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Differentially activated macrophages orchestrate myogenic precursor cell fate during human skeletal muscle regeneration

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Keywords: skeletal muscle, muscle stem cells, myogenic precursor, macrophage, myogenesis

ABSTRACT

Macrophages exert either beneficial or deleterious effects on tissue repair, depending on their activation/polarization state. They are crucial for adult skeletal muscle repair, notably by acting on myogenic precursor cells. However, these interactions have not been fully characterized. Here, we explored both *in vitro* and *in vivo*, in human, the interactions of differentially activated MPs with muscle precursor cells (MPCs) during adult myogenesis and skeletal muscle regeneration. We showed *in vitro* that through the differential secretion of cytokines and growth factors, pro-inflammatory macrophages inhibited MPC fusion while anti-inflammatory macrophages strongly promoted MPC differentiation by increasing their commitment into differentiated myocytes and the formation of mature myotubes. Furthermore, the *in vivo* time course of expression of myogenic and macrophage markers was studied in regenerating human healthy muscle after damage. We observed that regenerating areas containing proliferating MPCs were preferentially associated with macrophages expressing pro-inflammatory markers. In the same muscle, regenerating areas containing differentiating myogenin positive MPCs were preferentially coupled to macrophages harbouring anti-inflammatory markers. These data demonstrate for the first time in human that macrophages sequentially orchestrate adult myogenesis during regeneration of damaged skeletal muscle. These results support the emerging concept that inflammation, through macrophage activation, controls stem cell fate and coordinates tissue repair.

INTRODUCTION

Macrophages (MPs) are one of the main agents of innate immunity. They contribute to the host defence and phagocytosis of undesirable pathogens or tissue debris. As a consequence, MPs are mainly considered as "inflammatory" cells that are deleterious for the tissue and efforts are focused on decreasing or preventing their presence in the tissue. Indeed, several studies have shown that MPs enhance tissue damage. For example, MP depletion prevents or reduces the clinical signs of experimental autoimmune neuritis (1) or attenuates blood-nerve barrier disintegration during chronic nerve injury (2). MP depletion is also beneficial in the case of pulmonary and hepatic injury induced by toxicants (3). As the effects of MPs can be either beneficial or deleterious (4-6), depending on the concentration of MP supernatant used (for *in vitro* studies) or the time point when MP depletion is induced (*in vivo* models), it is clear that the actions of MPs are complex. In support of this, several studies have demonstrated beneficial roles for MPs in the context of tissue homeostasis and repair. For example, MP-secreted factors have been reported to promote the survival, regeneration and neurite extension of neurons (7-9), proliferation of hepatocyte (10) and skeletal muscle regeneration (11-13).

The multiplicity of MP effects is likely due to their inflammatory profile or state of activation. Indeed, MPs adopt various inflammatory profiles depending on their environment. To help in understanding these various phenotypes, MPs have been classified into four main subtypes (14, 15). Pro-inflammatory or classically activated or M1 MPs are induced by TH1 cytokines. They secrete pro-inflammatory cytokines and Reactive Oxygen Species (ROS) and exhibit microbicidal and tumoricidal activities. Alternatively-activated or M2a MPs are triggered by TH2 cytokines such as Interleukin (IL)4. These cells secrete a battery of specific markers (YM1, AMCase, Arginase 1 (Arg1)), are associated with extracellular matrix synthesis, angiogenesis, parasite containment and are observed during chronic fibrosis. Regulatory or M2b MPs are induced by a combination of TLR ligands and immune complexes and dampen inflammation. Anti-inflammatory or deactivated or M2c MPs are induced by anti-inflammatory compounds (*e.g.* IL10, glucocorticoids). They secrete anti-inflammatory cytokines

1
2
3 such as IL10 and Transforming Growth Factor (TGF) β . Harboring different phenotypes, it is likely
4
5 that the activated/polarized MPs exhibit various functions during innate immunity. Very few studies
6
7 have explored their functions on progenitor cells. Interferon (IFN) γ - and Tumor Necrosis Factor
8
9 (TNF) α -stimulated microglia blocks differentiation of adult neural progenitor cells while microglia
10
11 activated by IL4 or low levels of IFN γ stimulates these neural progenitor cells toward
12
13 oligodendrogenesis and neurogenesis, respectively (16). In skeletal muscle, we have shown that
14
15 Lipopolysaccharide (LPS)/IFN γ -activated MPs stimulate myogenic cell proliferation while IL4 and IL10-
16
17 activated MPs support their differentiation (11). Although MP activation states have been clearly
18
19 defined *in vitro*, the link between the activation states of MPs and their functions remains unknown
20
21 for most *in vivo* situations.
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24
25 Adult skeletal muscle regeneration is an excellent paradigm of sterile inflammation with complete
26
27 resolution. While the exact time course of human skeletal muscle regeneration has not been
28
29 determined due to the restrictions of being able to sample human muscle a limited number of times
30
31 using the biopsy technique, the time it takes for regeneration to be completed is known to vary
32
33 depending on the nature of the experimental model employed. For example it has been shown that
34
35 models of acute muscle damage employing neuromuscular electrical stimulation, in contrast to
36
37 voluntary contractions, are required if the goal is to induce damage and regeneration, clearly
38
39 observable by signs such as infiltration of macrophages into the myofibres, activation and
40
41 proliferation of satellite cells, and the presence of myofibres positive for embryonic myosin (17-20).
42
43 With regard to the time course of human muscle regeneration, studies aiming to examine the
44
45 endpoint are sparse, with one recent study demonstrating ongoing regeneration of the myofibres
46
47 and remodelling of the extracellular matrix as late as 30 days after a single bout of electrical
48
49 stimulation (17). Several studies have demonstrated the presence of damaged fibres from a range of
50
51 2 to 7 days post trauma (19-21), confirming the complexity of the sequence of events required for
52
53 successful and complete regeneration of damaged skeletal muscle. From the few studies
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55 investigating muscle regeneration in humans, thus, it appears that biopsy sampling approximately 7
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1
2
3 and 30 days post trauma would be suitable time points for studying processes of inflammation and
4
5 satellite cell activity during skeletal muscle tissue repair.

