathological conditions, such as atherosclerotic plaques or some tumours (*Leuschner et al., 2011*).

M2 macrophages express several markers, one of the most commonly used being CD163. CD163 is a receptor for hemoglobin and haptoglobin complexes, expressed both in human and murine macrophages. Ligation of these complexes can counter the oxidative tissue damage due to hemolysis and inhibit the production of free radicals. This process is thought to be important for the shift to pro-inflammatory to anti-inflammatory macrophages during injury healing (*Philippidis et al., 2004*). Another M2 marker expressed only in rodents is CD206, a mannose receptor that can bind and internalize sugar groups present in proteins in the damaged tissues. CD206 can also bind to myeloperoxidase, produced by the cell membrane lysis process operated by neutrophils, reducing tissue inflammation (*Shepherd and Hoidal, 1990*). In mouse, FIZZ1 (transcription factor found in inflammatory zone 1), Ym1 (chitinase-like 3 lectin) and ARG1 (arginase 1) can also be considered good M2 markers, but not for human (*Mantovani et al., 2009*).

Up to date, no good markers for the various sub-types of M2 macrophages have been identified. Tidball and colleagues consider CD163 an M2c-only marker, since its expression is induced by IL-10, that indeed is the stimulus that polarize macrophages towards the M2c phenotype (*Villalta et al., 2011*). On the other hand, other Authors point in the direction of considering CD163 more an M2a marker (*Mantovani et al., 2004*) and in a previous work Tidball and colleagues considered CD163 as a marker of both M2a and M2c (*Villalta et al., 2009*).

Altogether, it is clear that more work will be needed to better understand the complexity of macrophagic polarization and to identify good specific markers.

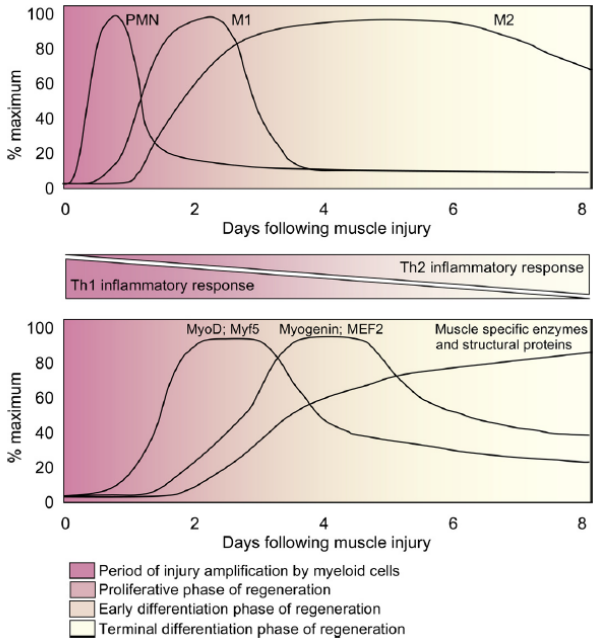


Figure 6: Progression of different inflammatory cell types during muscle regeneration (top) and changes in the expression of muscle-specific markers (bottom). PMN: neutrophils; M1: M1 macrophages; M2: M2 macrophages.
(Reprinted with permission from *Tidball and Villalta, 2010*)

As already said, and illustrated in the top section of Figure 6, macrophages invade the damaged muscle tissue 1 day after injury, when the neutrophil peak starts to decline. The first type of macrophages present in the injured tissue are M1, pro-inflammatory. The signals responsible for the beginning of the inflammatory response and attraction of pro-inflammatory cells are not fully known. It is thought that the release of intracellular chemoattractant can recruit inflammatory cells from the blood flow. In the case of muscle, a possible chemoattractant candidate is desmin, a muscle-specific intermediate filament protein that is rapidly lost during fiber disruption and it is able to activate the complement system (*Linder et.al, 1979*). Another factor involved in the recruitment of leukocytes is monocyte chemoattractant protein 1, also called CC chemokine ligand 2 (CCL2). CCL2 is released by macrophages resident in the tissue when there is an injury (*Moyer et al., 2011*).

M1 macrophages, once arrived at the site of injury, are able to phagocyte cellular debris and release pro-inflammatory cytokines that stimulates inflammation and cause further damage to the fibers via production of nitric oxide (NO) through the nitric oxide synthase (iNOS) (*Tidball and Villalta, 2010*). They express high

levels of the pro-inflammatory cytokines IL-12 and IL-23 and can produce reactive oxygen species as well as other inflammatory cytokines like IL-1β, TNFα, IL-6 (*Mantovani et al., 2005*).

Various studies on muscle injury have investigated whether blocking the acute phase of the inflammation to limit the secondary damage caused by neutrophils and in part by M1 macrophages could be detrimental or beneficial for final repair. These studies found that limiting the extent of the inflammatory response during neutrophil invasion can limit the damage to the fibers and decrease the pain (*Tidball, 2005*). On the other hand, depletion of macrophages greatly hinders muscle regeneration. Summan and colleagues targeted macrophages in mice using liposome-encapsulated clodronate and then proceeded to look at its effect on muscle injury, finding that treated mice showed impaired muscle regeneration (*Summan et al., 2006*). Tidball and Wehling-Henricks found that treating mice with an anti F4/80 antibody also led to impaired tissue repair. (*Tidball and Wehling-Henricks, 2007*). Similar results were found by Segawa and colleagues, using a specific antibody that blocks M-CSFR (macrophage colony stimulating receptor) leading to suppression of macrophages infiltration (*Segawa et al., 2008*). Several studies based on the use of non-steroidal anti-inflammatory drugs (NSAID) demonstrated that administering NSAID during the early repair phase can result in a modest reduction of inflammatory symptoms, but treatments longer than 3 days have a negative effect on the healing of the injured muscles, delaying regeneration and increasing fibrosis. Other studies using steroidal anti-inflammatory drugs showed that, no matter how long the treatment, their administration had detrimental effects, such as delayed debris removal, and longer regeneration (*Prisk and Huard, 2003; Smith et al., 2008*). These results, together with the data on impaired regeneration when macrophages are absent, show that the first acute inflammatory phase is indeed necessary for proper muscle healing.

The acute inflammatory phase reaches the maximum peak 2 days after injury, when macrophages shift towards an M2 phenotype. M2 macrophages express high levels of anti-inflammatory cytokines such as IL-10 and TGFβ. Macrophages stay at the site of the injury until complete healing. M2 macrophages can secrete many growth factors that can stimulate tissue regeneration, like Transforming Growth Factor-β1 (TGFβ1). Macrophage-derived TGFβ1 promotes fibroblast differentiation into myofibroblasts by enhanced expression of tissue inhibitors of metalloproteinases (TIMPs), that blocks the degradation of the extracellular matrix and stimulate the synthesis of collagen. They can also secrete factors that induce apoptosis in myofibroblasts after wound healing is reaching completion (*Murray and Wynn, 2011*).

This shift in macrophagic polarization is very important for correct regeneration, but little is known about its exact mechanism. Some *in vitro* studies suggest that the phagocytic process itself could contribute to the shift, since M1 polarized macrophages showed a reduction of TGFα secretion and an increased the production of TGFβ after phagocytosis of necrotic muscle cells (*Arnold et al., 2007*). Not all the necrotic tissue is able to stimulate this shift: necrotic neutrophils increase IL-10 production by macrophages, while lysed lymphocytes do not have this effect (*Fadok et al., 1998*). Another important factor might be the presence of apoptotic *versus* necrotic cells in the tissues: apoptotic neutrophils are able to suppress expression of various cytokines like IL-10 and TNFα, while increasing expression of TGFβ. This observation supports the hypothesis that apoptosis of neutrophils at the site of injury could be a signal to activate the switch in macrophagic polarization (*Fadok et al., 2001*).

During the shift from M1 to M2, macrophages reduce their expression of iNOS, while up-regulating arginase, causing a shift in the metabolism of arginine. At the site of injury, within 1-2 days after damage, M1 macrophages convert arginine into citrulline and NO, that can modulate the first part of the healing process and can increase the damage to the tissue, while after 3 days, the shift towards an M2 phenotype leads to the hydrolysis of arginine into ornithine and urea, that can increase tissue repair (*Albina et al., 1990*, *Shi et al., 2002*).

As mentioned above, macrophages are crucial for correct muscle regeneration: however, there is still no general consensus on the effects of M1 and M2 subtypes on myogenic cells. As shown in figure 6, M1 macrophages are present during the first part of the process, when satellite cells are activated and start to proliferate, while M2 macrophages appears at later stages, when myoblasts differentiate and fuse to form new fibers and repair the less damaged ones. Experiments performed by Chazaud and colleagues indeed found that M1 macrophages in co-culture with myoblasts stimulated their proliferation but not their differentiation, while M2 macrophages stimulated differentiation and not proliferation. Supporting this thesis, depleting macrophages at the time of the injury impaired regeneration, but depleting macrophages after 4 days from the damage had only little effect on muscle regeneration *per se*, but the diameter of the newly formed fibers was significantly smaller (*Arnold et al., 2007*). This effect was seen also for human myoblasts *in vitro* and in human muscle sections (*Saclier et al., 2012*).

This data agrees with what was found by Bencze and colleagues, who co-injected M1 macrophages with human myoblasts in immunodeficient mice, and found that pro-inflammatory macrophages improved the participation of the injected myoblasts in the host muscle regeneration, lengthening proliferation, increasing

migration and delaying differentiation. They also found by immunostaining of transplanted macrophages at different time points that they were able to switch to an M2 phenotype in vivo, speculating that they might stimulate differentiation during muscle regeneration (*Bencze et al., 2012*).

On the other hand, Tidball and colleagues found different results when studying macrophages population in the dystrophic *mdx* murine model of Duchenne Muscular Dystrophy (DMD). In fact, they found that M2 macrophages, when placed in co-culture with C2C12 murine myoblasts, were able to enhance their proliferation, while co-cultures of C2C12 myotubes with M1 macrophages led to the lysis of the myotubes (*Villalta et al., 2009*). This effect was seen also with dystrophic mouse myoblasts and macrophages (*Villalta et al., 2011*).

Murine macrophage conditioned medium (mMCM)

For the past years our group studied the myogenic effects of factors secreted by the murine macrophagic cell line J774. These cells, upon stimulation with LPS in a serum-free environment, produce a conditioned medium (murine macrophage-conditioned medium, mMCM) that proved to have a strong effect on myogenic cells, both *in vitro* and *in vivo*.

LPS stimulation should lead to a typical M1 polarization, but the effects of mMCM on myogenic and non-myogenic cells that we have found so far are broader than one could be expected under this hypothesis (*Cantini et al., 2002*).

mMCM was able to select and expand a sub-population of small, round, poorly adhesive and slow-growing stem-like cells, that showed mesenchimal differentiation plasticity. At the same time, it could inhibit the tendency of rat satellite cells to shift towards adipogenesis. Finally, intramuscular administrations *in vivo* of concentrated mMCM in a rat model of extensive surgical ablation dramatically improved muscle regeneration, increasing the amount of contractile tissue and decreasing fibrosis (*Malerba et al., 2009*).

mMCM was also able to enhance the proliferation rate of DMD myoblasts, while at the same time maintaining their myogenicity after many *in vitro* passages. Expanded cells were also injected in muscles of immuno-deficient mice, and we found that they were able to participate in the regeneration of the recipient muscles (*Malerba et al., 2010*).

Duchenne Muscular Dystrophy and its mdx *murine model*

Duchenne Muscular Dystrophy (DMD) is an inherited neuromuscular disorder that affects about 1 every 3500 live male births. Patients are usually asymptomatic until 3-5 years (*Matsuo, 1996*); the disease is then characterized by progressive muscle degeneration and the patients are invariably confined to a wheelchair by their mid-teens. Affected individuals usually die in the third decade of age due to heart and/or respiratory failure (*Blake et al., 2002, Chakkalakal et al., 2005*).

Fetal DMD muscle is histologically normal, but after birth, even before the onset of the first clinical signs, clusters of necrotic and regenerating fibers can be seen in DMD muscle biopsies. The muscle is subject to continuous cycles of degeneration and regeneration, but eventually the regenerative capacity of the muscle is lost and the muscle fibers are gradually replaced by adipose and fibrotic tissue. This progressive muscle fiber loss results in muscle wasting and weakness (*Blake et al., 2002*).

Both forms of muscular dystrophy are caused by mutations in the DMD gene, which encodes for \ dystrophin, a large rod-shaped protein of 427 kDa, member of the β-spectrin/α-actinin protein family. The DMD gene also encodes for various shorter isoforms that consist in truncated COOH-terminal proteins, all derived from different splicing patterns and internal promoters (*Blake et al., 2002*).

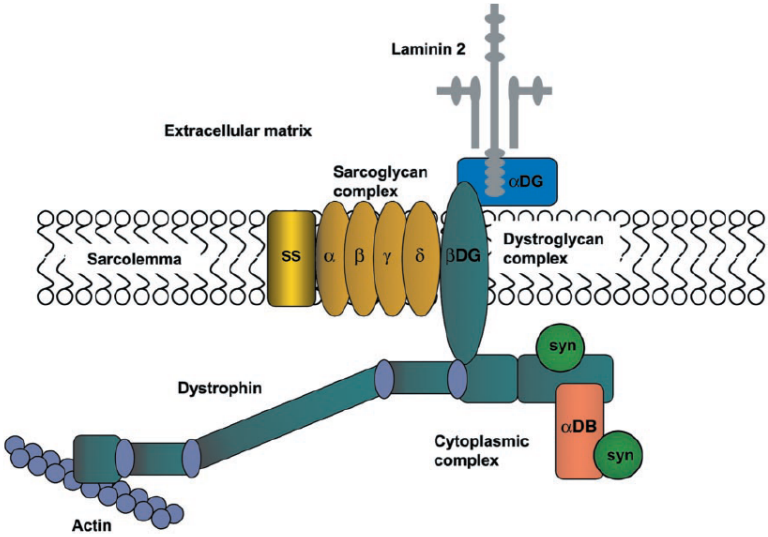


Figure 7: Schematic representation of dystrophin and the dystrophin-associated protein complex (DPC).. Dystrophin binds the actin cytoskeleton via its N-terminus, and via its C-terminus to α-dystrobrevin (αDB), syntrophins and β-dystroglican (βDG). β-dystroglican is linked to the sarcoglycan complex, that is composed by composed by α-, β-, γ- and δ-sarcoglycan and sarcospan (SS). β-dystroglican is also linked to α-dystroglycan (αDG), that is linked to laminin 2. (Reprinted with permission from *Blake et al., 2002*)

Dystrophin is localized to the cytoplasmic face of the sarcolemma, within a cytoskeletal lattice termed costamere. Costameres are transverse, rib-like structures composed of many different proteins (like vinculin and β-spectin) that overlie the Z lines of the sarcomere. They couple the sarcolemma with the Z disk (*Lapidos et al., 2004*). In the costameres, Dystrophin is part of a large protein complex called the dystrophin-associated complex (DPC) composed by at least 10 different proteins (Figure 7). This complex is inserted in the sarcolemma, and it links it through dystrophin to the actin cytoskeleton, and also to the extracellular basal lamina. Thus it can be said that DPC links the outside of the muscle fiber with the inside, stabilizing the cell membrane against the mechanical forces developed during muscle contraction (*Ervasti, 2007*).

The absence of dystrophin leads to the disarray of DPC, weakening of the sarcolemma and to the loss of a correct organization of the costameric structure. DMD muscle is hence characterized by abnormal fragility and leakiness of the sarcolemma, is more permeable to macromolecules and less resistant to mechanical stress. The influx of Ca^{2+} through the leaky sarcolemma is increased, leading to activation of proteases, especially calpains, that destroy the membrane, increasing the calcium influx even more in a constant loop that disrupts calcium homeostasis and eventually lead to cell death (*Deconinck and Dan, 2007*).

Dystrophic muscles are subjected to continuous cycles of degeneration, with the destruction of the muscle fibers and the subsequent regeneration that eventually fail to keep up with the necrosis of the muscle fibers, causing atrophy and accumulation of fat and fibrotic tissue due to the concurrent exhaustion of the myogenic potential of satellite cells.

At present there is no treatment for DMD and the only clinical options involve palliative measures. Since controlling and limiting inflammation has been shown to slow down the pathology (see below), patients can be treated with anti-inflammatory drugs, i.e., corticosteroids like Prednisolone or Deflazacort (*Deconinck and Dan, 2007*).

The mdx *mouse*

The dystrophic *mdx* mouse (C57BL/10ScSn-*Dmd*^{mdx}/J) was first identified in 1984, because of its increased creatine kinase serum concentrations (*Bulfield et al., 1984*). It is a spontaneous mutant that carries a nonsense mutation in exon 23 of the Dmd gene (*Sicinski et al., 1989*).

Despite carrying a genetic defect that in human would lead to DMD, the *mdx* mouse does not show a similarly severe phenotype; for one, it does not show

obvious weakness until late in life, when lumbar kyphosis, hind limb contracture and dropped head can be seen. Its life span is moderately reduced compared to *wild type* mice, with no *mdx* surviving after 24 months. *Mdx* muscles are hypertrophic and their strength starts to decline progressively after 20 weeks of age (*Connolly et al., 2001*). Muscle degeneration is not continuous, but it appears in subsequent waves. The first wave of degeneration and regeneration is observed at 2–4 weeks after birth, which results in necrosis and increase in the number of newly differentiating myofibers. After this inflammatory peak, *mdx* fibers show frequent central nucleation, but the expression of fetal myosin heavy chain declines. Necrotic fibers can be found at any age, especially with increasing frequency after 18 months of age. *Mdx* muscle tissue is slowly lost with age. The cycles of degeneration and regeneration continue but, differently from human patients, satellite cells maintain their potential to generate new fibers and continue to express markers of activation (*Nakamura and Takeda, 2011*).

Muscle weakness is usually not evident until at very old age. Fibrosis in most *mdx* mouse muscles is less pronounced than in DMD patients, with the exception of the diaphragm. *Mdx* mice also show abnormal cardiac function, similarly to DMD-associated cardiomyopathy. The *mdx* heart shows an increase in myocardial fibrosis and the occurrence of foci of myocardial necrosis and inflammation with age (*Willmann et al., 2009*).

Mdx muscles also show atrophy and fat accumulation like DMD patients, but less prominent. One of the most affected muscles of the *mdx* mice is the diaphragm (*Durbeej et al., 2002; Collins and Morgan, 2003; Willmann et al., 2009*).

As already said, the *mdx* dystrophic muscle is subject of continuous waves of degeneration and regeneration, and the muscles suffer from chronic inflammation. Macrophages and T cells are the primary infiltrating immune cell types, both in DMD patients and in the *mdx* mouse model. Both M1, M2a and M2c macrophages types were found in the dystrophic muscle (*Villalta et al., 2009*), and M1 macrophages, with their lytic action over the damaged tissue, further damage the already weak dystrophic environment. Indeed, it has been demonstrated that decreasing macrophage pro-inflammatory activity can alleviate the severity of the pathology, both histologically and functionally (*Wehling et al., 2001*). Tidball and colleagues demonstrated that ablation of the iNOS gene in *mdx* mice, thus interfering with the metabolism of M1 macrophages during inflammation, reduced muscle membrane lysis, caused by M1 macrophages themselves.