6
7 *In vivo* experiments in mouse have shown that MPs are very important for muscle repair (22). A
8
9 sequential involvement of pro-inflammatory and alternatively activated/anti-inflammatory MPs has
10
11 been delineated in this process. The first appearing pro-inflammatory MPs enter into the damaged
12
13 muscle and a few days later, they convert into anti-inflammatory MPs (11). Meanwhile, the main
14
15 muscle stem cells, satellite cells, undergo adult myogenesis through several sequential steps, each of
16
17 which must be correctly completed to accommodate successful achievement of the subsequent
18
19 steps. This process begins with the activation of the quiescent satellite cells on the myofibre by a
20
21 damage signal. Activated myogenic precursor cells (MPCs) proliferate and it is believed that most of
22
23 them enter into terminal myogenic differentiation, while a subset returns to quiescence to self-
24
25 renew the satellite cell pool (23). Adult myogenesis encompasses: i) activation of quiescent satellite
26
27 cells and expansion of MPCs; ii) the commitment of MPCs into terminal differentiation. This requires
28
29 cell-cycle withdrawal and expression of the myogenic programme including the expression of the
30
31 myogenic regulatory factors myogenin and MRF4 and contractile proteins such as myosins; iii) the
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33 migration of the cells towards each other to establish cell/cell contacts and iv) the fusion of the cells
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35 into plurinucleated and contractile structures that form myotubes *in vitro* and myofibres *in vivo*.
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40 In the present work, we studied for the first time in human the relationship between the
41
42 inflammatory profile of MPs and their regulatory role on the homeostasis of MPCs. We explored
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44 using human coculture *in vitro* which steps of adult myogenesis are controlled by differentially
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46 activated MPs and analysed the involvement of a series of MP-secreted effectors. *In vivo*, we
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48 investigated in parallel the kinetics of adult myogenesis and the presence of MP subtypes during
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50 human skeletal muscle regeneration.
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MATERIALS AND METHODS

Human MPC culture. Human MPCs were isolated from normal adult skeletal muscle sample according to the French legislation (protocol registered at the Agence de la Biomédecine in 2007 "Interrelations entre les cellules souches adultes du muscle strié squelettique et les macrophages" and Cochin Hospital Cell Bank, Paris, agreement n° DC-2009-944). Cells were obtained and cultured as previously described (24) in HAMF12 medium (Gibco) containing 15% Foetal Bovine Serum (FBS) and were sorted before the first passage with anti-CD56 magnetic beads (Miltenyi Biotec) according to the manufacturer instructions. The purity of the cells was assessed for CD56 expression (555518 BD Pharmingen) by flow cytometry (FC500 Beckman Coulter cytometer).

Human MP culture. MPs were obtained from human Buffy-coat (purchased from Etablissement Français du Sang according to INSERM-EFS agreement number C CPSL UNT n°09/EFS/024) as previously described (11, 25). MPs seeded in 12-well plates (36 000 cells/cm²) were activated/polarized for 2-3 days into M1 MPs with 1 µg/ml LPS and 10 ng/ml IFN γ , into M2c MPs with 10 ng/ml IL10 and 80 ng/ml Dexamethasone, or into M2a MPs with 10 ng/ml IL4. Cells were then extensively washed (4 times) with serum-free medium to remove the effectors. Conditioned media were obtained by incubating MPs for 24h in advanced RPMI medium (Gibco) containing 0.5% FBS. Analysis of surface antigen expression was performed by flow cytometry. Activated MPs were detached by non enzymatic dissociation solution (Sigma-Aldrich) and gently scraped before being incubated with anti- CD16, HLA-DR, CD80, CD206 and CD163 antibodies (BD Pharmingen).