Disrupting INFγ-mediated signaling also decreased the pathophysiology of the mouse, doubling the treadmill running time and reducing fiber injury in the mice.

The authors speculate that disruption of this signaling changed the balance between M1 and M2 macrophages, causing a shift towards the M2 phenotype and promoting muscle healing. The author also speculated that this change in the polarization of the macrophages is due to INFγ suppression of the M2 phenotype, more than by its inducing effect of M1 macrophages (*Villalta et al., 2011*).

Co culture of M1 and M2a macrophages derived from dystrophic mice showed that M2a macrophages can prevent cell lysis by M1 macrophages by expressing high levels of arginase-1 that compete with iNOS for L-arginine, reducing NO production (*Villalta et al., 2009*). Tidball and colleagues also speculate that IL-10 could be involved in the shift in macrophage phenotype during the progression of the *mdx* pathology. In the muscle of 4 weeks old *mdx* mice, there is a high production of IL-10. They found that IL-10 is able to induce phagocytosis in the *mdx* macrophages and stimulate expression of CD163, contributing to the macrophagic shift (*Villalta et al., 2011*).

After the wave of M1 macrophages in correspondence of the initial bout of muscle degeneration around the 4 weeks, even though the muscle obviously can never reach complete repair, an M2c population, capable of dampening the activity of M1 macrophages, then replaces the pro-inflammatory macrophages. M2 *mdx* macrophages, when placed in co-culture with C2C12 murine myoblasts, were able to enhance their proliferation, while co-cultures of C2C12 myotubes with M1 *mdx* macrophages led to the lysis of the myotubes (*Villalta et al., 2009, 2011*).

At the same time, macrophages are also involved in the fibrosis process of the dystrophic pathology in the *mdx* mouse: TGFβ, a cytokine that can increase the expression of connective tissue proteins, has an important role in the fibrotic deposition process. Studying a fibrinogen deficient mouse, Pura Muñoz-Cánoves and colleagues concluded that fibrinogen deposition, a part of the fibrotic process, can induce a pro-inflammatory macrophages response that stimulates synthesis of IL-1β that stimulates the secretion of TGFβ itself, in a sort of positive feedback mechanism that in the end enhances fibrotic deposition itself (*Vidal et al., 2008*).

Fibrosis in the *mdx* muscle is also promoted by arginine metabolism by M2 macrophages, as demonstrated by James Tidball and colleagues. They generated a line of arginase-2-null mutants *mdx* mice, finding that these mice showed reduced fibrosis, while supplying arginine in the diet of the mice for a long period of time enhanced it (*Wehling-Henricks et al., 2010*).

The *mdx* mouse show many features similar to the human pathology, but at a later time point, but the reason of this difference needs to be still completely elucidated

(*Blake et al., 2002*). Despite these differences, it is nonetheless still a very good and useful model for the study of the DMD pathology.

Aim of the thesis

The aim of this thesis is to investigate the interplay between macrophages and muscle regeneration, using both *wild type* and dystrophic (*mdx*) mouse models, with *in vitro* and *in vivo* experiments.

To do so, we used a murine tumour macrophages conditioned medium (mMCM) that we have already shown to have relevant pro-myogenic effects, bth *in vitro* and *in vivo*, in rat and human. Beside the murine model, we will also investigate its action on the proliferation of muscle-derived fibroblasts and on the polarization of primary human macrophages. Last but not least, we will continue the work towards the characterization of its biochemical composition.

Our ultimate goal is to gain better knowledge of the interactions between macrophages and muscle cells, both in the normal and in the dystrophic muscle, to then apply it towards the development of therapeutic tools for muscle pathologies.

Materials and methods

mMCM production

The murine macrophage conditioned medium (mMCM) was prepared using the murine macrophage cell line J774A.1, that was expanded in 10% fetal bovine serum (FBS) in high glucose Dulbecco's Modified Eagle Medium (DMEM) with 100 U/ml penicillin, 100 mg/ml streptomycin (P-S) until confluence was reached. Cells were then washed 2 times with 1X PBS, and one time with DMEM, and then they were left with serum-free DMEM containing 0,01 μg/ml lipopolysaccharide (*E. coli* LPS, Sigma-Aldrich) for 24 hours. They were then washed again 2 times with PBS, one more time with DMEM and then they were left in serum-free DMEM for 72 hours. All cell culture media were from Life Technologies.

The conditioned medium (mMCM) was then collected, centrifuged at 5000 rpm for 30 minute at 4°C, filtered with a 0,22 μm filter to remove cellular debris and then concentrated tenfold with an Amicon Ultra-15 centrifugal filter unit with a 3 kDa cut-off (Millipore). Both the 1X and 10X mMCM were aliquoted and stored at -20°C until use.

mMCM test

To test if a mMCM batch had the desired biological activity we used primary cultures from neonatal rat limbs, as described in *Malerba et al., 2009*. Specifically, whole limbs of 1-2 days old neonatal rats were cut off and placed in PBS. After removing the feet, samples were minced, removing the cartilage skeleton but not the the skin. The resulting material was then collected in a 50 ml Falcon tube and centrifuged 10 minutes at room temperature at 400 xg, to remove the PBS. It was then resuspended in 2-4 ml (depending on the quantity of the material) of a mixture of 2.4 mg/ml dispase (Life Technologies) and 1.5 Wursh Units/ml collagenase I (Life Technologies), 2,5 mM $CaCl_2$ in DMEM and incubated for 30 minutes at 37°C, vortexing every 10 minutes. 4 ml of DMEM 10% FBS was then added to deactivate the enzymes and the cell suspension was filtered with a 20 μm filter to remove tissue debris. Cells were centrifuged 10 minutes at 400xg at room temperature and then they were plated at a density of about 1000 cells/^{cm2} in DMEM 10% FBS and P-S, with and without mMCM at 20%. In order to minimize the amount of 'spent' medium, we actually added 2% v/v of 10x mMCM. We then left the cells proliferating until they reached

confluence, and pushed their fusion by switching to fusion medium (2% horse serum, HS, in DMEM). With this culture protocol, in the absence of mMCM the non-myogenic fraction takes over the myoblasts, yielding virtually no myotube formation. Conversely, in the presence of mMCM there is a massive pro-myogenic effect that leads to the formation of large numbers of myotubes (Figure 1).

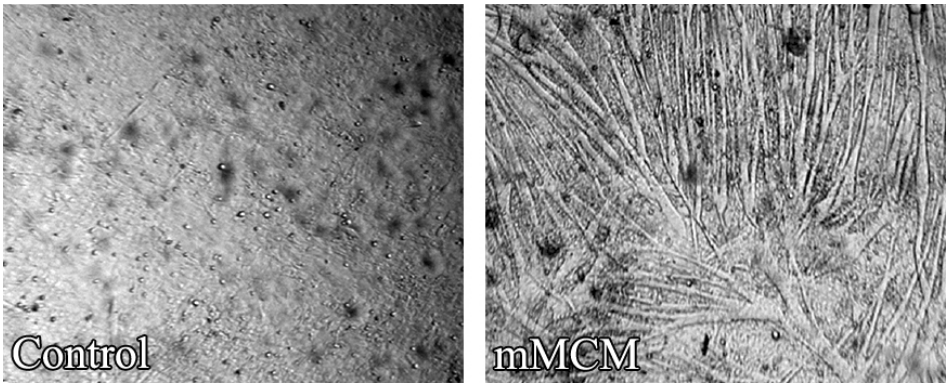


Figure 1: mMCM test with rat neonatal cells, with and without mMCM.

Satellite cells and fibroblasts isolation

Whole *extensor digitorum longus* muscles from C57BL/10ScSnJ (*wt* mice) C57BL/10ScSn-*Dmd*^{mdx}/J (*mdx*) or C57BL/6-Tg(ACTB-EGFP)1Osb/J (GFP transgenic) mice were carefully collected with maximum attention to preserve both tendons and not to damage or stretch the muscle. They were subsequently placed in a solution of collagenase I (Life Technologies) at 0,8% v/v in DMEM with 100 U/ml penicillin, 100 mg/ml streptomycin for about 90 minutes, until single fibers started to dissociate from the whole muscle. After that, they were placed in a cell culture plate with the plating medium, composed by 20% fetal bovine serum, 10% horse serum, 0,5% chicken embryo extract (Seralab) in DMEM with 100 U/ml penicillin, 100 mg/ml streptomycin. The muscle fibers were gently dissociated by pipetting the medium on the muscle. Fibers were then collected by pipetting them under the microscope and put in a new culture plate with plating medium, to wash them from muscle debris.The passage was then repeated 2-3 times. Fibers were then collected, counted and placed in an eppendorf-type tube; finally, fibers were mechanically broken by passing the suspension through a 21G syringe needle for 20-25 times.

Satellite cells were then plated on a gelatin-coated plate and left proliferating for 1 or 2 days in 20% FBS, 25 ng/µl FGFb (Fibroblast Growth Factor basic, Immunotools) in Ham's F12 medium (Euroclone) with P-S. After that, the

medium was filtered with a 20 μm filter to remove fibers' debris while keeping the floating cells. To separate satellite cells from the fibroblast, cultures were treated by citrate buffer ($C_6H_5Na_3O_7 \cdot 2H_2O$ 14.5 mM; KCl 134 mM) to detach the former while leaving the latter behind. Floating cells were then transferred in a new gelatin-coated plate and expanded. This passage was repeated for 3 or 4 times, always keeping the supernatant cell fraction with floating cells, until only satellite cells were present in culture. Their identity was confirmed by immunostaining for Pax7 and MyoD.

The cells that remained adherent to the plate were left proliferating in 20% FBS in DMEM, until confluence was reached and myoblasts fused. They were then trypsinized with 0,05% trypsin with EDTA 0,2 g/l for 1 or 2 passages, until only fibroblasts remained. Their identity was confirmed by immunostaining for TCF4.

Isolation of chicken primary myoblasts

These cells were prepared essentially with the same protocol described for neonatal rat myoblast, starting from pre-hatching chicks at E18-19.

Time course cell proliferation

All the analyzed cells were left proliferating for some days in different media:

- Mouse satellite cells: 20% FBS, 25 ng/μl FGFb in Ham's F12 medium with 100 U/ml penicillin, 100 mg/ml streptomycin;
- Mouse fibroblasts: 20% FBS in DMEM with 100 U/ml penicillin and 100 mg/ml streptomycin;
- Chicken fetal myoblasts: 10% FBS in DMEM with 100 U/ml penicillin and 100 mg/ml streptomycin;
- Rat fetal myoblasts: 10% FBS in DMEM with 100 U/ml penicillin and 100 mg/ml streptomycin;

Cells were then trypsinized with 0,05% trypsin with EDTA 0,2 g/l and counted by Burker chamber. Cells were plated at a density of 800 cells/^{cm2}, with and without 2%v/v concentrated mMCM, and left proliferating until reaching 70-80% of confluence. They were detached and counted by Burker chamber, to calculate their duplication time.

Duplication time was calculated with the formula:

$$t_d = \frac{t_{TOT}}{\log_2\left(\frac{x_f}{x_i}\right)}$$

where t_d is the duplication time, t_{TOT} is the total time cells were in culture, x_f is the number of cells counted after t_{TOT} and x_i is the initial number of plated cells.
After that, an aliquot of cells was re-plated again at the initial density and left proliferating for another 2-3 days, after which their duplication time was calculated again. This was repeated at least 3 times.

Satellite cells differentiation

Satellite cells were grown for at 6-7 days without FGFb in Ham's F12 20% FBS and then plated at very high density (150.000 cells in a 24-wells plate well) in gelatin-coated wells. 12 hours later, after complete cell adhesion, the medium was switched to fusion medium (2% HS in DMEM) to induce myotubes formation. After at least 2-3 days of fusion, cells were lysed for protein or RNA isolation with RIPA buffer (50 mM Tris pH 7.5, 100mM NaCl, 1% Triton X-100, 1% deoxycholate, all from Sigma-Aldrich) added with the Complete Mini protease inhibitor cocktail (Roche) or TRIzol reagent (Life Technologies), respectively.

Blood macrophages isolation and culture

Monocytes from the human blood were isolated from human blood buffy coat, supplied by the Transfusion Center of Padova Hospital. The blood was stratified by Ficoll (Ficoll/Hypaque, density 1.077 ± 0.001) centrifugation, diluting blood 1:2 with PBS and Medium 199 (Euroclone), then 20 ml of blood were mixed with 15 ml of Ficoll 1077. The sample was then centrifuged 30 minutes at room temperature at 600xg. Peripheral blood mononuclear cells were then collected with a Pasteur pipette and washed in PBS - 5mM EDTA by centrifuging 20 min at room temperature, then eliminating the supernatant. The pellet was then washed with PBS and Medium 199, centrifuging at 300xg for 15 minutes at room temperature. Cells were then counted and plated $15*10^6$ cells/100mm plate in 20% FBS in RPMI (Euroclone). After 2 hours monocytes were already adherent to the plate, while the non-adherent blood cells were removed by washing 2 times with PBS and fresh medium was added. After 7 days, the cells had differentiated into macrophages.

Macrophages polarization

Adherent macrophages were polarized towards the M1 phenotype by adding to the medium 1 μg/ml LPS and 10 ng/ml IFNγ, and towards the M2 phenotype with 20 ng/ml IL4 and 5 ng/ml IL13 or mMCM. Cells left in contact with the

polarization medium for three days were then collected and analyzed. When needed, concentrated mMCM was added in the usual amount (2% v/v final volume) at the indicated times.

Cytofluorimeter analysis

Cells were washed with PBS and then scraped with a cell scraper in PBS - EDTA 5 mM with 8 mg/ml lidocain. They were then collected and centrifuged at 300xg for 5 minutes at room temperature. The pellet was then resuspended in 100µl of PBS containing 2% FBS, cells were counted and incubated for 30 minutes in the dark with the necessary antibodies. The antibodies used were anti-CD68-FITC (BD Biosciences, 5 µl every 10^6 cell) anti-CCR2-PE (R&D System, 10 µl every 10^6 cell), anti-CD163-PE (BD Biosciences, 20 µl every 10^6 cell) and anti-CD206-FITC (BD Biosciences, 20 µl every 10^6 cell). Cells were incubated also with the corresponding isotypic antibody as a negative control. After incubation, cells were washed with PBS 2% FBS, resuspended in 250 µl of the same solution, and stored in ice until analysis at the cytofluorimeter (FACS Canto II, Becton Dickinson and Beckman Coulter Epics XL).

Haematoxylin and eosin staining

Muscles were snap-frozen in liquid nitrogen cooled iso-pentane and then cut with the cryostat in 20µm sections that were placed onto gelatin-coated slides. These were rehydrated briefly with PBS, fixed in 2% paraformaldehyde (PFA) in PBS for 8 minutes, then washed briefly with PBS, stained with Harris' haematoxylin for 2 minutes followed by eosin, dehydrated in alcohols and then mounted using the Eukitt mounting reagent (O. Kindler GmbH).

Immunostaining protocols

Immunostaining of single cells

Cells were seeded in a 24-wells plated on gelatin-coated glass coverslips. At the desired times they were quickly washed with PBS and then fixed in 2% paraformaldehyde in PBS for 10 minutes. They were then washed 5 minutes with PBS, permeabilized 15 minutes with 0,5% Triton X100 (Sigma-Aldrich) in PBS, washed again 5 minutes with PBS and then incubated overnight at 4°C with the different antibodies: anti-Pax7 monoclonal mouse antibody (produced by us with the hybridoma cell line kindly provided by Dr. Rudnicky) diluted 1:5, anti-MyoD

and anti-miogenin antibody (Dako) 1:50, anti-desmin antibody (Dako) 1:75, anti-TCF4 antibody (Cell Signaling Technology) 1:100.
Coverslips were then washed 5 minutes with PBS, 5 minutes in 0,5% Triton in PBS, 5 minutes with PBS and then incubated 1 hour at 37°C with the appropriate secondary antibody diluted in PBS 1% Bovine Serum Albumine (BSA, Life Technologies): Cy3 anti-mouse antibody (Jackson) 1:400, Cy2 anti-mouse antibody (Jackson) 1:200, Cy3 anti-rabbit antibody (Jackson) 1:200. Coverslips were then washed 5 minutes with PBS, 5 minutes in 0,5% Triton in PBS, 5 minutes with PBS. Nuclei were counterstained with DAPI contained in the Fluorescent Mounting Medium (Sigma-Aldrich) and observed with the epi-fluorescence microscope Leica DMR5000.

Immunostaining of muscle section

Frozen muscles were cut with the cryostat and 9µm sections were placed on gelatin-coated slides. These were washed briefly with PBS, fixed in 2% PFA in PBS for 8 minutes, then washed briefly with PBS, saturated with 10% HS in PBS for 1 hour at room temperature and then incubated with the appropriate primary antibody diluted in 1% BSA in PBS: anti-dystrophin antibody (GeneTex) 1:100 1 hour at 37°C, anti-GFP antibody (Life Technologies) 1:100 overnight at 4°C. Slides were then washed 5 minutes with PBS for 3 times, and then incubated with anti-rabbit secondary antibody (Jackson), 1:200. They were washed again 5 minutes with PBS for 3 times, nuclei were counterstained with DAPI contained in the Fluorescent Mounting Medium (Sigma-Aldrich) and the sections were observed with observed with the epi-fluorescence microscope Leica DMR5000. When necessary, the areas of the sections were determined using Photoshop CS3 (Adobe).

Exosomes extraction

Exosomes were purified by differential centrifugation at 4 °C, starting with a centrifugation at 1000xg for 15 min, then 12,000 xg for 20 min, 18,000–20,000 xg for 20 min and 100,000 xg for 70 min. The resulting microvesicles pellets were washed in 13 ml PBS, filtered through a 0.22 µm filter, collected by ultracentrifugation at 1100,000 xg for 70 min and resuspended in PBS.

RNA extraction

Samples were lysed in the appropriate TRIzol® Reagent (Life Technologies) volume, following manufacturer's protocol and resuspended in RNAse-free water.

Real-Time analysis

The samples' RNAs were retrotranscribed and amplified with the SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase kit (Life Technologies). The amplification was carried out with the Corbett Rotor-Gene 6000. The results were analyzed with the Corbett Rotor-Gene 6000 software.