***In vitro* myogenesis.** MPCs were seeded on glass coverslips at 12000 cells/cm² and incubated with or without MP-conditioned medium for 3 days. In some experiments (Fig.1), MPCs were seeded on activated MPs. In other experiments (Fig.5), blocking antibodies were added at the time of addition of MP-conditioned medium. Antibody concentrations were chosen based on the ID50 according to the manufacturer and the concentration of secreted effectors by MPs, if known, from the literature: anti-TNF α 1/100 and 1/1000, anti-IL1 β 1/200, anti-TGF β 1/200, anti-IL13 1/100 (Abcam), anti-VEGF 1/200, anti-IL6 1/100, anti-TWEAK 1/100 (R&D) (11, 26-28).

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3 **Commitment into myocytes.** MPCs were seeded on glass coverslips at low density (500
4 cells/cm²). In this condition, contact between MPCs was minimal, so they could not fuse. MP-
5 conditioned medium was then added and the cells were cultured for 72h.
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9 **Myogenic fusion.** MPCs were differentiated into myocytes for 3 days. These differentiated cells
10 were seeded at high density: 50000 cells/cm², in 96 well plates. In this condition, the differentiation
11 process and migration events were prevented. MP-conditioned medium was added and cells further
12 cultured for 72h.
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16 **Immunolabellings.** Cells were labelled with anti-desmin (Ab32362 Abcam) and anti-myogenin
17 (556358 BD Pharmingen) primary antibodies, revealed by Cy3- and biotinylated-conjugated
18 secondary antibodies and streptavidin-DTAF (Jackson Immunoresearch, Vector Laboratories). Each
19 experiment was repeated at least 3 times with different primary cultures and performed in triplicate.
20 About 5-7 pictures (x20 magnification) were taken from the triplicates, in order that about 200-400
21 (low cell density) or 1000 (high cell density) cells were counted per condition per experiment.
22
23 Differentiation was measured as the number of myogenin positive nuclei divided by the total number
24 of nuclei. The number of myotubes (cells containing 2 or more nuclei) was expressed as a percentage
25 of the total cell number (Figs.1,5). Fusion index was the number of nuclei within myotubes divided by
26 the total number of nuclei (Fig.4). Myotubes were also classified according to the number of
27 myonuclei they contained and their distribution was expressed as a percentage of the total number
28 of myotubes (Fig.4).
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44 **MPC motility.** MPC were seeded in 24 well plates at 1500 cells/cm², MP-conditioned medium was
45 added and cells were imaged every 30 min for 16 h using a Zeiss Axiovert 200. Cell velocity was
46 calculated using Image J software with the Manual Tracking plug-in. About 50 cells were analysed per
47 condition per experiment.
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52 **MPC migration.** Migration was performed using two chambers Ibidi inserts (Ibidi GmbH). One
53 chamber was filled with 4500 MPCs in growth medium. The second chamber was seeded with 25000
54 MPs. MPs were activated for 3 days. The silicone walls of the device were removed and cells were
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3 cultured in advanced RPMI medium containing 0.5% FBS. The gap between the two cell types was
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5 imaged at time 0 and 24 h. The distance that each cell had moved into the gap was analysed using
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7 Image J software. About 100 cells were analysed per condition per experiment.
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10 **Human muscle samples.** The human muscle biopsy sampling study was approved by the Regional
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12 Scientific Ethical Committees of Copenhagen in Denmark (Ref: HD-2008-074) and conformed to the
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14 standards set by the Declaration of Helsinki. Volunteers gave written informed consent before
15
16 inclusion. Three young healthy untrained men (mean \pm S.D. age 25 ± 2 yrs; height 182 ± 6 cm; weight
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18 80 ± 4 kg) participated in the study. Briefly, the exercise protocol utilized 10 sets of 20 maximal
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20 voluntary lengthening contractions of the quadriceps muscles, performed on an isokinetic
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22 dynamometer (KinCom KC125AP, Chattanooga group Inc.). Maximal percutaneous electrical
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24 stimulation was delivered to the *vastus lateralis* muscle during the contraction by a handheld
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26 stimulator (Elpha II 3000, Danmeter, Biotin). Muscle biopsies were obtained from the stimulated
27
28 *vastus lateralis* on days 7 and 30 after the exercise protocol. A biopsy was also collected from the
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30 non-exercised leg on day 0 as a control. Muscle biopsies were collected under local anaesthesia (1%
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32 lidocaine) with a Bergstrom biopsy needle. After collection of the specimen, the fibres were aligned,
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34 embedded in Tissue-Tek (Sakura Finetek Europe) and frozen by immersion in isopentane, precooled
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36 by liquid nitrogen. Samples were stored at -80°C . For each biopsy, 10 serial transverse sections (10
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38 μm) were cut at -24°C using a cryostat and transferred onto SuperFrost Plus glass slides (Menzel-
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40 Gläser).
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44 **Human muscle immunolabellings.** Serial sections of human muscle were **treated for hematoxylin-**
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46 **eosin (HE) staining** or stained with antibodies raised against the following molecules: CD56 1/100
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48 (347740; Becton Dickinson), ki67 1/200 (CP249; Biocare Medical), myogenin 1/50 (F5D;
49
50 Developmental Studies Hybridoma Bank), Pax7 1/100 (MO15020; Neuromics), CD68 1/100 (ab955
51
52 Abcam), iNOS 1/25 (ab15323 Abcam), CD206 1/50 (ab8918 Abcam), CD163 1/500 (sc-20066 Santa
53
54 Cruz), Arginase 1 1/25 (sc-18355 Santa Cruz), Cox2 1/25 (ab2372 Abcam), revealed by FITC- or Cy3-
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56 conjugated secondary antibodies (Jackson Immunoresearch). Pictures were taken with a Zeiss Axio
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3 observer.Z1. The slide was scanned with a tile scan camera which automatically collects overlapping
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5 focal stacks and processes them to reconstitute the entire muscle section. To confirm coexpression
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7 of CD68 labelling with pro- or anti-inflammatory markers, pictures were taken with an sp2 confocal
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9 microscope Leica TCS SP2 AOBS with a 1 μm depth.
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11 **Scoring inflammation and myogenesis in human regenerating muscle.** Calculations were made at
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13 day 7 post-electrostimulation. Post-injury regeneration was visible at the myofibre level. Discrete
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15 "regenerating areas" – corresponding to necrotizing/regenerating myofibres – which contained
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17 either CD56^{pos}ki67^{pos} or myogenin^{pos} cells were quantified and evaluated for all the stainings. Zones
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19 containing numerous neighbouring regenerating myofibres were excluded to avoid confusion along
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21 the 10 serial sections. A total of 250 of regenerating areas was counted from 3 different biopsies. For
22
23 each regenerating area, the number of cells positive for CD56, myogenin, ki67, CD68/iNOS,
24
25 CD68/COX2, CD68/Arg1, CD206 and CD163 was evaluated.
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27

28 **Statistics.** All *in vitro* experiments were performed at least 3 times using independent MP and
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30 MPC primary cultures. *In vivo* analyses have been performed on 3 different subjects. Results are
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32 expressed as mean \pm s.e.m. Means were compared using t-test or Anova for statistical analyses. $P <$
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34 0.05 was considered significant.
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RESULTS

Human MPC cultures were used in the *in vitro* study. More than 97% of these cells expressed CD56, a canonical marker for myogenic cells in human (Suppl.Fig1). After an expansion phase during which they proliferate, the majority of human MPCs enter into terminal differentiation and express myogenin (29). A subset of 15-20% of MPCs returns to quiescence to constitute a reservoir (29, 30). Differentiation is associated with migration of the cells into "areas" where fusion takes place (31). As previously shown, human MP activation triggers specific inflammatory profiles: M1 MPs secrete high levels of TNF α and IL1 β (11). Flow cytometry analysis revealed that, as expected, M1 MPs expressed high levels of the co-stimulatory antigen CD80, M2a MPs expressed CD206 and M2c MPs expressed CD163. However, M2c MPs also expressed high levels of CD206 (Suppl.Fig1).

Polarized MPs exert their effect on MPCs through soluble factors

We checked whether the effects of activated/polarized MPs were triggered by soluble factors or whether contact between the two cell types was essential for the regulation of myogenesis. MPCs were either cocultured with MPs or treated with MP-conditioned medium for 3 days, and expression of myogenin and fusion of the cells were measured as final outcomes of *in vitro* myogenesis. Fig.1 shows that M1 MPs slightly decreased cell fusion ($p < 0.05$) while M2a MPs and M2c MPs stimulated both the expression of myogenin (Fig.1A,C) and myotube formation (both $p < 0.05$) (Fig.1B,C). Myogenin expression (Fig.1A,C) and myotube formation (Fig.1B,C) by MPCs were similar in coculture and with MP-conditioned medium, indicating that MPs mainly exerted their effects on myogenesis through soluble factors. The following experiments were then performed with MP-conditioned media.

M2a MPs and M2c MPs stimulate MPC commitment into myocytes

Commitment into myocytes, which is the entry into terminal differentiation, was analysed through the expression of myogenin in low-density cell cultures, to prevent migration/fusion of the cells. M1 MP-conditioned medium did not alter the expression of myogenin by MPCs while M2a MP- and M2c MP-conditioned media strongly increased myogenin expression, by 2.7 and 4.2 fold,

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3 respectively ($p < 0.05$) (Fig.2). These data show that M2a MPs and M2c MPs stimulate the
4
5 differentiation of MPCs into myocytes.
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7 **M1 MPs stimulate MPC motility**

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9 Fig.3A shows that a M1 MP-conditioned medium did not alter MPC motility compared to
10
11 untreated cells. However, the speed of the cells was lower under M2a MP- and M2c MP-conditioned
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13 media (22 to 24% less, $p < 0.05$). The median velocity was lower in cells treated with M2a MP- and
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15 M2c MP-conditioned medium (Fig.3B). Mean velocities of the cell populations above and below the
16
17 median were also decreased (data not shown), indicating a decreased motility of the entire MPC
18
19 population and not of a subset of MPCs.
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21