Western blot protocols

Western blot on myotubes

For the MyHC western blot the samples were run in a 10% Bis-Tris acrylammide gel and blotted on a nitrocellulose membrane, that was saturated 2 hours at room temperature in 1% BSA in TBS (50 mM Tris, 150 mM NaCl). The membrane was washed 3 times for 10 minutes with TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) and incubated with a mix of different MyHC isoforms antibodies (F8 1:10, BF32, 1:4000, F1.652 anti-Emb 1:500, BFF3 2B 1:750, RTD9 2B+2X 1:2000, all kindly provided by Prof. Schiaffino) diluted in TBS 1% BSA for 1 hour at room temperature. The membrane was washed 3 times for 10 minutes with TBST and incubated with an anti-mouse secondary antibody (Dako) conjugated with HRP diluted 1:2000 in 1% BSA in TBS. The membrane was then washed 2 times for 10 minutes with TBST and 1 time for 10 minutes in TBS. The chemioluminescence was assayed with the Santa Cruz western blotting luminol reagent (Santa Cruz) and acquired with the Kodak 4000mmPro image station.

Western blot on muscle lysates

Intervening muscle sections obtained when preparing the microscope slides were kept aside and then lysed in an appropriate volume of RIPA lysis buffer. The concentrations of the samples were determined by Bradford assay.

20-40 μg of total proteins were then run in a NuPAGE® Novex 4-12% Bis-Tris Gel (Life Technologies) and blotted on a nitrocellulose membrane (Trans-Blot transfer Medium, Biorad). The membranes were then stained with Ponceau Red stain (Roche), photographed, de-stained in water and saturated in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, all from Sigma-Aldrich) with 10% milk overnight at 4°C. The membrane was then washed 3 times with TBST and incubated with the primary antibody for the appropriate time. It was then washed other 3 times with TBST and incubated 1 hour at room temperature with the corresponding secondary antibody, conjugated with HRP. The membrane was

washed 3 times with TBST and the chemioluminescence was assayed with the Santa Cruz western blotting luminol reagent and acquired with the Kodak 4000mmPro image station.
Antibodies used: anti-GFP (Life Technologies) 1:1000 overnight at 4°C, anti-rabbit HRP-conjugated antibody (Life Technologies) 1:2000 1 hour at room temperature.

Western blot on exosomes

The samples were mixed with Laemmli sample buffer (1:1 ratio) and loaded onto a 12% gel. The proteins were then transferred to a nitrocellulose membrane (GE Healthcare). The membrane was saturated in TBST with 5% milk for 1 hour at room temperature. The primary antibody used was anti-Tsg101, diluted 1:2000 (clone 4A10 Abcam), overnight at 4 °C. The membrane was then washed 3 times for 15 minutes. The secondary HRP-conjugated antibody (Pierce), diluted 1:5000, was left 1 hour at room temperature. The luminescence was visualized using the Supersignal Dura reagent (Pierce).

Densitometry analysis

The densitometry analysis of all the blots was carried out with the ImageJ software, freely available at http://rsbweb.nih.gov/ij/

2D gel electrophoresys

Isoelectrofocusing and SDS-PAGE

Samples were precipitated overnight at -20°C in acetone (sample:acetone ratio 1:4), then they were centrifuged at 15.000xg for 15 minutes at 4°C. They were then resuspended in the denaturation/solubilization solution composed by Urea 8M, CHAPS 4% (w/v), DTE 1% (w/v), 6 ml of distilled water and 100 µl of Bromophenol Blue (all Sigma-Aldrich). Samples concentration was then read with the Bradford assay.
The isoelectrofocusing was carried out with the IPGphorTM instrument (GE Healthcare), using 2D precast gels and isolectrofocusing strips with a non-linear pH range of 3-10 (BioRad Laboratories, Hercules-California), loading 60 µg of proteins *per* sample.
After the focusing process, strips were equilibrated leaving them for 12 minutes in a reducing solution of Tris-HCl pH 6.8 0.5 M, Urea 6M, SDS 2% (w/v), DTE 2% (w/v). Then they were moved 5 minutes into an alkylating solution composed by

Tris-HCl pH 6.8 0.5 M, Urea 6 M, glycerol 30% (v/v), SDS 2% (w/v), 2-Iodoacetamide 2,5% (w/v) and traces of bromophenol blue.
The electrophoresis was carried out with a linear gradient of 9-16% T, 2.6% C of acrylamide-PDA.

Silver Staining

The gels were left 1 hour in a fixing solution of 40% ethanol, 10% acetic acid in distilled water at room temperature, and then washed for a minimum of 3 hours and a maximum of 3 days in 5% ethanol, 5% acetic acid in distilled water at room temperature. Then they were left 5 minutes at room temperature in distilled water, washed 3 times for 10 minutes at 4°C in distilled water, 30 minutes in NDS 0,05% in distilled water at 4°C, 4 times for 15 minutes at 4°C in distilled water at 4°C, 30 minutes at room temperature in 8g/l $AgNO_3$, 2 ml/l NaOH 10N, 13.3 ml/l NH_3 25% in distilled water, 4 washes of 4 minutes each with distilled water at room temperature, 12 minutes in the development solution (0,05 g/l citric acid, 1 ml/l formaldehyde 37% in distilled water at room temperature and then the development reaction was blocked putting the gel into a solution of 5% acetic acid in distilled water at room temperature for 15 minutes.
The gels were ten read with the Image Scanner III (GE Healthcare) and analyzed with the ImageMaster 2D platinum v 7.0 software (GE Healthcare).

Surgical protocols

All mice were anesthetized with isofluorane during every surgical procedure and received analgesic treatment (caprofen) for three days post-op.

Effect of mMCM on intact muscles

The TA muscles of *wt* mice were injected every 2 days for 3 times with 50 µl of concentrated (10X) mMCM in the left TA and 50 µl of DMEM as a control in the right TA. After that mice were sacrificed, the muscles were collected and frozen in liquid nitrogen-cold iso-penthane and stored at -80°C until sectioning.

Satellite cells transplant

For the first two experiments, the TA muscles of 2x3 adult (4-6 months) *mdx* mice were injected with 300.000 *wt* satellite cells in 50 µl of either DMEM or DMEM containing 10%v/v 10x mMCM (mMCM in the left TA and DMEM in the contralateral muscle as a control). Cells were grown in the medium described above and were detached in citrate buffer before injections. In order to remove all FBS, which could have caused an acute immune response), cells were washed

three times in DMEM before being pelleted and resuspended in the injection volume. Mice then received 4 subsequent 50 µl mMCM 10X/DMEM injections every 2 days, and were sacrificed after 13 days. Muscles were collected and frozen in liquid nitrogen-cold isopenthane and stored at -80°C until sectioning.
For the second set of experiments, 50.000 GFP^+ *wt* satellite cells suspended in 50 µl of either DMEM or DMEM containing 10%v/v 10x mMCM (mMCM in the left TA and DMEM in the contralateral muscle as a control) were injected in the TA muscles of 2x3 adult (4-6 months) *mdx* mice. Mice then received 3 subsequent injections of 50 µl of concentrated (10X) mMCM and plain DMEM every 2 days. Mice were sacrificed 20 days after the last injection and muscles were collected, washed briefly in PBS, fixed 1 hour at 4°C in 2% PFA in PBS and left overnight at 4°C in 10% sucrose in H_2O. Muscles were then frozen in liquid nitrogen-cold iso-penthane and stored at -80°C until sectioning. The sections were placed both on gelatin-coated coverslips or collected in a tube for protein analysis.

Effect of mMCM on muscle regeneration upon glycerol damage
The TA muscles of 6 *wt* mice were injured by injection of 50 µl of 50% glycerol in PBS. After 3 (three mice) or 10 days (three mice), 50 µl of concentrated (10X) mMCM was injected in the left TA and 50 µl of DMEM was injected as a control in the right TA every 2-3 days for 15 days. Mice were then sacrificed and the muscles were collected and frozen in liquid nitrogen-cold isopenthane and stored at -80°C until sectioning. The sections were placed both on gelatin-coated coverslips or collected in a tube for RNA analysis.

Mesoscale analysis
The cytokines contained in mMCM 1X and in the conditioned media of non-stimulated J774 cells, C2C12 cells and *wt* fibroblasts were measured using the MesoScale Discovery multi cytokine approach (Meso Scale Discovery, Gaithersburg, USA). The measurement procedure was performed as described by the manufacturer's protocol. The plate was read by using the MSD Sector Imager 6000 and the data were analyzed using the MSD Workbench software.

Mass spectrometry analysis
50 µg of 10X mMCM-contained proteins and 50 µg of 10X non-stimulated J774 conditioned medium contained proteins were then run in a NuPAGE® Novex 4-12% Bis-Tris Gel (Life Technologies). At the end of the run, the gel was stained

with the SimplyBlue™ SafeStain reagent (Life Technologies). Bands that were differentially stained in the two samples were identified and cut from the gel.
After dehydration with acetonitrile (ACN, Sigma), gel pieces were reduced with 10 mM dithiothreitol (DTT, Fluka) in 50 mM NH_4HCO_3 for 1 hour at 56°C, alkylated for 45 minutes in the dark at room temperature with 55 mM iodoacetamide (Sigma) in 50 mM NH_4HCO_3.
Samples were washed 4 times alternatively with 25 mM NH_4HCO_3 and acetonitrile, digested overnight at 37°C with 40 µl of 12.5 ng/µl Trypsin (Promega) in 25 mM NH_4HCO_3, and then the extracted peptides were washed with 3 changes of 50% ACN, 0.1% formic acid (FA, Fluka).
Samples were then dried under vacuum and dissolved in 0.1% FA to be analyzed in an LTQ-Orbitrap XL mass spectrometer (ThermoScientific), coupled with a nano-HPLC Ultimate 3000 (Dionex). The samples were loaded into a homemade pico-frit column packed with C18 material and separated using a 40 min linear gradient of acetonitrile/0.1% formic acid (from 3% to 50% acetonitrile in 23 min), at a flow rate of 250 nl/min. Capillary voltage was set at around 1.3-1.5 kV and source temperature at 200 °C.
The obtained raw data files were analyzed with the Proteome Discoverer 1.2 (ThermoScientific) software, using Mascot (version 2.2.4, Matrix Science, London, UK) as search engine.
The database used for protein identification was SwissProt (version 2011.05, 528048 sequences), and the enzyme specificity was set to trypsin with 1 missed cleavage. Mass tolerance window was set to 10 ppm for parent mass and to 0.6 Da for fragment ions. Carbamidomethylation of cysteine residues was set as fixed modification and methionine oxidation as a variable modification.
The False Discovery Rate (FDR) calculated by Proteome Discoverer was set so that only peptides classified as medium or high confidence (95% and 99% confidence respectively) were considered as positive hits. The results were then analysed with the DAVID software available at http://david.abcc.ncifcrf.gov/.

Statistical analysis

The statistical significance of all the results was calculated by the appropriate t test using the online tool available at http://www.graphpad.com/quickcalcs/ttest1.cfm

Results

Mouse model

Effect of mMCM on murine satellite cells

Wild type *satellite cells*

Previous data had shown that mMCM could greatly enhance rat satellite cells proliferation and differentiation, delaying their senescence and preventing their trans-differentiation (*Malerba et al., 2009*). Switching to the mouse model, we initially wanted to assess if its effects on murine satellite cells were the same.

To this aim, we initially conducted a series of experiment on satellite cells isolated from single fibers using the mechanical separation protocol described by Collins and colleagues (*Collins et al., 2005*) to determine the effect of mMCM on their duplication time.

Cells were checked at the beginning and at the end of the experiment by immunostaining with the two myogenic markers Pax7 and MyoD, to ensure that the preparations had indeed yielded suitably pure cultures of myogenic cells and that no loss of myogenicity had occurred in time; these analyses consistently yielded percentages higher than 90%? (see Figure 1 for an example). For the proliferation experiments cells were grown either in straight growth medium or in growth medium containing 20% mMCM (as described in M&M, in all our experiments we actually used a ten-fold concentrated mMCM, added in the needed amounts). Duplication times were calculated by averaging the values obtained from three culture passages.

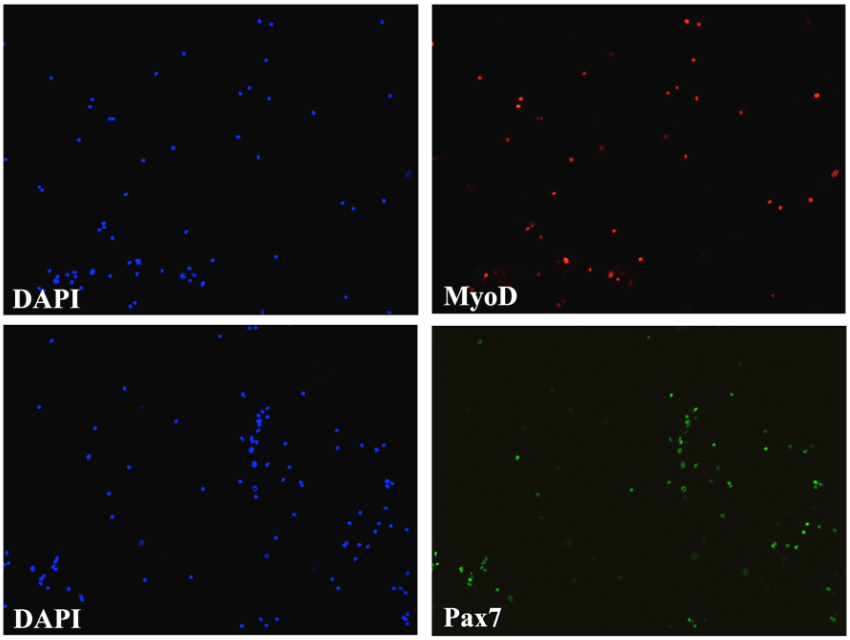


Figure 1: Immunostaining of *wt* satellite cells in culture. Cells remained positive for Pax7 and MyoD for the whole time of culture.

As shown in Figure 2, cells treated with mMCM always proliferated faster than controls and mMCM almost halved the duplication time (from 90 to 48 hours, p<0,01).

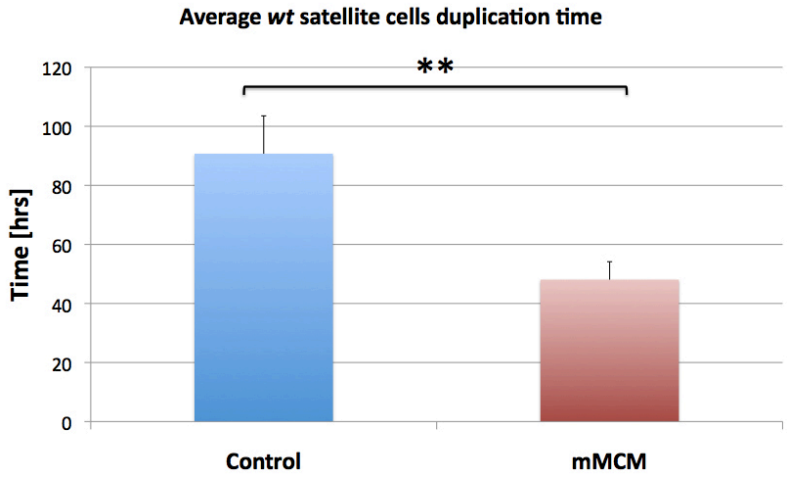


Figure 2: Average duplication time of *wt* satellite cells isolated from single fibers. mMCM significantly reduced duplication time of treated cells.. (** p<0.01; n=3 independent experiments, with different cell preparations.)

We then assessed if mMCM, added in higher concentrations, could impair or stimulate even more satellite cells proliferation. To do so, we conducted a proliferation experiment with 3 different conditions: control cells without any macrophagic factors, 20% mMCM and 100% mMCM .

Results clearly showed that mMCM, even at high concentrations in the growth medium, did not have any visible effect on cell viability and pushed cell proliferation even more (Figure 3). Given the relatively small difference between the two concentrations, however, for all subsequent experiments we used the 20% concentration.

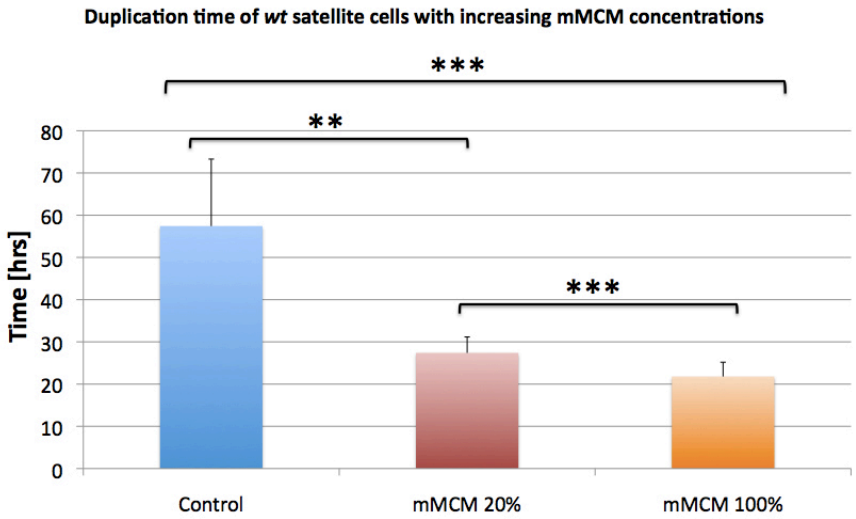


Figure 3: Average duplication time of *wt* satellite cells isolated from single fibers with mMCM at two concentrations in the growth medium. As already demonstrated, mMCM at 20% significantly reduced duplication time of treated cells, and mMCM at 100% reduced it even more. (n=6 two independent experiments with different cell preparations, ** p<0,01, *** p<0,001).

As mentioned above, these experiments were conducted used satellite cells isolated from single fibers. There is however another method widely used to obtain satellite cells, i.e., using FACS with specific markers combination (α7-integrin$^+$, CD34$^+$, sca1$^-$, CD45$^-$ CD11b$^-$ and CD31$^-$) starting from whole muscle (*Pasut et al., 2012*). Even though we did not have routine access to such protocol, we could test FACS-sorted *wt* mouse satellite cells kindly provided by Dr. A. Pasut (Sprott Center for Stem Cell Research, Ottawa Hospital Research Institute). As shown in Figure 4, also in this case cells treated with mMCM proliferated faster than controls (duplication time decreased from 45 to 31 hours).

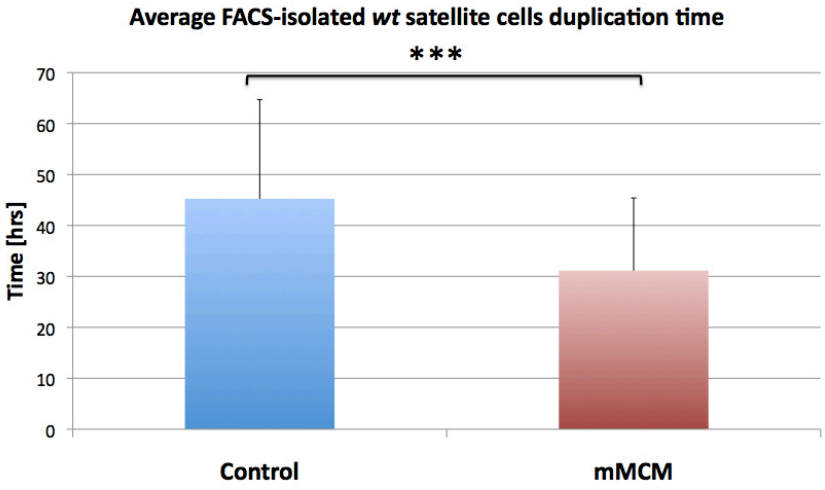


Figure 4: Average duplication time of FACS-sorted murine *wt* satellite cells. The presence of mMCM in the growth medium decreased duplication time. (*** p<0,001, two independent replicas using a single batch of cells.)