22 **M1 MPs do not attract MPCs while they migrate towards MPCs**

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24 Cell migration towards a gradient/other cell type was analysed in a double chamber silicone
25
26 device (Fig.3C). Under M2a MPs and M2c MPs-conditioned media, MPCs displayed the same degree
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28 of migration as towards other MPCs, while their migration toward M1 MPs was much lower (-45%,
29
30 $p < 0.05$) (Fig.3D). Conversely, when examining MP migration, M1 MPs were strongly attracted by
31
32 MPCs, compared to M2a MPs and M2c MPs (Fig.3E) (2.5 and 2 fold, respectively, $p < 0.05$).
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35 **M1 MPs inhibit MPC fusion**

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37 Already differentiated myocytes (preventing the differentiation parameter) were plated at high
38
39 density (preventing the migration parameter) in MP-conditioned media to evaluate fusion after
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41 desmin immunolabelling (Fig. 4A). Fusion index was strongly decreased in MPCs cultured with M1
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43 MP-conditioned medium (63%, $p < 0.05$) (Fig.4B). The myotube distribution was significantly different
44
45 under all MP conditions tested compared to MPC alone ($p < 0.05$). In particular, M1 MP-conditioned
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47 medium inhibited the formation of large myotubes (75% inhibition for myotubes containing over 10
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49 nuclei, $p < 0.05$) while the formation of small myotubes (3 to 5 nuclei) was unaffected (Fig.4C). As a
50
51 whole, the fusion index of MPCs was not affected by M2a MP- and M2c MP-conditioned media
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53 (Fig.4B). However, the distribution of myotubes was significantly different from that of MPCs
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55 cultured alone (both $p < 0.05$). M2c MP-conditioned medium stimulated the formation of large
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3 myotubes (68% of increase for myotubes over 10 nuclei, $p < 0.05$) (Fig.4C). Similarly, M2a MP-
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5 conditioned medium stimulated the formation of large myotubes (52%, $p < 0.05$) (Fig.4C). In both
6
7 cases, the formation of small myotubes was not altered. These results suggest that activated MPs
8
9 differentially control the formation of mature myotubes.
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11 **Effectors secreted by activated MPs regulate in vitro myogenesis**

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14 Some MP-released effectors are known or believed to have an impact on MPCs. However, the role
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16 of these effectors in the regulation of myogenesis through secretion by MPs has not been
17
18 established. We tested the involvement of a series of 7 molecules in the regulation of myogenesis by
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20 MPs through the use of blocking antibodies. Blocking IL6 (Fig.5A) increased myotube formation (by
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22 38%, $p < 0.05$) only in MPCs cultured with M1 MP-conditioned medium. Blocking IL1 β similarly
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24 increased myotube formation only in MPCs cultured with M1 MP-conditioned medium (+49%,
25
26 $p < 0.05$) (Fig.5B). The blockade of VEGF (Fig.5C) increased myotube formation in MPCs treated with
27
28 all MP-conditioned media, although the effect was more pronounced for M1 MPs (+58%, +20% and
29
30 +26% for M1 MPs, M2a MPs and M2c MPs, respectively, all $p < 0.05$). As TNF α is known to have
31
32 differential effects on myogenesis depending on its concentration (32), we used two dilutions of
33
34 blocking antibodies (Fig.5D). At a low dilution, anti-TNF α decreased myotube formation in M2a MP-
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36 and M2c MP-conditioned medium treated MPCs (30 and 31%, $p < 0.05$) while it had no effect on M1
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38 MP-conditioned medium treated cells (Fig.5D, white bars). At a higher concentration, anti-TNF α still
39
40 decreased myotube formation in M2a MP- and M2c MP-conditioned medium treated cells - to the
41
42 same extent ($p < 0.05$) - and increased myotube formation of MPCs incubated with M1 MP-
43
44 conditioned medium (+27% of fusion, $p < 0.05$) (Fig.5D, gray bars). Blockade of the TNF α -family
45
46 member TWEAK did not affect the formation of myotubes by MPCs in all conditions (Fig.5E). Anti-
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48 IL13 antibodies induced an increase of myotube formation in MPCs cultured only in the presence of
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50 M1 MP-conditioned medium (+50%, $p < 0.05$) (Fig.5F). Adding blocking anti-TGF β antibodies
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52 significantly reduced the formation of myotubes in MPC cultures treated with M2a MP- and M2c MP-
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54 conditioned media (-26 and -27%, respectively, $p < 0.05$) (Fig.5G). Except for the TWEAK antibody,
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3 none of these blocking antibodies affected the formation of myotubes by MPCs cultured alone
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5 (Fig.5H). Although it is known that MPCs synthesize some of these cytokines and growth factors (33,
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7 34), MPs secrete much higher levels of these compounds (11, 26). These results show that effectors
8
9 differentially secreted by activated MPs regulate myotube formation according to the state of
10
11 activation of MPs.
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14 **During *in vivo* human muscle regeneration, MPs sequentially express pro and anti-inflammatory**
15
16 **markers and are associated with proliferating and differentiating MPCs, respectively**

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18 To explore inflammation during *in vivo* human muscle regeneration, we used a protocol
19
20 combining lengthening contractions and electrostimulation (20). This resulted in myofibre damage,
21
22 and the kinetics of tissue repair can be followed during the ensuing muscle regeneration. Suppl.Fig.2
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24 shows that in normal untreated human muscle (day 0), CD56 positive cells (CD56^{pos}) were observed
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26 throughout the muscle, at the normal place of satellite cells, while no staining was observed for the
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28 embryonic form of myosin heavy chain, which is a characteristic of regenerated/regenerating
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30 myofibres (35). On day 7 after the damage induction, the myogenic marker CD56 stained numerous
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32 areas while embryonic myosin heavy chain stained several regenerating myofibres (Suppl.Fig.2, day
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34 7). These areas associated with CD56^{pos} cells and young new myofibres were observed in regions of
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36 damaged myofibres and where regeneration took place. These regions, which we called regenerating
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38 areas, were distributed throughout the muscle biopsy cross section (Suppl.Fig.2, day 7). On day 30
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40 after damage, the muscle had recovered its normal histology, except for very rare myofibres with a
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42 centrally located myonucleus (Suppl.Fig.2, day 30). That the dynamic processes of damage and
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44 regeneration can be followed so clearly in this model makes it suitable for studying the relationship
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46 between MP activation and myogenesis *in vivo*.
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51 To assess MP activation *in vivo*, we analysed on serial sections the expression of various pro and
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53 anti-inflammatory markers, together with myogenic markers, during normal human skeletal muscle
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55 regeneration. Pax7 is a transcription factor expressed by quiescent and cycling, but not
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57 differentiating, MPCs. CD56 is expressed by quiescent, cycling, differentiating MPCs and young
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3 regenerated myofibres. Myogenin is a transcription factor only expressed by differentiating
4 myogenic cells. In human, CD68 is believed to widely label MPs while expression of CD206 and CD163
5 has been associated with anti-inflammatory phenotypes. When expressed by MPs, iNOS and
6 cyclooxygenase 2 (COX2) are pro-inflammatory markers while Arg1 is expressed by M2a MPs (14, 15).
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11 In normal muscle at steady state, almost no CD56^{pos} cells were ki67^{pos} indicating that they were
12 quiescent (Fig.6, d0), as previously reported (18). As expected, no labelling for myogenin was
13 observed (data not shown). Few cells were observed in the interstitial space that were positive for
14 CD68, CD206 or CD163; no positivity was observed for iNOS, COX2 or Arg1 (Fig.6, d0). At day 7 after
15 damage, numerous regenerating areas were present, containing proliferating cells (ki67^{pos}CD56^{pos})
16 and/or differentiating cells (myogenin^{pos}) (Fig.6, d7, Fig.7). The vast majority (over 80%) of the
17 regenerating areas was associated with MPs that were positive for pro- and for anti-inflammatory
18 markers. Less than 20% of the regenerating areas were associated with only one type of MPs: 17%
19 contained MPs expressing only anti-inflammatory markers while 0.4% to 2% was associated with only
20 pro-inflammatory MPs (when considering 3 and 2 markers, respectively). This suggests that MPs
21 expressing pro- and anti-inflammatory markers coexisted in the same regenerating areas. To analyse
22 whether early and later regenerating areas were preferentially associated with different types of
23 MPs, they were divided according to the absence (about 28% of the total regenerating areas) or the
24 presence (about 72%) of myogenin^{pos} cells, independently of the number of myogenin^{pos} cells. The
25 number of MPs per myofibre did not differ between myogenin^{pos}- and myogenin^{neg}- containing
26 regenerating areas. An MP/MPC ratio of 2.5 ± 0.7 was found in regenerating areas, the median being
27 1.8 (the absolute range was from 0 to 12 MPs/area). When the regenerating areas were divided
28 according to the presence or absence of myogenin^{pos} cells, MP inflammatory markers segregated
29 (Fig.6, d7, Fig.7). Pro-inflammatory markers such as iNOS and COX2 were expressed by more MPs in
30 regenerating areas containing only myogenin^{neg} cells compared to those containing at least one
31 myogenin^{pos} MPC: 51.3 vs. 22.2 and 44.6 vs. 18.6%, respectively ($p < 0.05$) (Fig.6, d7, Fig.7).
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33 Conversely, Arg1-expressing MPs were more numerous in regenerating areas containing myogenin^{pos}
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3 MPCs, compared to those without differentiating MPCs: 52.8 vs. 30.9% ($p < 0.05$) (Fig.6, d7, Fig.7). The
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5 actual expression of iNOS, COX2 and Arg1 by CD68 MPs was assessed by confocal microscopy (Fig.7).
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7 Immunostainings showed that most of the regenerating areas contained MPs expressing CD206 and
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9 CD163. However, regenerating areas containing myogenin^{pos} MPCs were associated with 1.8 fold
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11 more CD206^{pos} MPs and CD163^{pos} MPs ($p < 0.05$) (Fig.6, d7, Fig.7). It has been shown that murine
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13 myogenic cells express CD206 (36). Colabelling of regenerating human muscle with CD68 and CD206
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15 showed that almost all CD206^{pos} cells observed in the regenerating areas were costained with CD68
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17 indicating their macrophagic origin (Suppl. Fig.3). Thirty days after damage, no cycling or
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19 differentiating cells were observed (Fig.6, d30). With regard to the untreated muscle at d0, few MPs
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21 expressing CD68, CD206 or CD163 were observed between the myofibres, and they did not express
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23 iNOS or COX2. Some MPs expressed Arg1 (Fig.6, d30). Taken together, these data reveal that
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25 different MP subsets are preferentially associated with areas containing proliferating or
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27 differentiating MPCs.
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DISCUSSION