When culturing FACS-isolated cells, though, we noticed something that we had not seen with cells isolated from single fibers, that is, the duplication time of both control and treated cells decreased over time. At the beginning of the proliferation experiments control cells had an average duplication time of 65 hours, which decreased to 46 in mMCM-treated cells, but these values decreased at each passage, reaching 33 hours for controls and 21 hours treated cells after six passages. For this reason we decided to check the expression of Pax7 and MyoD at each passage, to verify if the change in proliferation was linked to a loss of myogenicity. As shown in Figure 5, virtually all cells were positive for Pax7 at the first passage; its expression then tended to decrease in control cells while it varied very little in the presence of mMCM. On the contrary, more $MyoD^{+}$ cells were present in control cells for most of the passages (Figure 5). These findings indicated that the decreased doubling time was not related to a loss of myogenic commitment. Besides, they also suggested the possibility that cells grown in the presence of mMCM maintained a more "stem" phenotype, as they were almost all Pax7 positive and expressed less MyoD; this finding would have been in agreement with what we had already found with rat satellite cells (*Malerba et al., 2009*),

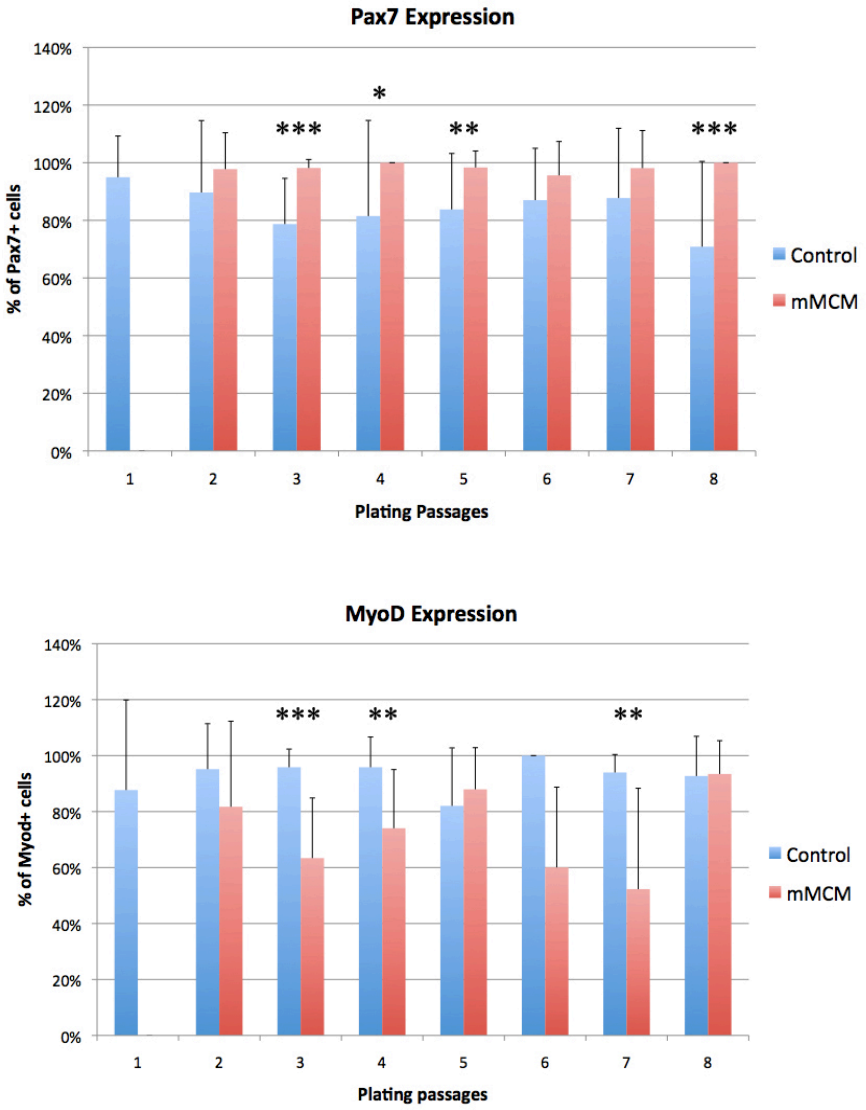
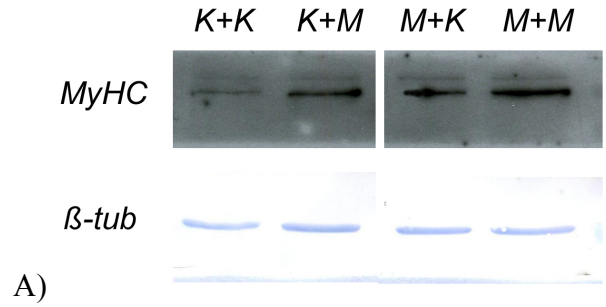


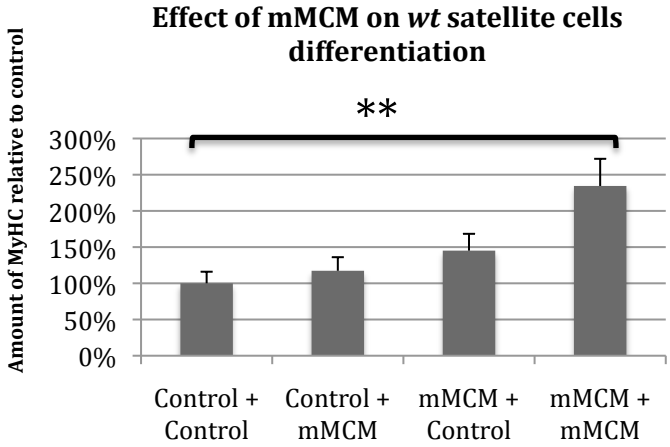
Figure 5: Percentage of FACS-isolated *wt* satellite cells positive for Pax 7 or MyoD at each plating passage. Passage 1 is the starting point at which cells were counted and plated with and without mMCM, thus only control cells values are present. (** p<0,01, *** p<0,001 n=15)

Our previous results had shown that mMCM was also able to enhance the differentiation of rat satellite cells (*Malerba et al., 2009*); on the other hand, as described in the Introduction, there is still a debate on the role played by macrophagic factors in differentiation. For this reason we went on to investigate mMCM effects on the differentiation of our *wt* murine satellite cells. The experimental design comprised satellite cells expanded in growth medium with and without mMCM and then switched to fusion medium, again with and without mMCM. The level of differentiation in each condition was evaluated by measuring the expression of myosin heavy chain (MyHC) *via* western blot. The presence of mMCM, no matter when it was added, did not inhibit the expression

of MyHC, and if mMCM was always present in the growth medium MyHC was more expressed (Figure 6).



A)

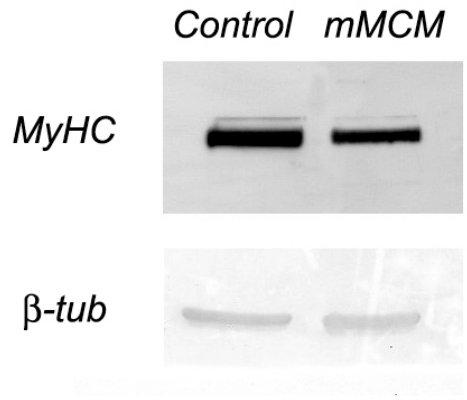


B)

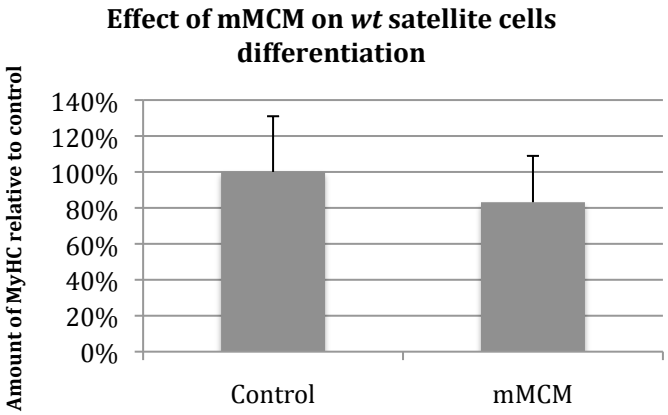
Figure 6: A) Example of a western blot of myotubes from *wt* satellite cells. K+K are control cells, grown and differentiated without mMCM; K+M are cells that proliferated without mMCM but that had mMCM added in the fusion medium; M+K are cells that proliferated with mMCM but that received fusion medium alone; M+M are cells that proliferated and differentiated always in the presence of mMCM. In order to avoid the bias caused by the difference in proliferation rate, control cells were initially seeded at higher density. β-tubulin was used as housekeeping gene to verify equal loading of protein in the blot. B) Densitometry analysis averaging two independent experiments. (** $p<0,01$)

The effect of mMCM was also assessed using a different experimental design, in which satellite cells grown without mMCM were plated at very high density and twelve hours after seeding growth medium was switched to fusion medium, with and without mMCM. This allowed to determine the effect of mMCM on the fusion process alone. Cells were allowed to completely differentiate for three days before being lysed to obtain both RNA and proteins. We then analyzed the expression of different myogenic markers to assess fusion.

The addition of the macrophagic factors to *wt* satellite cells at high density (Figure 7) did not significantly affect the differentiation level.



A)



B)

Figure 7: A) Western blot of myotubes from *wt* satellite cells plated at high density with the subsequent addition of fusion medium without and with macrophagic factors. β-tubulin was used as housekeeping gene to verify equal loading of protein in the blot. B) Densitometry analysis that illustrates the amount of MyHC relative to the control cells. No significant differences in the MyHC levels were found. (n=2)

In one instance, we also analyzed the expression of MyoD and miogenin using qRT-PCR, quantifying their expression by comparison with expression levels of the glyceraldheide-3-phosphate dehydrogenase (GAPDH) housekeeping gene. Once again, no significant differences were found between controls and mMCM-treated cells (Figure 8).

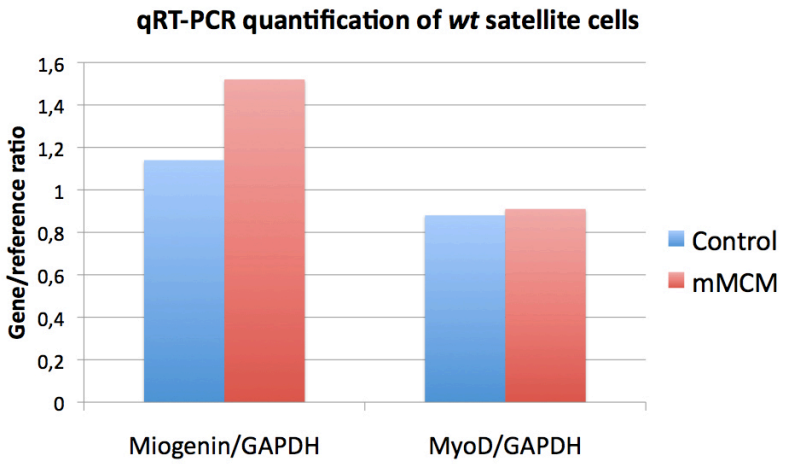


Figure 8: qRT-PCR quantification of myogenin and MyoD expression in differentiated *wt* satellite cells. Expression levels are expressed through their ratio with GAPDH housekeeping gene expression. Statistical analysis done with the REST tool did not find any significant difference between the two samples.

Mdx *satellite cells*

The same set of experiments on proliferation and differentiation described above were performed on *mdx* satellite cells, also isolated from single fibers. Again, cells were preliminarily checked by immunostaining and we confirmed that they remained Pax7+ and MyoD+ for the whole culture period.

In the case of *mdx* satellite cells (Figure 9) mMCM was able to significantly ($p<0,01$) decrease the duplication time too and the effect was even more pronounced than that seen with *wt* cells (from 108 to 28 hours).

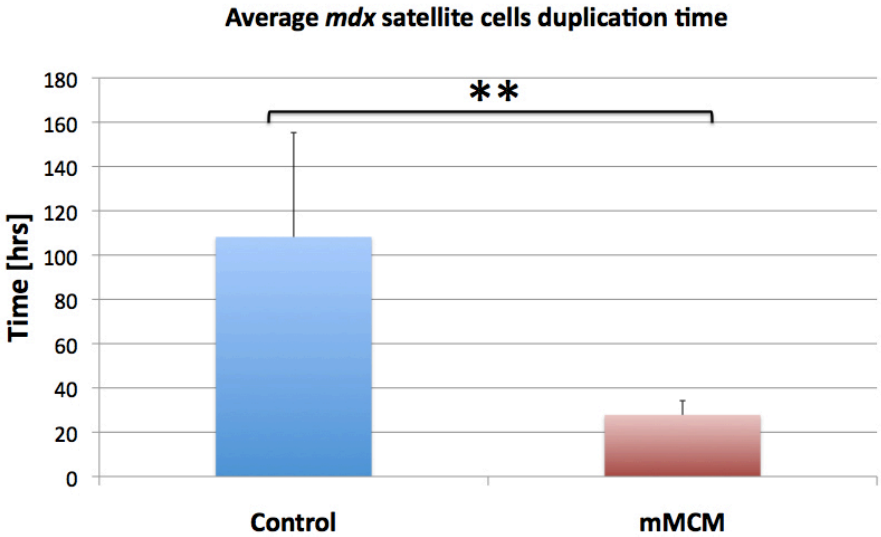
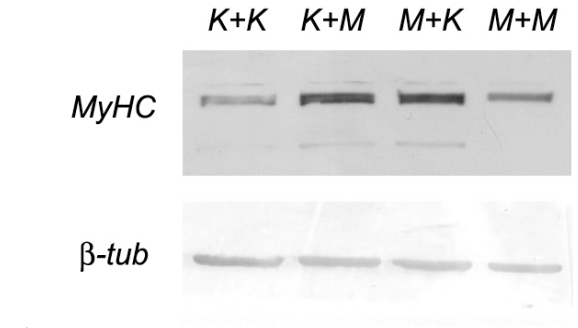


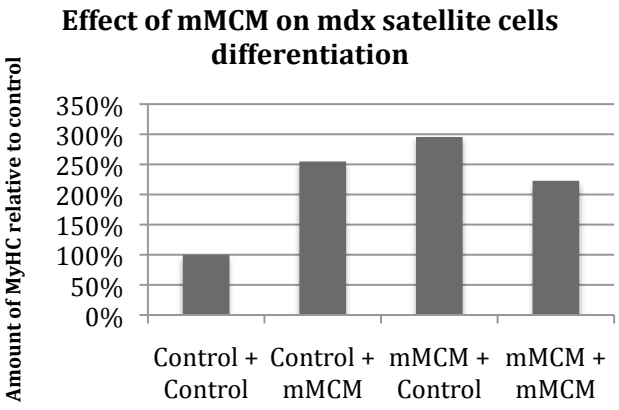
Figure 9: Average duplication time of *mdx* satellite cells isolated from single fibers. mMCM significantly reduced duplication time of treated cells. (n=4 independent experiments with 4 different cell preparations. ** $p<0,01$)

As for *wt* satellite cells, we then assessed the effects of mMCM at higher concentrations, performing an experiment with control cells without any macrophagic factors, 20% mMCM and 100% mMCM. This time the results were identical to those found in *wt* cells, that is, 100% mMCM led to a marginal, albeit statistically significant, increase in proliferation rate (data not shown).

We also investigated mMCM effects on *mdx* satellite cells differentiation, using the same experimental design described above.
Once again, the presence of mMCM did not hamper the differentiation process (Figure 10); besides, the difference between control and treated cells was even more pronounced than that seen in *wt* cells.



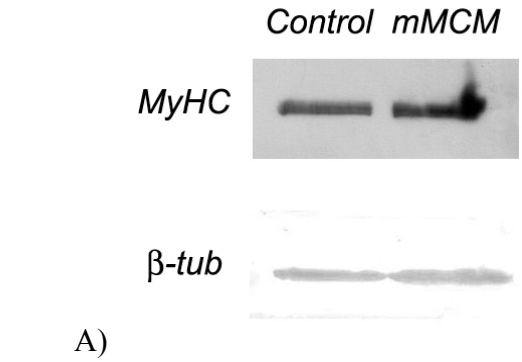
A)



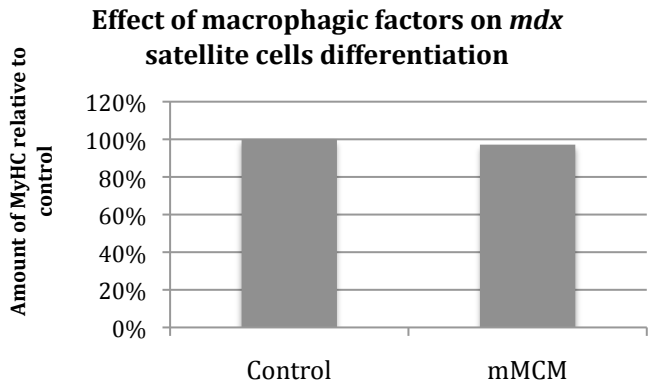
B)

Figure 10: A) Western blot of myotubes from *mdx* satellite cells. K+K are control cells; K+M are cells that proliferated without mMCM, but that had mMCM added in the fusion medium; M+K are cells that proliferated with mMCM but that received fusion medium alone; M+M are cells that proliferated and differentiated always in the presence of mMCM. β-tubulin was used as housekeeping gene to verify equal loading of protein in the blot. B) Densitometry analysis that illustrates the amount of MyHC relative to the control cells (replicas of the experiment are presently in course).

We also investigated mMCM effect on differentiation alone, as we did with *wt* satellite cells, plating them at high density and switching to fusion medium with and without macrophagic factors 12 hours after plating.
The presence of mMCM did not change the expression of MyHC (Figure 12).



A)



B)

Figure 11: A) Western blot of myotubes from *mdx* satellite cells plated at high density with the subsequent addition of fusion medium without and with macrophagic factors. Control are control cells; mMCM are cells that had mMCM added in the fusion medium. β-tubulin was used as housekeeping gene to verify equal loading of protein in the blot. B) Densitometry analysis that illustrates the amount of MyHC relative to the control cells. MyHC level did not change when mMCM was added with the fusion medium.