The present study shows for the first time in human that MPs differentially act on MPCs depending on their activation/polarization state. They exert these trophic roles through the secretion of a series of cytokines and growth factors, thereby controlling the fate of the muscle progenitors at each step of *in vitro* myogenesis. We also show for the first time in human that these *in vitro* activated MPs correspond to MP subsets *in vivo*. Indeed, during human muscle regeneration, regenerating areas are first preferentially associated with MPs bearing pro-inflammatory markers. Then, at the time of myogenic differentiation, MPs expressing anti-inflammatory markers are preferentially found in the regenerating areas.

Trophic activities for MPs have recently emerged in various contexts of tissue development, morphogenesis and angiogenesis (37, 38). In normal adult tissues, recent advances have been made in identifying supportive roles for MPs in stem cell homeostasis. MPs promote erythroblast survival and differentiation through very late activation antigen 4 (VLA-4) and CD163 (39-41). MPs support stem cell homeostasis in the bone marrow, and in intestinal and mammary epithelia (42-45). In the nervous system, microglia rescues neurogenesis in neural stem cells by the secretion of neurogenic factors (46). MPs deliver anti-inflammatory cues beneficial to spinal cord recovery (47). They secrete TGF β which promotes oligodendrocyte differentiation and myelinogenesis (48). Microglia activated by pro-inflammatory cues block neural progenitor differentiation while IL-4-activated microglia stimulates the formation of oligodendrogenesis, possibly through the production of IGF-1 (16, 49). In skeletal muscle, we have shown *in vitro* that MPs rescue MPCs from apoptosis and stimulate their growth and proliferation through a panel of secreted and membrane-bound effectors (25, 33).

In our *in vitro* coculture system, based on a 1:3 MP:MPC ratio, we here showed that MPs mainly exerted their effects on myogenesis through the secretion of soluble factors. We have previously shown *in vitro* that M1 MPs stimulate MPC proliferation while M2a MPs and M2c MPs do not (11). Here we explored each step of *in vitro* myogenesis using an experimental setup that allows for analysis of each step independently of the others: commitment into myocyte, migration and fusion

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3 (36). During *in vitro* myogenesis, MPCs migrate to the injured area and then toward each other in
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5 order to form myotubes. Differentiating and differentiated MPCs express a wide range of chemokine
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7 receptors that bind to chemokines in a redundant way (50). Activated MPs have been shown to
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9 secrete specific panels of chemokines (14, 15) and likely other attracting molecules. Chemotaxis is a
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11 global integrated cell response to signalling by several individual receptors. Therefore, it is difficult to
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13 decipher which signals secreted by MPs govern MPC motility and chemotaxis. However, our results
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15 present a new property of human MPs in the regulation of progenitor motility. Anti- and M2a MPs
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17 were observed to decrease the motile properties of MPCs and were better attractants for MPC
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19 directional migration. We have previously shown *in vitro* that MPCs attract circulating monocytes
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21 (33). It is therefore tempting to propose, knowing that M1 MPs are the first to enter the damaged
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23 muscle in mouse (11), that monocyte-derived M1 MPs have a great migratory capacity toward MPCs
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25 at the beginning of muscle regeneration. It can be hypothesised that these M1 MPs would maintain
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27 MPC migratory capacities thus preventing fusion from occurring too early, a high migratory speed
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29 being detrimental for good fusion (51). M2a MPs and M2c MPs would further attract MPCs, decrease
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31 their motility, thus favouring contact between cells, and deliver pro-differentiating cues. Further
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33 analyses are required to confirm these hypotheses.