Taken together, these experiments confirmed that mMCM had a strong effect also on *mdx* satellite cells proliferation; it was able to drastically reduce duplication time, even more than what was seen with *wt* cells, and not only it did not impair the differentiation process but it might have actually enhanced it.

Effect of mMCM on muscle-derived fibroblasts

Previous data had indicated that mMCM was able to inhibit the proliferation of the non-myogenic fraction of primary muscle cultures from neonatal rat (*Malerba et al., 2009*). Starting from this observation, we decided to study its effect on fibroblasts proliferation, both *wt* and *mdx*.

Fibroblasts were obtained by modifying the protocol for satellite cells preparations (as described in M&M), thanks to their different adhesion properties. Their identity was then confirmed by immunostaining with TCF4, a recently identified fibroblasts marker (*Mathew et al., 2011*) (Figure 12).

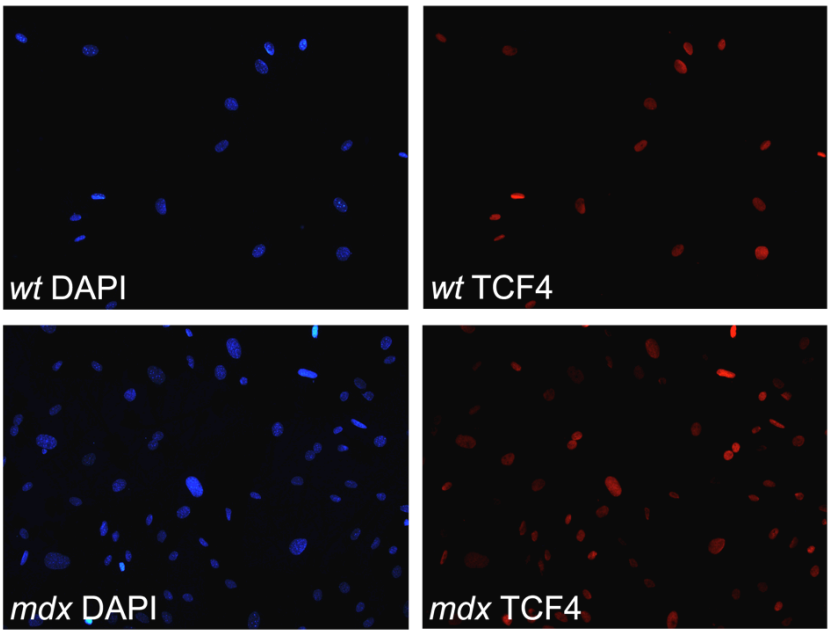


Figure 12: Mouse fibroblasts isolated from preparations of single muscle fibers. Both *wt* (upper panel) and *mdx* (lower panel) fibroblasts were all positive for TCF4, a fibroblasts marker.

We assessed mMCM effects on the proliferation rate of both fibroblasts types, conducting the same experiment already performed on satellite cells.

mMCM did not have any significant effect on the proliferation of *wt* fibroblasts, with control and treated doubling time being very close. On the contrary, mMCM significantly ($p<0,05$) reduced the proliferation rate of *mdx* fibroblasts, increasing their duplication time from 53 to 67 hours (Figure 13).

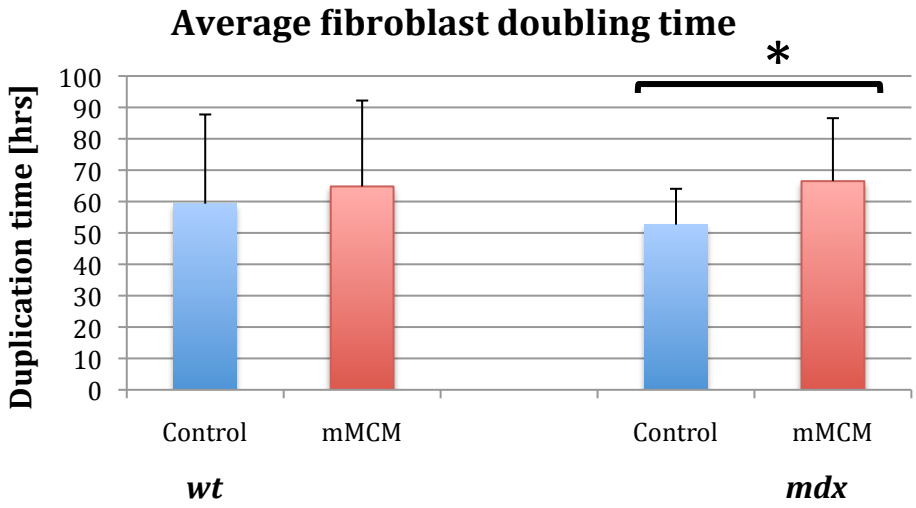


Figure 13: Average duplication time of *wt* and *mdx* fibroblasts. mMCM did not have any significant effect on the proliferation rate of *wt* cells, while it significantly reduced duplication time of treated *mdx* cells. (n_{wt}=6 independent experiments with three different cell preps; n_{mdx}=5 independent experiments with three different cell preparations.)

Effect of mMCM on satellite cells trans-differentiation

One of the most intriguing effects of mMCM we had seen in the past was the ability of preventing the trans-differentiation of rat satellite cells when seeded at sub-clonal density (*Malerba et al., 2009*). We therefore set out to verify if the same finding could be seen on murine satellite cells. Satellite cells, both *wt* and *mdx*, were isolated from single fibers, plated at low density (50 cells/cm^2) with and without mMCM, allowed to proliferate to confluence and then switched to fusion medium.

In the case of *wt* satellite cells, 15 days from plating, control cells showed virtually no myotubes, wide areas of fibroblasts-like cells and many clusters of adipocyte-like cells (Figure 14, upper panels). In mMCM treated cells, on the other hand, we found many myotubes, fewer fibroblast-like areas and almost no adipocyte-like cells.

In the case of *mdx* satellite cells, 15 days from plating control cells showed few myotubes, large areas of fibroblasts-like cells and the presence of some clusters of adipocytes, albeit clearly less numerous than those seen for the *wt* cells. In the mMCM treated well we found many myotubes, few fibroblast-like cells and virtually no adipocyte-like cells (Figure 14, lower panels).

Altogether, these results seem to point in the same direction of the effect of mMCM on rat.

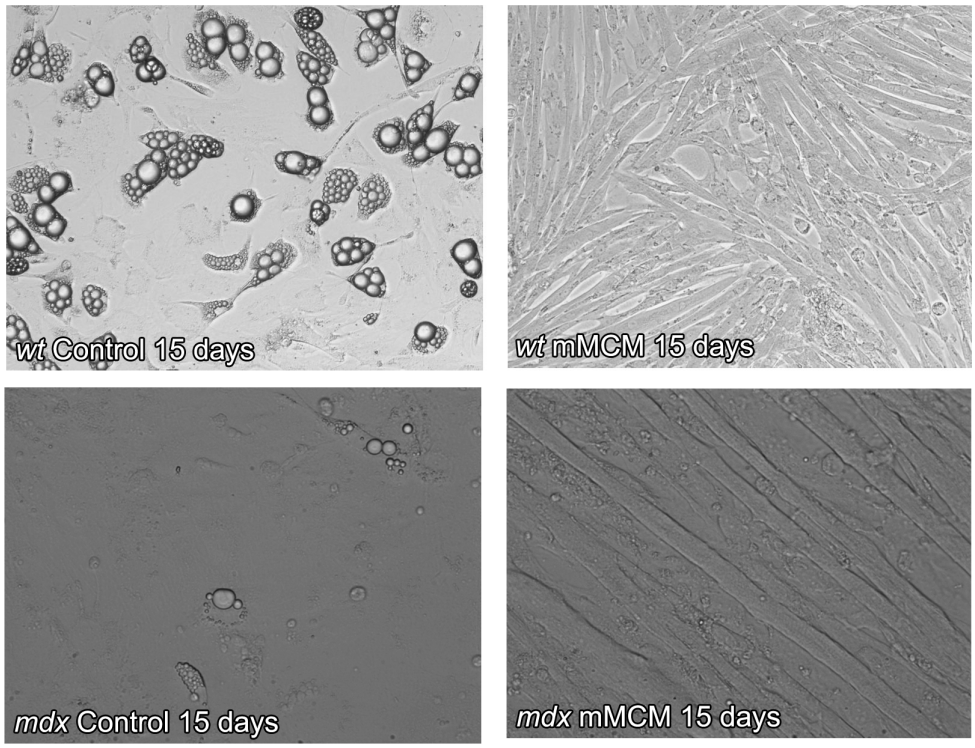


Figure 14: Upper panels: Culture of mouse *wt* satellite cells isolated from single fibers plated at low density. 15 days after plating in the control wells we found many clusters of adipocyte-like cells, while there were almost none in mMCM treated cells, where instead we found large numbers of myotubes. Lower panels: Culture of mouse *mdx* satellite cells isolated from single fibers plated at low density. 15 days after plating, in the control wells we found some adipocytes clusters, while there was almost none in mMCM treated cells, were we found a huge number of myotubes.

In vivo *effects of mMCM in mouse muscles*

In the past we had tested mMCM *in vivo*, but only in the context of tissue reconstruction upon surgical removal of muscle mass. Now we set out to investigate its possible effects in models of muscle regeneration upon non-ablational damage and in cell transplantation.

At first, we determined if mMCM alone had a pro-inflammatory effect in intact muscle. To do so, we injected concentrated (10X) mMCM in the *tibialis anterior* (TA) muscle of *wt* mice, using plain DMEM as a control in the contralateral muscle. We then sacrificed the mice and analyzed the muscles to detect signs of

inflammation. The control and the treated muscles were almost the same (Figure 15), and we detected no sign of acute inflammation.

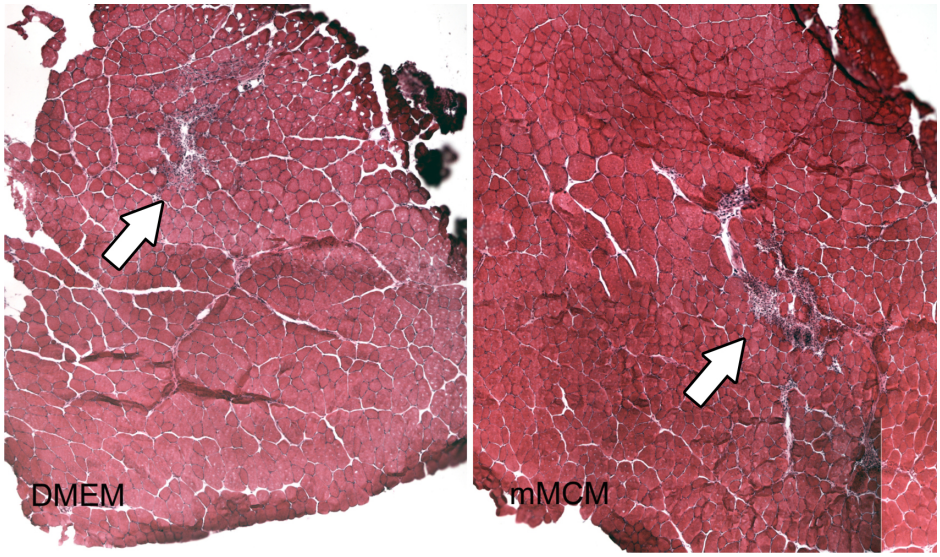


Figure 15: Hematoxylin and eosin staining of *wt* muscles injected with either concentrated mMCM or DMEM as a control. After receiving three intramuscular injections in four days, neither treatment led to obvious signs of local inflammation, save for the small areas of needle damage (arrowheads).

After this, we decided to proceed in two directions, analyzing mMCM effects on cell transplant in dystrophic mice and on muscle damage repair.

Effect of mMCM on wt *cell transplant in* mdx *mice*

To assess if mMCM could facilitate cell transplantation in a dystrophic environment, we used C57BL/10ScSn-*Dmd*^{mdx}/J mice.

In two distinct experiments, we injected in TA muscles of 3 *mdx* mice 300.000 *wt* satellite cells, derived from mice of the same strain, resuspended either in concentrated 100% mMCM or in DMEM (left and right muscles, respectively). Mice then received 8 subsequent injections of concentrated mMCM or DMEM every 2 days, and then they were sacrificed 2-3 days after the last injections. Upon harvesting, muscles were sectioned, immunostained and the dystrophin-positive fibers in each section were counted and normalized for the area of the sections. Unexpectedly, we did not find any positive effect of mMCM (Table 1).

	Dystrophyn$^+$ fibers/mm^2			
	Exp 1		Exp 2	
	Control	*mMCM*	*Control*	*mMCM*
Mouse 1	11,54	8,60	14,53	12,77
Mouse 2	25,22	14,14	16,95	10,63
Mouse 3	17,75	25,35	23,89	27,40

Table 1: Dystrophin$^+$ fibers per mm^2 counts for the two transplant experiments of *wt* satellite cells in *mdx* recipients.

However, we noticed that the repeated injections had caused significant damage and regeneration in treated muscles, something that we had never found in *wt* animals. It then seemed that our protocol was introducing two confounding factors: the formation of new revertant fibers (i.e., occasional dystrophin-positive fibers that are found both in DMD and *mdx* muscles and whose appearance is known to be linked to regenerative processes) and the possible effects of mMCM on regeneration itself.

We then changed the experimental design, reducing the number of injections and prolonging the gap between end of treatment and harvesting of the muscles. Besides, we decided to inject satellite cells derived from transgenic GFP+ mice, so that we would have been able to follow cell grafting without having to rely only on dystrophin expression. 50.000 GFP$^+$ *wt* satellite cells were injected in the TA muscles of *mdx* animals, one leg with 100% mMCM and the contralateral with DMEM as a control. Muscles then were injected 3 times every 2 days with concentrated (10X) mMCM or plain DMEM. Animals were sacrificed 16 days after the last injection, to allow resolution of inflammation.

This time, mMCM treated muscles clearly showed more GFP$^+$ fibers compared to the controls (Figure 16), indicating that mMCM had improved cell grafting.

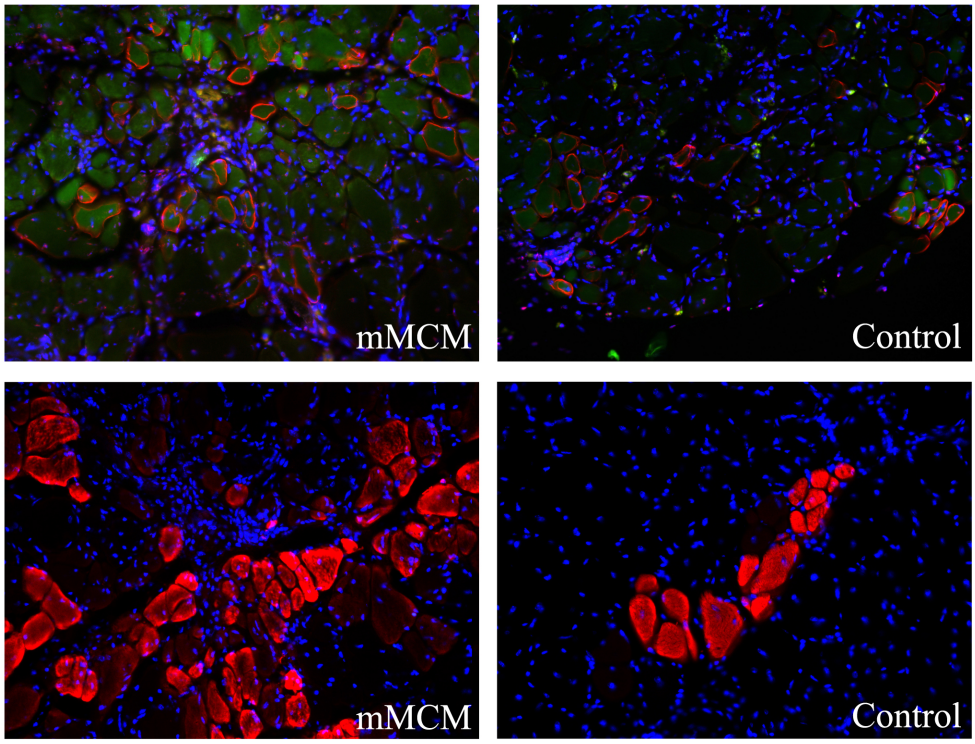


Figure 16: Sections of *mdx* TA muscles injected with GFP$^+$ *wt* satellite cells with mMCM and DMEM as a control. The upper images show dystrophin stained in red, and native GFP in green. The lower images show examples of sections immunostained with an anti-GFP antibody. mMCM treated muscles always showed more GFP$^+$ fibers.

These findings were then confirmed by western blot analysis, measuring GFP expression in the total muscle (Figure 17).

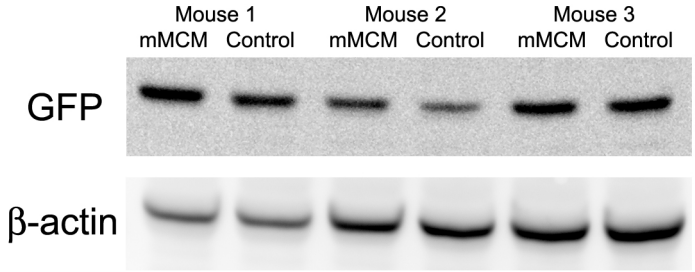


Figure 17: Western blot analysis of GFP expression in the muscles of *mdx* mice that received the transplant of GFP$^+$ satellite cells.

Effects of mMCM on muscle regeneration upon injury

To assess mMCM effects on muscle regeneration we injected it in the TA muscles of *wt* mice that had been pre-damaged *via* glycerol injections (50% glycerol in PBS 1X). This type of damage is characterized by a slow kinetic of regeneration and fat accumulation (*Pisani et al., 2010*); in our hands, necrosis was still present

at 6 days after injury and complete resolution of inflammatory infiltrate was not achieved before day 20. We then injected 10X mMCM (or DMEM as a control in the contralateral muscle) starting 3 days after injury, every 2-3 days for 15 days. After that, mice were sacrificed and the muscles were collected and analyzed.
We found no apparent differences between the sections of control and treated muscles (Figure 18).

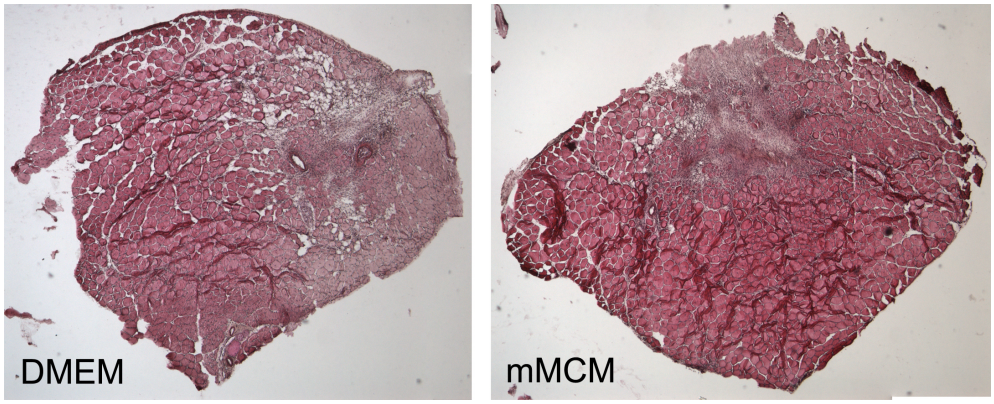


Figure 18: Hematoxylin and eosin staining of *wt* muscles damaged with glycerol and then treated 3 days after injury with concentrated (10X) mMCM or DMEM as a control. In both muscles we detected the presence of large inflammatory infiltrates, clearly showing that regeneration was not completed yet.

We decided to repeat the same experiment, but this time we damaged the muscles and waited for 10 days (i.e., the time point at which our preliminary analyses had shown that all necrotic tissue had been removed) before injecting concentrated (10X) mMCM and DMEM in the contralateral muscle as a control, every 2-3 days for 20 days. The rationale of this choice was to begin the treatment at a different phase of regeneration, when the peak of M1 activity should have begun to subside.
This time also, we found no particular histological differences between treated and control muscle: regeneration seemed to have progressed normally in either case (Figure 19).

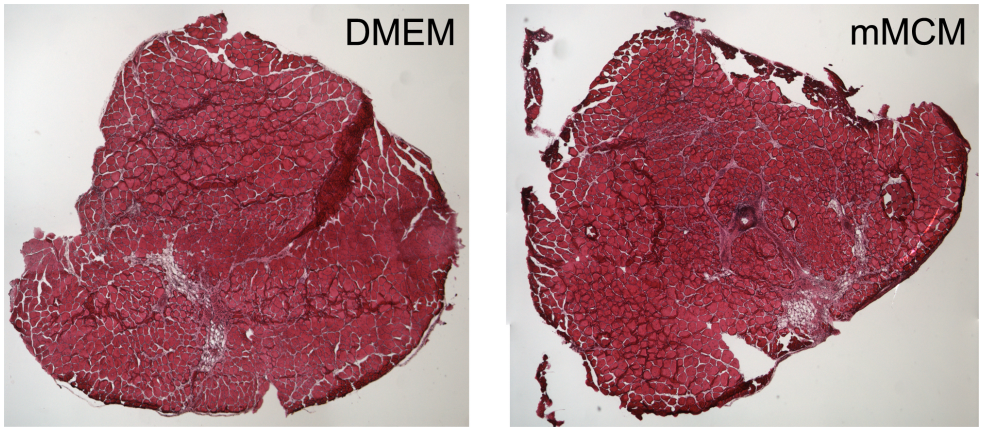


Figure 19: Hematoxylin and eosin staining of *wt* muscles damaged with glycerol and then treated 10 days after injury with concentrated (10X) mMCM or DMEM as a control. No evident difference was seen between control and treated muscles.

In order to verify if mMCM could still have had an effect on the dynamic of macrophage populations during regeneration we initially try to use western blot to quantify M1 and M2 markers. Unfortunately, this proved to be unfeasible, due to the poor performance of the commercially available antibodies we tried. We then resorted to qRT-PCR and monitored the expression of five macrophagic markers: F4/80, for both classes, CD68 and CCR2, for M1 macrophages, and CD163 and CD206, for M2 macrophages.

For all markers, changes in the expression of the RNA followed the same trend (except for one animal), i.e., mMCM treatment appeared to reduce their expression, thereby hinting at a general decrease in the quantity of macrophages present in the muscles.

Such effect was more pronounced after 3 days, while the differences in the values for the mice treated 10 days after injury were smaller (Figures 20, 21 and 22).

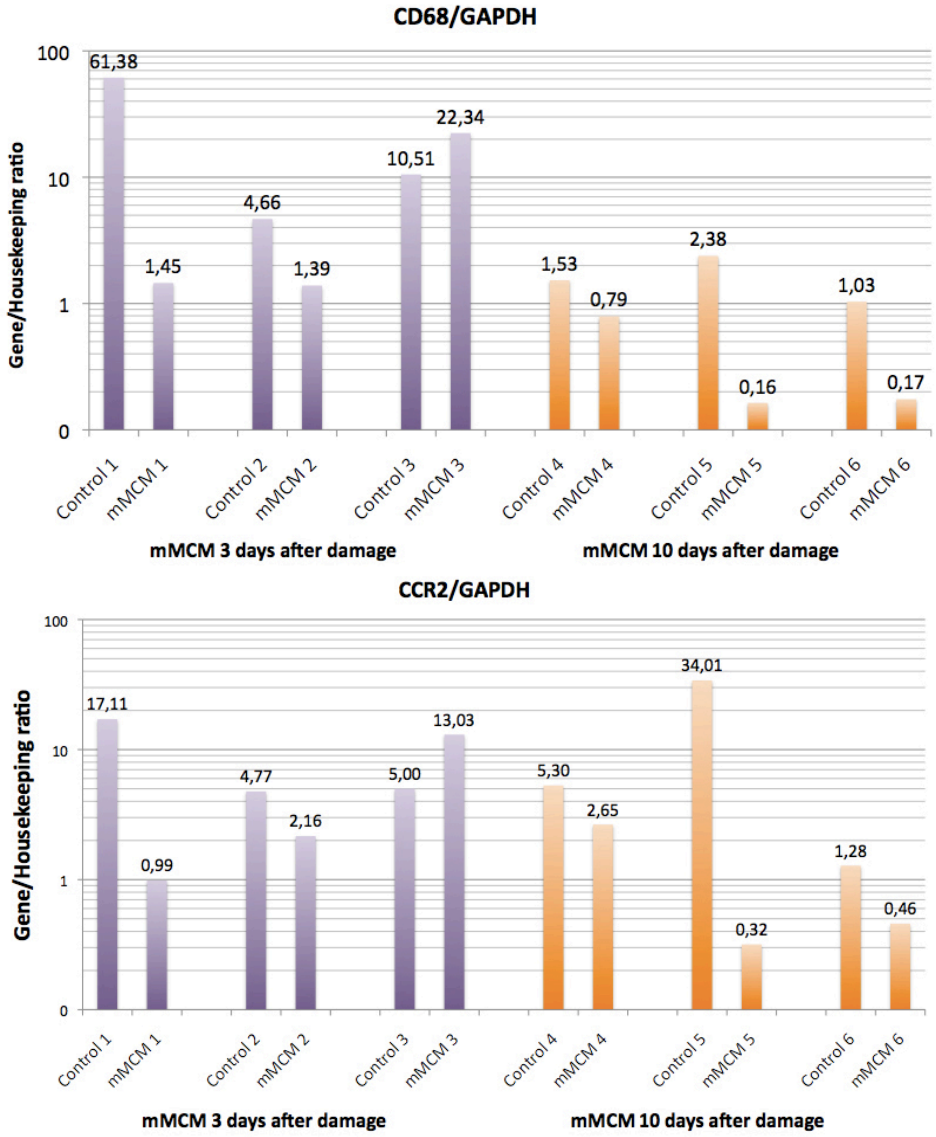


Figure 20: qRT-PCR quantification of the M1 macrophages' markers CD68 and CCR2 expression in glycerol-injured *wt* muscles that received mMCM injections 3 days after damage (mice 1, 2 and 3, purple bars), and 10 days after damage (mice 4, 5, and 6, orange bars). Expression levels are expressed through their ratio with the GAPDH housekeeping gene expression.

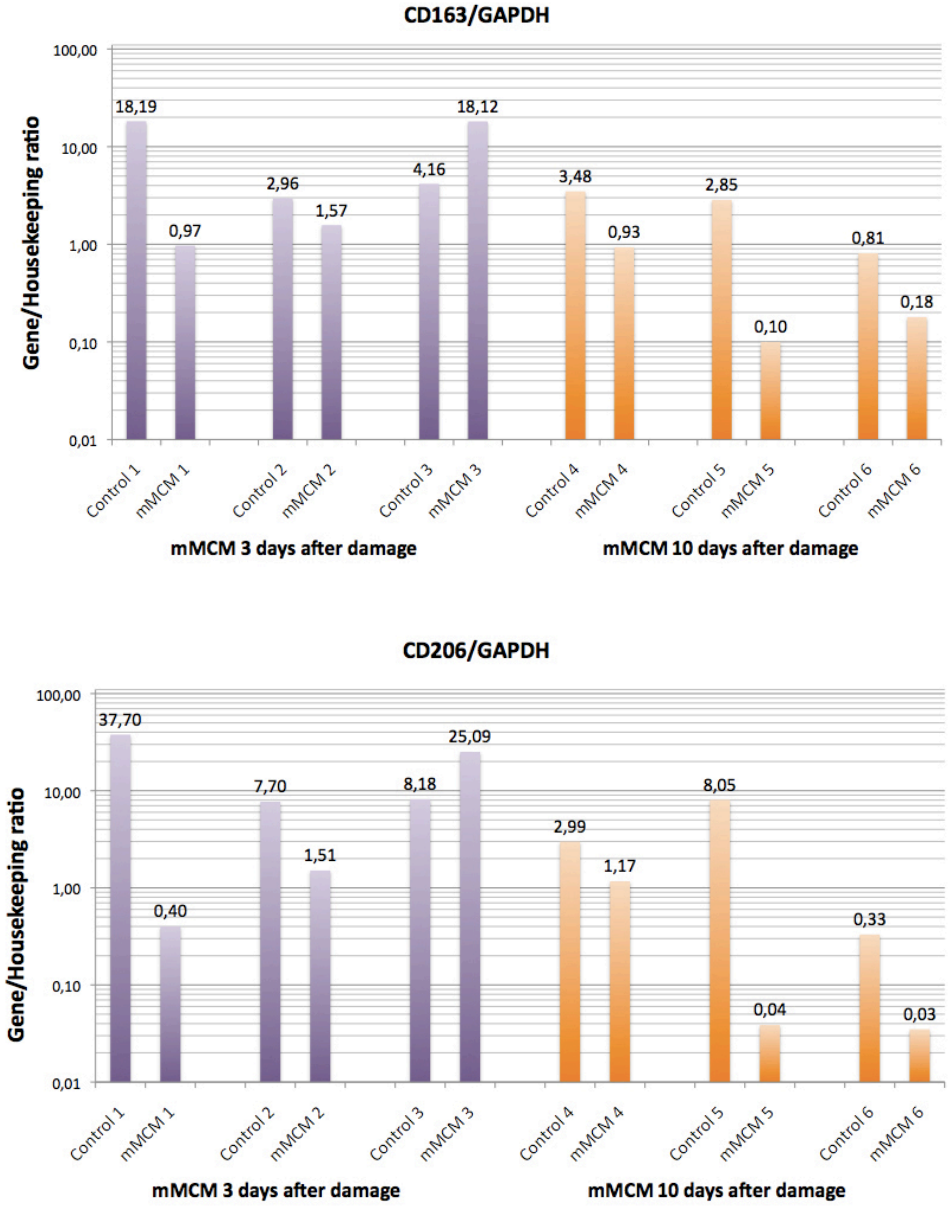


Figure 21: qRT-PCR quantification of the M2 macrophages' markers CD163 and CD206 expression in glycerol-injured *wt* muscles that received mMCM injecions 3 days after damage (mice 1, 2 and 3, purple bars), and 10 days after damage (mice 4, 5, and 6, orange bars). Expression levels are expressed through their ratio with the GAPDH housekeeping gene expression.

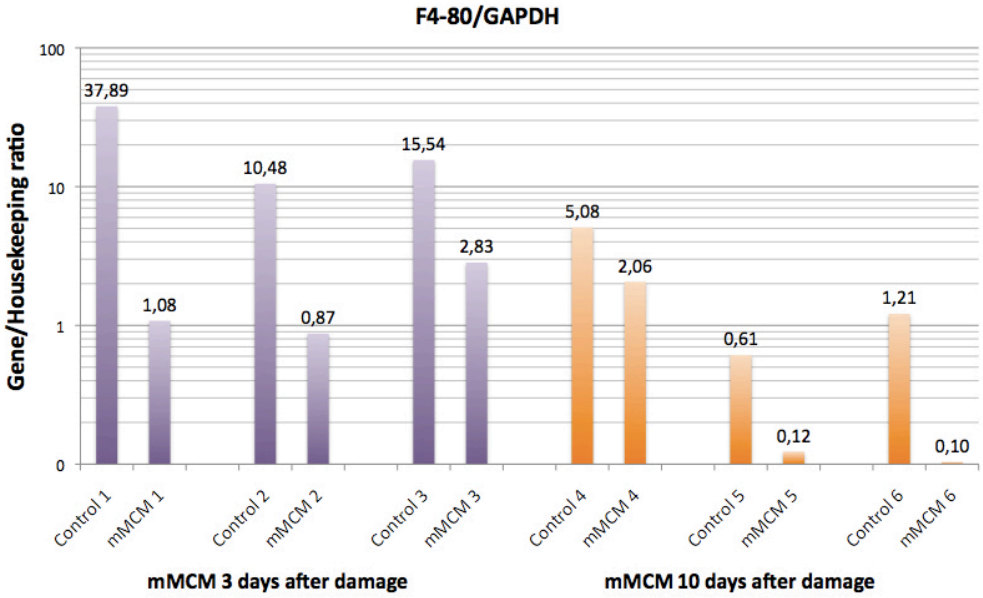


Figure 22: qRT-PCR quantification of the general macrophages' markers F4/80 expression in glycerol-injured *wt* muscles that received mMCM injecions 3 days after damage (mice 1, 2 and 3, purple bars), and 10 days after damage (mice 4, 5, and 6, orange bars). Expression levels are expressed through their ratio with the GAPDH housekeeping gene expression.

Effect of mMCM on non-mammalian myogenic cells

Mammals, and rodents in particular, are the most commonly used models for studies on myogenesis; however, chicken cells are also used, especially in developmental studies. During the course of this work we had access to pre-hatching chicks and we took this chance to assess if and how mMCM could affect non-mammal myogenic cells. To this aim, we prepared primary fetal myoblasts isolated from the legs of fetal chickens. Once again, we investigated mMCM effect on both their proliferation and expression of myogenic markers.
Differently from rodent and human myoblasts, mMCM did not have any significant effect on the proliferation of chicken primary myoblasts (Figure 23), as the duplication time of treated and control cells were almost the same (35 hours).

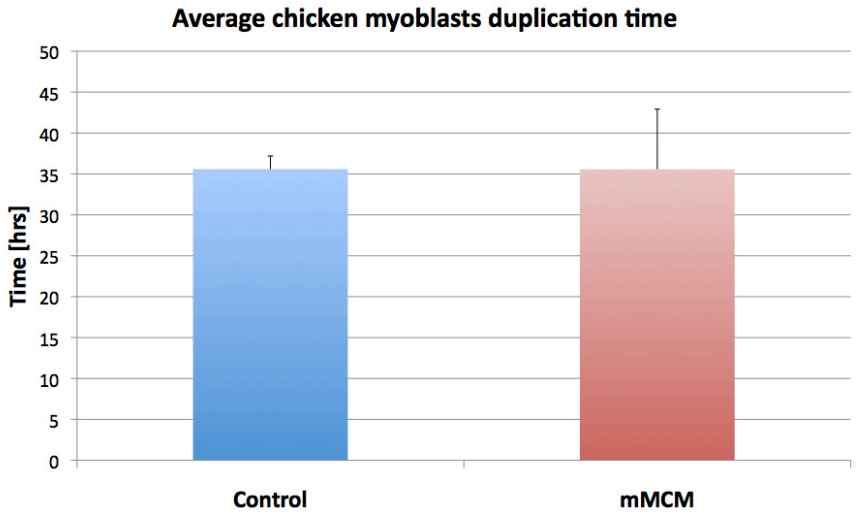


Figure 23: Average duplication time of chicken fetal myoblasts. mMCM did not reduce the duplication time of treated cells. (n=5 independent experiments with different cell preparations.)

Despite not having any effect on proliferation, however, mMCM had a clear effect on the expression of MyoD, myogenin and Pax7. In particular, the percentage of MyoD- and myogenin-positive cells resulted always higher in the presence of mMCM, and the difference was statistically significant (Figure 24). The effect of mMCM on Pax7 was less evident, since the percentage of Pax7 positive cells was significantly higher after one plating passage, but then decreased at the following passage (Figure 24).

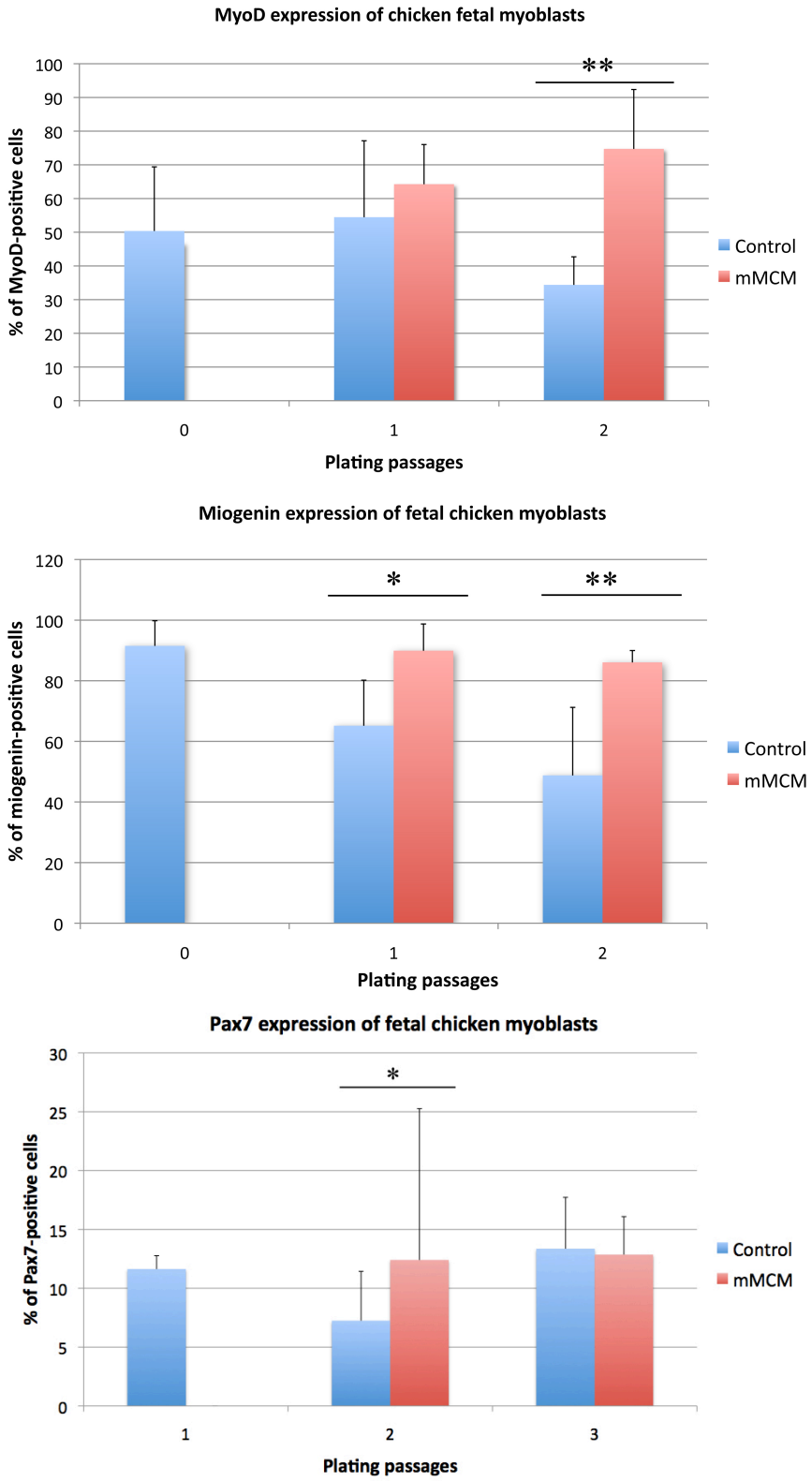


Figure 24: MyoD, myogenin and Pax7 expression in chicken fetal myoblasts during the time course experiment to assess their duplication time. When mMCM was present, MyoD, miogenin, and Pax7 positive cells were always more than control cells. Passage 0 indicates cells at the beginning of the experiment. (n=3 independent experiments with 3 batches of cells)

Effect of mMCM on the polarization of macrophages

mMCM is the product of activated macrophages, and the results of the qRT-PCR from the *in vivo* experiments on muscle injury hinted that it could have an effect on the inflammatory process. This led us to collaborate with Dr. Bolego (Dept. of Pharmacology, University of Padova), to assess if mMCM could have an effect on macrophages polarization.

Dr. Bolego and her group provided us with an established model for the study of macrophage polarization, in which primary human monocytes are isolated from the blood and differentiated *in vitro* into macrophages before being stimulated with different cytokines to acquire either M1 or M2 phenotype. In particular, M1 macrophages are obtained by stimulation with LPS and INFγ, while M2 macrophages are obtained with IL4 and IL13 (*Ambarus et al., 2012*). Polarization is then assessed by cyto-fluorimetry using various markers. It should be noticed, however, that although cytokine stimulation does push macrophages towards specific phenotypes, this is not an absolute fate. Macrophages have a high degree of plasticity and not all macrophages will change towards a specific polarization, so the sum of the various percentages will never reach 100%.

The first preliminary experiment we performed was aimed at understanding what was mMCM effect on naïve macrophages, adding it in the place of the polarization stimuli. We stimulated for 1 day the cells with LPS and INFγ, IL4 and IL3 or mMCM, then after 3 days we collected and analyzed the cells, using an anti-CD68 antibody. As mentioned in the Introduction, CD68 is a macrophagic marker that is expressed mostly, but not exclusively, by M1 macrophages.

Table 2 illustrates the results of this first experiment. Cells polarized towards the M1 phenotype showed an increase in the percentage of CD68+ macrophages (19,1%), while macrophages polarized towards the M2 phenotype showed a decrease even lower than untreated cells (0,9%). mMCM did not have any effect on the percentage of CD68+ macrophages, as the percentage was almost equal to that of untreated cells (1,8% *vs* 1,6%).

Effect of mMCM on naïve macrophages	
	% of $CD68^+$ cells (M1)
Unstained	0.2
Untreated	1.6
LPS/INFγ (M1)	19.1
IL4/IL13 (M2)	0.9
mMCM	1.8

Table 2: Cell sorter counts for $CD68^+$ cells in the total population after stimulation of human macrophages with cytokines or mMCM.

After this first experiment, we decided to verify if mMCM could affect macrophages identity if it was added after the polarization stimuli. This time, we were able to add also an M2 marker, CD206. mMCM was added 3 days after polarization, and was left for 3 more days before collecting and analyzing the cells.

When added to already polarized macrophages, mMCM seemed to push them towards the M2 phenotype (Table 3). When considering M1 polarization, the percentage of CD206-positive cells resulted higher upon mMCM treatment (9,0% *vs* 5,4%); at the same time, when considering M2 polarization the presence of mMCM decreased the expression of CD68 (1,6% *vs* 6,3%).

Effect of mMCM after macrophages' polarization		
	% of $CD68^+$ cells (M1)	**% of $CD206^+$ cells (M2)**
Unstained	0.2	0.7
LPS/INFγ (M1)	12.6	5.4
LPS/INFγ (M1)+mMCM	12.4	9.0
IL4/IL13 (M2)	6.3	45.6
IL4/IL13 (M2)+mMCM	1.6	45.8

Table 3: Cell sorter counts for $CD68^+$ (M1) and $CD206^+$ (M2) cells in the total population after stimulation of human macrophages towards the M1 or the M2 phenotype, with and without the addition of mMCM after polarization. (Untreated cells were unavailable due to experimental problems.)

A similar experiment was performed adding mMCM before polarizing macrophages.
mMCM was added 16 hours before polarization, and cells were analyzed 3 days after the polarization. This experiment (Table 4) seemed to confirm the previous results: in the presence of mMCM. CD68 expression resulted lower (35,5% *vs* 30,9% for cells polarized towards the M1 phenotype, 3,9% *vs* 2,6% for M2 polarization), and the percentage CD206-positive cells always resulted higher (31,5% *vs* 36,4% for cells polarized towards the M1 phenotype, 24,2% *vs* 26,9% for M2 polarization).

Effect of mMCM before macrophages' polarization		
	% of $CD68^+$ cells (M1)	**% of $CD206^+$ cells (M2)**
Unstained	0.5	0.5
Untreated	7.1	17.2
LPS/INFγ (M1)	35.5	31.5
mMCM+LPS/INFγ (M1)	30.9	36.4
IL4/IL13 (M2)	3.9	24.2
mMCM+IL4/IL13 (M2)	2.6	26.9

Table 4: Cell sorter counts for $CD68^+$ (M1) and $CD206^+$ (M2) cells in the total population after stimulation of human macrophages towards the M1 or the M2 phenotype, with and without the addition of mMCM before polarization.

Given the recently published data questioning the validity of CD68 as a true M1 marker (*Ambarus et al., 2012*), we decided carry out more analyses eliminating CD68 and adding another M2 marker, CD163. We also added dexamethasone, an anti-inflammatory drug that pushes macrophages towards the M2 phenotype (*Ehrchen et al., 2007*, *Graversen et al., 2012*), as a positive control. We repeated the experiments twice, adding mMCM alone and before and after polarization. While we confirmed that mMCM alone does not have any effect on macrophages polarization, the result we obtained for the other two experiments were discordant with what we saw when measuring $CD68^+$ cells.
Adding mMCM before polarization (Table 5) always decreased the percentage of CD206/CD168 positive cells, no matter which polarization was stimulated; such effect was opposite of that obtained when dexamethasone was added. Adding

mMCM after polarization had less clear effects, since there was no change when mMCM was added after M1 polarization, while adding it after M2 polarization increased the percentage of M2 macrophages (Table 6).

Effect of mMCM before macrophages' polarization		
	Average % of $CD206^+$ $CD163^+$ cells (M2)	**Standard deviation**
Unstained	1,3	0,4
Untreated	19,6	12,0
Dexamethasone	51,5	16,4
mMCM	21,0	3,0
LPS/IFNy (M1)	4,9	5,4
Dexa + LPS/IFNy (M1)	17,9	12,6
mMCM + LPS/IFNy (M1)	3,3	2,8
IL4/IL13 (M2)	31,2	33,3
Dexa + IL4/IL13 (M2)	59,7	28,3
mMCM + IL4/IL13 (M2)	27,8	23,1

Table 5: Average values of cell sorter counts for $CD206^+CD163^+$ (M2) cells in the total population after stimulation of human macrophages towards the M1 or the M2 phenotype, with and without the addition of mMCM before polarization (t n=2; each experiment was carried out with a different preparation of primary human macrophages).

Effect of mMCM after macrophages' polarization		
	Average % of $CD206^+$ $CD163^+$ cells (M2)	**Standard deviation**
Unstained	1,3	0,4
Untreated	9,4	5,2
Dexamethasone	27,9	25,6
mMCM	10,4	5,6
LPS/IFNy (M1)	2,3	2,1
Dexa + LPS/IFNy (M1)	12,4	16,5
mMCM + LPS/IFNy (M1)	2,1	2,4
IL4/IL13 (M2)	11,2	10,9
Dexa + IL4/IL13 (M2)	39,4	32,8
mMCM + IL4/IL13 (M2)	12,9	16,1

Table 6: Average cell sorter counts for $CD206^+CD163^+$ (M2) cells in the total population after stimulation of human macrophages towards the M1 or the M2 phenotype, with and without the addition of mMCM after polarization (n=2; each experiment was carried out with a different preparation of primary human macrophages).

Analyses of mMCM composition

Mesoscale

We analyzed mMCM composition by assessing the presence of cytokines by Mesoscale analysis, thanks to the collaboration of Dr. Galvagni (Dept. of Biotechnologies, University of Siena).

Mesoscale is a very precise ELISA-based platform that can detect and determine the concentration of a given set of cytokines in a sample (http://www.mesoscale.com).

We performed two rounds of analyses (the summary of the two experiments is reported in Table 7) with a slightly different set of cytokines for each round, analyzing the concentration of cytokines in 4 different samples: mMCM, medium conditioned by non-stimulated J774 cells, medium conditioned by C2C12 cells (a stable line of murine myoblasts), and medium conditioned by murine fibroblasts (3T3 cell line). We know from previous experiments that conditioned medium prepared from non-stimulated J774 cells does not have any myogenic effect.

Concentration pg/ml				
	MCM	**Unstimulated J774**	**C2C12 myoblasts**	**3T3 Fibroblast**
IFNγ	2	1	-	1
IL-1b	38	-	-	-
IL-2	17	-	-	8
IL-4	13	9	18	9
IL-5	22	10	-	13
IL-6	6313	-	47	80
IL-8	42	-	1859	2747
IL-10	636	89	35	47
IL-12p70	131	-	58	62
IL-12	485	9	-	12
TNFα	1501	17	-	-

Table 7: Mesoscale analysis of cytokine concentration in mMCM, non-stimulated J774 medium, C2C12 myoblasts medium and 3T3 fibroblasts medium.

The results showed that mMCM contains several cytokines, both pro- and anti-inflammatory. The most abundant cytokines present specifically in mMCM are

IL-6 (both a pro-inflammatory and an anti-inflammatory cytokine), TNFα (a pro-inflammatory cytokine), IL-10 (an anti-inflammatory cytokine) and IL-12 (pro-inflammatory).

We decided to verify if one or the mix of the 3 more concentrate cytokines (TNFα, IL-6 and IL-10), could somehow mimic the effect of mMCM if present at the same concentrations in the growth medium. To do so, we used our standard mMCM evaluation assay with rat neonatal cells (see the Materials and Methods section). Neither the single cytokines alone, nor the mix of all the 3, had any effect on the proliferation or differentiation of the cells (data not shown), thus indicating that these cytokines alone are not the cause of mMCM biological activity.

Mass Spectrometry analysis

In the beginning, we wanted to analyze mMCM composition by a combination of 2D gel electrophoresis and mass spectrometry, by separating both mMCM and the unstimulated J774 conditioned medium, that does not have any activity on myogenic cells, with a 2D gel, to spot differences in their composition and to identify factors that are present only in mMCM or that are differentially expressed. These factors could be those that are responsible for mMCM biological action and identifying them would be of great interest for further research. Figure

We performed a preliminary 2D gel separation of both mMCM and non-stimulated J774 conditioned medium, illustrated in Figure 25. The two conditioned media shows indeed various different spots that indicate differentially expressed proteins.

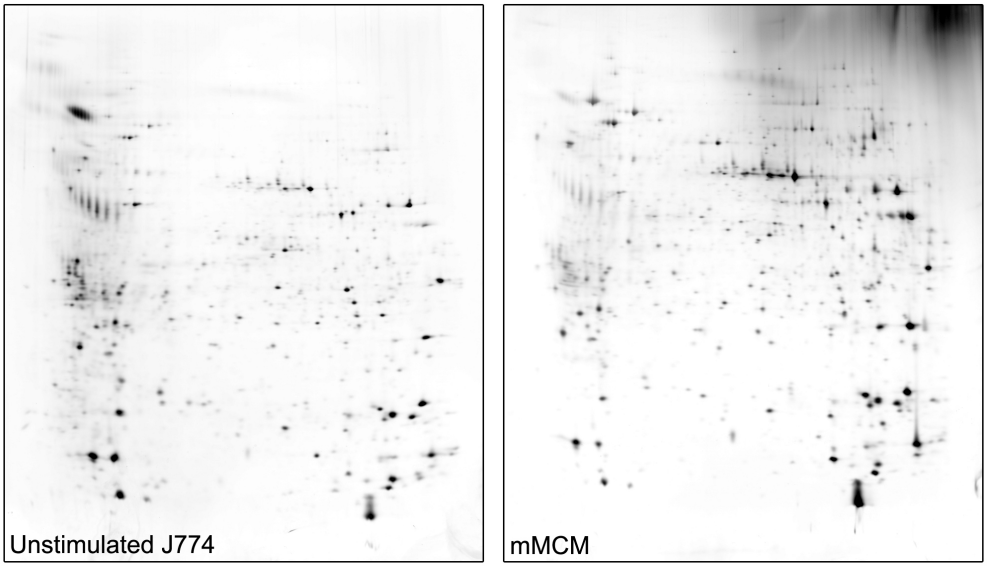


Figure 25: 2D gels of non-stimulated J774 conditioned medium and mMCM. The results clearly show differences in their composition.

The ideal follow-up to these results should have been the mass spectrometry analysis of the individual, differentially expressed spots. Unfortunately, we could not proceed further with the analysis by 2D gel electrophoresis, so we decided to use a single-dimension gel instead. mMCM and the non-stimulated J774 medium were run on a 1D gel, that was then stained with Coomassie blu. We identified 4 bands (with approximate MW of 45, 60, 90 and 95 kDa) that appeared stronger in mMCM, and one (35 kDa) that appeared stronger in the non-stimulated J774 lane (Figure 26).

Bands were not pure, for obvious technical reasons, and the analysis on them identified more than 150 proteins *per* bend. These proteins were clusterized using the DAVID software, to identify proteins involved in processes of interest. The clustering analysis was then investigated.

A preliminary analysis spotted some proteins that are now being considered as candidates for further analyses. One of them is prosaposin, a macrophagic secreted glycoprotein that was recently found to be involved in the muscle regeneration process, both in *wt* and *mdx* mouse, specifically during the regeneration and remodelling phase of the process, and to be expressed by myoblasts and myotubes (*Li et al., 2013*).

Another interesting protein is talin 1, involved in the stability of the myotendinous junctions, whose ablation with its homologous talin 2 *in vivo* can lead to defects in myoblast fusion and sarcomere assembly, by the disruption of the interaction of integrins with the cytoskeleton, mediated by talins (*Conti et al., 2009*).

We also found various cathepsins, one of the most represented is cathepsin B, a member of the family that is induced by the IFNγ signalling that is overexpressed in the first stages of the pathology of the *mdx* mouse (*Fang et al., 2000*), and that is involved in the process of muscle atrophy (*Gallardo et al., 2001*). Another interesting candidate that we found is cathepsin D, that is upregulated in differentiating myoblasts (*Johnston et al., 2011*).
Finally, another possible candidate is properdin, a complement protein that is expressed in adipose tissue, localized to the adipocyte plasma membrane. Properdin knock-out mice show great fat accumulation in all the tissues, because fat accumulation in the adipose tissue is deregulated, because lack of properdin may release its block on the insulin-mediated stimulation of fatty acid uptake (*Gauvreau et al., 2012*).

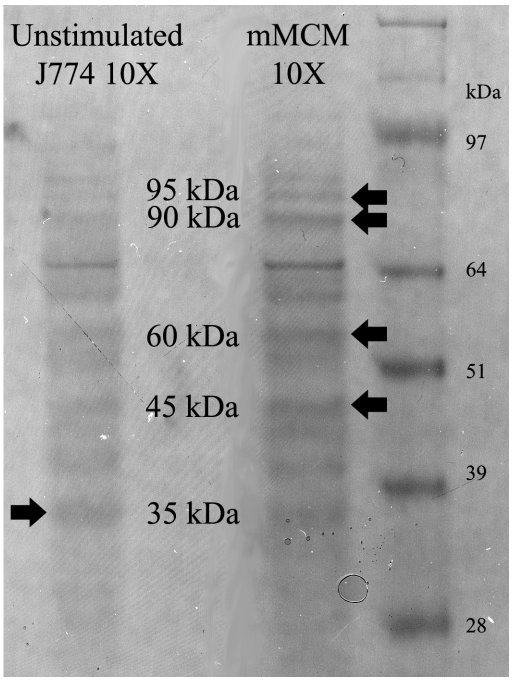


Figure 26: Coomassie Blue staining of the 1D gel loaded with mMCM and unstimulated J774 medium. The squares indicate the bands that were excised for the mass spectrometry analysis.

Exosomes

Immune system cells can also release exosomes, vesicles that are formed from the cell's endosomal membrane system and that are then secreted in the surrounding environment; they can contain many different molecules, such as proteins, mRNA, miRNA and even DNA. They can aid antigen presentation and exhibit either pro-inflammatory or anti-inflammatory properties, depending on the cell that generates them (*Pant et al., 2012*).

Myogenic cells release exosomes, both C2C12 cells (*Guescini et al., 2010*) and human myoblasts (*Marie-Catherine Le Bihan, personal communication*) but their role and content are still largely unknown.

To evaluate if mMCM could contain exosomes, we conducted a preliminary western blot experiment in collaboration with Dr. Guglielmo Guescini from Urbino University.

mMCM resulted positive for Tsg101, an exosomal marker (Figure 27), strongly suggesting the presence of exosomes. Further studies are now planned to understand the possible role of exosomes in mMCM.

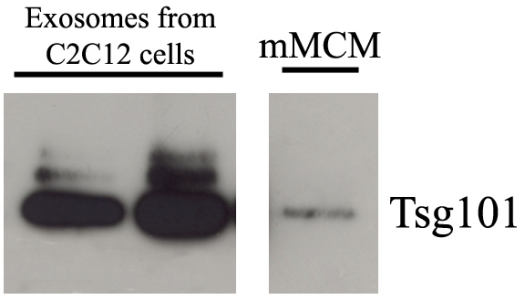


Figure 27: Western blot analysis of the presence of exosomes. mMCM resulted positive for Tsg101, an exosomal marker, thus strongly suggesting that mMCM contains exosomes. The 2 bands on the left are exosomes released from C2C12 myoblasts. The total protein quantity loaded for mMCM was 5 times lower than myoblasts exosomes extracts.

Discussion

Muscle repair is greatly dependent on immune system cells, especially macrophages. Many studies highlighted the necessary role of macrophages (*Leibovich and Ross, 1975*, S*umman et al., 2006*, *Tidball and Wehling-Henricks, 2007*, *Segawa et al., 2008*); still many questions are left unanswered. Investigating the effect of macrophages on muscle regeneration, especially on satellite cells' proliferation and differentiation, will be of great help for a better understanding of muscle healing and eventually for the setting up of therapies for muscle pathologies, both acquired, such as traumas of or inborn malformations, and inherited, like Duchenne Muscular Dystrophy (DMD).

To this aim, our group has been working on a model based on the conditioned medium obtained from a murine macrophage cell line, which we already demonstrated to have a very potent effect on the proliferation and the myogenicity of both rat satellite cells and human dystrophic myoblasts (*Cantini et al., 2002, Malerba et al., 2009, Malerba et al., 2010*).

In the present work we decided to switch to the mouse model, both on wild type (*wt*) and dystrophic (*mdx*) mice. This not only allowed us access to a wider range of research tools, but also to a very well known model for muscular dystrophy.

We first investigated mMCM effect on the proliferation of *wt* satellite cells isolated from single fibers by assessing their duplication time. mMCM was able to greatly enhance their proliferation rate, as the average duplication time decreased from 90 to 48 hours. Since satellite cells are a heterogeneous population (*Biressi and Rando, 2010*), we wanted to be sure that our results were reproducible using cultures obtained by FACS as α7-integrin$^+$, CD34$^+$, sca1$^-$, CD45$^-$ CD11b$^-$ and CD31$^-$, the other commonly used method for their preparation. Our findings showed that mMCM could also decrease the doubling time of FACS-isolated cells (from 45 to 31 hours), thereby confirming that its effect is exerted on satellite cells in general.

We repeated the experiment also with *mdx* satellite cells. Compared to those from *wt* animals, satellite cells from dystrophic muscle invariably have more cycles of activation and proliferation; besides, recent evidences suggest that epigenetic differences may be involved in the dystrophic pathology (*Consalvi et al., 2011*). These data hint that *mdx* satellite cells might have a different behavior than *wt* cells, and indeed mMCM had on them an even more pronounced effect in terms of duplication time, decreasing it from 108 to 28 hours.

From previous data we knew that mMCM also had an effect on the differentiation of rat myogenic cells and hence we investigated whether this was true also for murine satellite cells, by differentiating *wt* and *mdx* satellite cells in the presence or in the absence of mMCM. The presence of mMCM, no matter in what part of the growth or differentiation process, tended increased satellite cells differentiation, both *wt* and *mdx*.

From all these data, we clearly showed that mMCM has unique properties different from the action of the two macrophagic populations that participate in muscle regeneration. It is known that macrophages can be divided into two main populations: M1 and M2. These two types of macrophages release and express different markers and are involved in different aspects of the inflammatory processes (*Mantovani et al., 2004*). During muscle injury, M1 macrophages are the first macrophagic population that can be found in the injured muscle, and only after about 2 days from injury, they switch to an M2 polarization, changing their action on the regenerating environment and allowing resolution of inflammation (*Smith et al., 2008*). Several groups have tested M1 and M2 macrophages on myogenic cells, but no general consensus has been reached on their specific effects are. Chazaud and colleagues reported that M1 macrophages could stimulate proliferation of myoblasts but inhibit their differentiation, while M2 macrophages have the opposite effect (*Arnold et al., 2007*), but the group led by Tidball found opposite results: M2 macrophages stimulated the proliferation of myoblasts, while M1 macrophages caused cellular lysis (*Villalta et al., 2009*).

Our results clearly show that no matter what M1 or M2 macrophages effects on satellite cells are, mMCM behaves differently from the factors release by polarized macrophages, since it has a positive effect on both proliferation and differentiation. Besides, mMCM also does not appear to have any adverse effect on the cells, even if added at very high concentrations in the growth medium. Last but not least, the fact that mMCM seems to be particularly active on cells derived from dystrophic muscle could make it even more relevant as a therapeutic tool.

The replacement of muscle fibers with fibrotic tissue is one of the main pathological features both in dystrophic patients and in cases of large muscle ablations. Fibroblasts are crucial for muscle repair, as they are necessary for a correct reformation of the connective tissue, but if they proliferate too much they can form scar tissue. Lowering the extent of fibrotic deposition would therefore be very beneficial for clinical applications.

We already knew that mMCM was able to slow down the proliferation of non-myogenic cells in whole muscle cultures from neonatal rat (*Malerba et al., 2009*). We now assessed if this was true also for muscle fibroblasts. Our data showed that

mMCM was able to slow down the proliferation of *mdx* fibroblasts, increasing their duplication time from 53 to 67 hours, while it had no effect on *wt* cells.
Another intriguing feature of mMCM was its ablility to prevent the trans-differentiation of rat satellite cells into adipocytes (*Malerba et al., 2009*), so we investigated if this effect could be seen with mouse satellite cells too. Indeed, when we we placed *wt* and *mdx* satellite cells in the same conditions used in the original experiments (i.e., we plated them at sub-clonal density and left them proliferate), control cells showed the presence of various clusters of adipocyte-like cells, which were not seen when cells were treated with mMCM. Even though the effect was in agreement with our previous data, it must be pointed out that recently some researchers have questioned the capability of actual satellite cells to trans-differentiate towards adipogenesis. If on one hand some researchers say that cloned satellite cells can generate adipocytes if stimulated with the proper signals (*Holterman & Rudnicki, 2005* and references therein), while other reports indicate that such conversion can happen spontaneously (*Rossi et al., 2010*), some others maintain instead that such phenomenon is actually due to other cell types presents in the preparations (*P. Gilbert, personal communication*). At any rate, our results showed that, regardless of the origin of such adipocyte-like cells, when mMCM is present in the medium their formation is inhibited. The fact that *mdx* preparations showed less adipocytes that *wt* cells was somewhat unexpected, considering that fat deposition is one of the features of DMD, but on the other hand it is also true that the *mdx* muscle presents a much less prominent fat accumulation than human patients. It will therefore be of extreme interest to repeat this experiment with human satellite cells.
In summary, these data shows that mMCM has a very potent proliferative effect on satellite cells, it is able to slow down the proliferation of fibroblasts from dystrophic muscle and can prevent the formation of adipocyte-like cells in primary myogenic cultures, thereby confirming its potential as a therapeutic tool.
To test this possibility, and to improve our understanding of the mechanism(s) involved in its actions, we carried out various *in vivo* experiments.
Alone, mMCM was not pro-inflammatory, as we assessed by injecting it in an intact muscle, and finding no sign of inflammation and no differences from a negative control injected with plain DMEM.
As mMCM enhanced both the proliferation and the differentiation of satellite cells, we then tested its ability to ameliorate the grafting of *wt* myogenic cells in a dystrophic muscle by supplying mMCM with the injections of *wt* satellite cells, repeating the administration of the factors after the grafting. It has just been reported that co-injection of human M1 macrophages and myoblasts in

immunodeficient mice improved the participation of the latter in the host muscle regeneration, lengthening proliferation, increasing migration and delaying differentiation. This effect, though, was found for M1 but not M2 macrophages (*Bencze et al., 2012)*.

The first results we obtained from our experiments were not clear, given the unexpected damage that the repeated injections caused in the *mdx* muscle. We therefore changed the experimental conditions, limiting the number of injections to avoid excessive damage and transplanting GFP^+ cells to distinguish between grafted fibers and revertant fibers. These new experiments showed much less regeneration, and indeed a better grafting when mMCM was supplied with the cells and at a later time, as the GFP^+ fibers and the total GFP expression level were always higher in treated muscles.

Given that macrophages are known to release many cytokines that have powerful autocrine and paracrine effects, and our data found with the experiments on muscle ablations in rats (*Malerba et al., 2009*), we wanted to investigate the effects of mMCM on muscle regeneration processes upon a damage that destroyed the fibers *in situ*, without any tissue ablation. This type of damage mimics both the processes found in dystrophies and in many traumatic injuries. We used the glycerol damage model (*Pisani et al., 2010*) because of its slow kinetics, which could allow us to better understand mMCM effects during the various phases of the regeneration process. Somewhat disappointingly, when mMCM was supplied in the destructive phase of the inflammation (when the necrotic processes were still ongoing), we did not found any clear histological differences. However, when we analyzed the expression levels of various macrophagic markers by qRT-PCR at the same time point we found that in the presence of mMCM the expression levels of both M1 and M2 markers were lower than control muscles in almost all cases, hinting that those muscles contained fewer total macrophages. The same trend was found also in muscles that received mMCM injections at a later time point (10 days, when the necrotic phase was completed and the repair phase was ongoing), although the differences were less prominent.

We had been studying the effects of mMCM on the myogenic cells of at least 3 mammals species: human, rat and mouse (*Malerba et al., 2009, 2010 and this work*). During the course of this thesis we had the possibility of preparing primary myoblasts from pre-hatching chicks, a species that has also been used for myology studies. Interestingly, when we tested mMCM on chicken foetal myoblasts we found that its effects were partially different from what we had seen in mammals. In particular, it was not able to increase the proliferation of the

cultures, but was able to increase the percentage of cells that expressed MyoD, myogenin and, albeit not as much, Pax7. These results suggest that the macrophagic factors contained in mMCM can maintain the myogenicity of the cultures but do not affect cell proliferation. This could hence prove as a useful model for studying the molecular mechanisms of mMCM action(s).

mMCM effects are various and promising for further investigations, but one of the crucial data that will push this research further will be the determination of its active composition.

Being a conditioned medium, the composition of mMCM is undoubtedly very complex. In the past we have attempted some biochemical characterization by western blot, measuring some of the most likely candidates amongst cytokines, but with little success (*Malerba et al., 2009*). Now we decided to use different methods to investigate this matter, and the first step was using an automated ELISA-based platform, the MesoScale technology, that is able to detect the concentration of a definite set of cytokines in a sample. The results of the analysis showed that mMCM contained a mix of both pro- and anti-inflammatory cytokines.

The cytokine that resulted the most abundant in the analysis was IL-6. IL-6 is secreted by macrophages in a various range of immune processes, both in tissue injury and in infections and it can act both as a pro-inflammatory cytokine that stimulates B cells (*Rohleder et al., 2012*) and as an anti-inflammatory myokine that is released by the muscle itself. During exercise, IL-6 is produced by muscle fibers by a TNF-independent pathway and is able to regulate muscle metabolism during exercise, functioning as an energy sensor (*Pedersen, 2012*). IL-6 also stimulates the production and the release in the circulation of other anti-inflammatory cytokines, such as IL-1ra and IL-10. It can also inhibit the production of the proinflammatory cytokine TNF-α, and enhances lipid turnover, stimulating lipolysis and fat oxidation (*Petersen et al., 2005*).

Another abundant cytokine present in mMCM is TNFα. This is a pro-inflammatory cytokine that is secreted by M1 macrophages and other immune system cells and regulates the expression of other pro-inflammatory cytokines (*Palladino et al, 2003*). Stimulation of macrophages with TNFα pushes them towards the M1 polarization (*Mantovani et al., 2004*).

mMCM also contained high levels of IL-10, an anti-inflammatory cytokine that can inhibit the activation and the function of T cells, as well as monocytes and macrophages (*Moore et al., 2001*). Stimulation of macrophages with IL-10 causes them to shift to the M2c phenotype (*Mantovani et al., 2004*).

Finally, the last highly concentrated cytokine we found in our analysis is IL-12, a pro-inflammatory cytokine that modulates the action of T cells, stimulating the production of pro-inflammatory T helper type 1 cells (*Vignali and Kuchroo, 2012*). It should be noticed that the concentrations of cytokines we found in mMCM were similar or higher (up to tenfold) than those reported in the literature for media conditioned by primary monocytes/macrophages (*Arnold et al., 2007*).
We were able to test the effects of 3 of these cytokines (TNFα, IL-6 and IL-10), using our standard model for testing mMCM batches described in M&M. We found that neither of these cytokines alone, nor the mix of them could mimic mMCM effect on myoblasts, either on proliferation or differentiation. Interestingly, it has been recently reported that IL-6 deficiency *in vivo* impairs muscle regeneration and myoblasts proliferation (*Zhang et al., 2013*), and previous works reported that IL-6 and TNFα (at a concentration about tenfold higher than that present in mMCM) could enhance the proliferation of human myoblasts and C2C12 myoblasts (*Wang et al., 2008*, *Al-Shanti et al., 2008*). On the contrary, IL-10 alone was reported to have no effect on the proliferation of myoblast, but in the IL-10 mutant mouse apparently the transition from the proliferative to the early differentiation stages of myogenesis occurs earlier than normal (*Deng et al., 2012*).
The more complete approach that would allow us to determine mMCM composition is of course total mass spectrometry. With this method, we would have a precise and reliable list of all the proteins contained in our conditioned medium, even though it would not give quantitative indications, which would require more complex and long experiments, like stable isotope labeling by amino acids in cell culture (SILAC).
Another possible approach would be a combination of 2D gel separation and mass spectrometry to spot differentially expressed proteins between mMCM and non-stimulated J774 cell medium, which does not have any myogenic effect. This could be feasible also with a simple 1D SDS-Page, but with loss of resolution.
We initially started the 2D approach with our collaborators at the University of Siena, but we had to abandon it due to equipment problems in their mass facility. We then switched to the 1D gel approach, and we started by selecting some bands that we found stronger in the mMCM or in the non-stimulated J774 medium.
The analysis of the initial results – by means of the David bio-informatic tool– identified a set of potentially interesting proteins. The first one is prosaposin, a secreted glycoprotein that can stimulate myoblast fusion (*Rende et al., 2001*). Prosaposin is a precursor of four small lysosomal proteins: saposin A, B, C, and D, which are required for intracellular degradation of sphingolipids and that is

able to induce activation of the sphingosine kinase (*Minasi et al., 2001*, *Sandhoff and Kolter, 2003*). Sphingolipid signaling, especially the generation of sphingosine-1-phosphate by the sphingosine kinase, is necessary for satellite cells activation. If its synthesis is blocked, satellite cells remain quiescent and the muscle regeneration process is impaired (*Nagata et al., 2006*). Prosaposin was very recently found to be involved both in the muscle regeneration process, both in *wt* and *mdx* mouse, and to be expressed by myoblasts and myotubes, but its precise role in the regenerative process is still unknown (*Li et al., 2013*).

Another interesting protein is talin 1, a protein that regulates the stability of the myotendinous junctions by mediating the interaction between the cytoskeleon with integrins. Ablation of talin 1 and of its homologous talin 2 leads to defects in myoblast fusion and sarcomere assembly, because the interaction between integrins with the cytoskeleton is lost (*Conti et al., 2009*).

We also found properdin, a complement protein that was recently found to have a role in fatty acid metabolism, as it probably blocks the response to the insulin-mediated stimulation of fatty acid uptake (*Gauvreau et al., 2012*). Our mMCM is able to prevent the trans-differentiation into adipocytes of rat and mouse satellite cells, so this protein could be an interesting candidate, being able to inhibit the accumulation of fatty acids in the adipocytes.

Finally, we found several cathepsins, lysosomal proteases involved in the inflammation process. One of the most interesting and represented is cathepsin B, a member of the family that is induced by the IFNγ signaling and that is overexpressed in the first stages of the pathology of the *mdx* mouse (*Fang et al., 2000*). Cathepsin B is also involved in the process of muscle atrophy (*Gallardo et al., 2001*). Another interesting member is cathepsin D, that is up-regulated in differentiating myoblasts (*Johnston et al., 2011*).

Knowing that mMCM contained different cytokines and that it could affect inflammation progression *in vivo*, we speculated that it could have an effect also on macrophages themselves. To verify this hypothesis, we used, in collaboration with Chiara Bolego's laboratory in Padova, an established model for the study of macrophage polarization, in which primary human monocytes are isolated from the blood, differentiated into macrophages in culture and then stimulated to make them acquire either M1 or M2 phenotype (with LPS and INFγ or with IL4 and IL13, respectively). Cells are then labeled with specific anti-CD antibodies and analyzed by cytofluorimeter, to determine the percentage of M1 or M2 macrophages in the total population. First, we analyzed if mMCM alone could have an effect on macrophagic polarization, but various experiments, considering different markers, showed that it did not. We then decided to add mMCM before

and after polarization with the cytokines, and the first results we obtained hinted towards the fact that mMCM could push macrophages towards the M2 phenotype. In these experiments, however, we used CD68 as an M1 marker, and just CD206 as an M2 marker. Assessing a good marker for polarized macrophages has so far proved very difficult for the great plasticity of macrophages themselves (*Mantovani et al., 2009*), and even though CD68 is widely used as an M1 marker (*Villalta at al., 2009*, *Tidball and Villalta, 2010*) recent data suggest that it should be regarded as a more general macrophage antigen (*Ambarus et al., 2012*). To bypass this uncertainty we then decided to modify our experimental setting, by analyzing the population that resulted positive for both CD163 and CD206, two unquestioned M2 markers. Interestingly, these new experiments, conducted using mMCM both before and after macrophagic polarization, went in the opposite directions, indicating that mMCM pushed macrophages slightly towards the M1 phenotype. Such discrepancies, together with the findings on proliferation and differentiation of satellite cells, are a further indication that stimulated J774 cells do not reflect the activity of primary macrophages but rather appear to be a combination of M1 and M2. In any case, the fact that mMCM can also increase the expression of two myogenic markers in chicken myoblasts indicates that at least some of its biological effects are mediated by well-conserved pathways.

Conclusions and future prospects

mMCM, with its ability to enhance both the proliferation and differentiation of satellite cells, to slow down *mdx* fibroblasts growth and its ability to ameliorate myogenic cell grafting, proved itself a promising candidate for developing therapeutic approaches for muscle diseases as well as a tool to better understand the interplay between macrophages and muscle regeneration.

To reach a point in which mMCM could be translated into practical applications, though, more work is needed, especially to characterize its composition and dissect its active components. For this reason, we would like to try and continue mass spectrometry analysis, exploiting the difference between mMCM and non-stimulated supernatant to identify the active components. Of course, it is quite possible that the properties of mMCM originate from the synergy of many factors rather than on the additive action of few main players, something that would further complicate the task of dissecting it.

These data should be coupled with a microarray analysis on control and mMCM-treated cells that should highlight the up- and down-regulated pathways. The two sets of data would then be cross-linked to identify possible candidates, which would then be analyzed alone on the cells, and by immuno-depletion from the total mix, to confirm their biological activity in mMCM.

Aside from the biochemical characterization, there are experiments that we will be able to carry out in the immediate future to better define the picture of mMCM biological activity. One of them will be to perform more cell transplant experiments at shorter time points, coupled to labeling of proliferating cells. This will tell us if the improved grafting is due to an increased proliferation of the injected cells, to a better survival upon injection or both.

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