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37 Myogenic commitment into myocytes is the expression of the myogenic programme after cell
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39 cycle withdrawal and precedes fusion, which is the last step of *in vitro* myogenesis. In all the
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41 functional assays we tested *in vitro*, M2a MPs and M2c MPs displayed opposing effects to those of
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43 M1 MPs. M1 MPs inhibited MPC fusion, particularly the formation of large myotubes while M2a MPs
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45 and M2c MPs strongly stimulated the commitment of MPCs into myocytes and promoted the
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47 formation of large myotubes. Our results suggest that the formation of nascent myotubes is not
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49 controlled by MP-conditioned media. Most of the molecular systems involved in pre-fusion migration
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51 and myocyte fusion are transmembrane proteins and a few soluble effectors have been identified
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53 (52). Among them IL4 is involved in the formation of mature myotubes in mouse (53). IL4 production
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3 by lung MPs has been associated with the presence of M2a MPs (54, 55). IL4 may, therefore, be one
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5 of the effectors of the stimulation of fusion by M2a MPs and M2c MPs.
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8 Few molecules that can account for the effects of MPs on muscle precursors are known. Recent
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10 work in mouse showed that G-CSF is beneficial to skeletal muscle regeneration by stimulating MPC
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12 proliferation (56). This study did not explore which cells were the source of G-CSF. However, MPs
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14 secrete G-CSF, particularly M1 MPs, and G-CSF has been shown to be associated with inflammation
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16 and activation by Th-1 cytokines (57, 58). Exploring the role of CCR2 expressed by MPs, Lu et al. (59)
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18 have shown that MPs are likely to be the main source of IGF-1 in regenerating muscle; they further
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20 showed that the intramuscular Ly6C^{neg} MP subset, which presents an anti-inflammatory profile,
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22 expresses high levels of IGF-1, which promotes muscle repair. Here we show the involvement of
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24 several cytokines and growth factors in the regulation of myogenesis by activated MPs. The pro-
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26 inflammatory cytokines IL6 and IL1 β are mainly secreted by M1 MPs compared to M2a MPs and M2c
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28 MPs (11, 26), and blocking IL6 or IL1 β affected only this MP subtype by increasing the formation of
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30 myotubes. Accordingly, IL6 has a proliferative effect on MPCs (60, 61), while IL1 β has been shown to
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32 induce IL6 production (62) and to reduce myogenic differentiation (63, 64). TNF α is also produced in
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34 greater amounts by M1 MPs (11, 26). The effects of TNF α inhibition depended on the concentration
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36 of antibodies used, in accordance with the dose-dependent effect of TNF α on muscle cell
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38 proliferation (32). At low concentrations, TNF α stimulates myogenic differentiation (32). Accordingly,
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40 decreased myotube formation was observed upon TNF α inhibition of M2a MPs and M2c MP-
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42 conditioned media, which contain low levels of TNF α . Conversely, high levels of TNF α stimulate
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44 myogenic cell proliferation (32) and M1 MPs increased myotube formation when the cytokine was
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46 completely inhibited by blocking antibodies. VEGF stimulates MPC growth (65) and differentiation
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48 (66, 67). M1 MPs produce more VEGF than M2a MPs and M2c MPs (26). However blocking VEGF
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50 induced an increase in myotube formation in all conditions, suggesting that the effect of VEGF
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52 on MPCs is mainly on cell growth. IL13 regulates the secondary recruitment of reserve cells into
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54 myotubes, particularly on IGF-1 stimulated human cells (68) and increases fusion into myotubes (69).
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3 Unexpectedly, blocking IL13 induced increased myotube formation in MPCs cultured with M1 MP-
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5 conditioned medium, suggesting that IL13 participates in the non-differentiating property of M1
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7 MPs. TGF β is expressed to a greater extent by M2a MPs and M2c MPs (11). TGF β inhibits cell
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9 myogenesis at the time of differentiation (70-72) while it decreases cell growth and activates
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11 myogenesis when added to proliferating myoblasts (73). Here, TGF β participated in the pro-
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13 differentiating cues delivered by M2a MPs and M2c MPs to MPCs since its blockade reduced
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15 myotube formation in these two conditions. These results identify an initial series of effectors
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17 differentially secreted by activated MPs which are involved in the regulation of *in vitro* myogenesis
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19 through their effects on MPCs.
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23 It is likely that the MP activation states used for *in vitro* studies are not fully representative of MP
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25 subsets found *in vivo* (14, 74). Therefore, we followed, to our knowledge for the first time in human,
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27 the regeneration of skeletal muscle in muscle biopsies obtained from healthy young men who had
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29 performed maximal lengthening contractions of the quadriceps muscles – a model that has
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31 previously been used in many human studies of muscle damage (21, 75-77). However, not all studies
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33 using this model have observed damage in muscle biopsies collected in the days after exercise (20,
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35 78). Furthermore, in a previous study (20), electrical stimulation was reported to result in greater
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37 muscle damage than voluntary contractions. In light of this, and in order to ensure initiation of
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39 damage and regeneration processes, we increased the load delivered to the muscle by electrically
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41 stimulating the *vastus lateralis* muscle while the subject was contracting the muscle voluntarily.
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43 Analysis of muscle biopsies collected before and after this stimulus to the muscle enables distinct
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45 muscle regenerative events to be followed clearly in humans *in vivo*.
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49 We investigated the expression of a series of MP markers, together with myogenic markers,
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51 during the time course of regeneration in human muscle. The first lesson from this study is that MP
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53 subsets cannot definitively be identified by a sole marker, and that multiple labellings are required to
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55 attempt to define MP subsets *in vivo*. We analysed the expression of iNOS, COX2 and Arg1 by CD68^{pos}
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57 cells. Colabelling and analysis at the single cell level are necessary since many other cells, including
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3 endothelial cells, interstitial cells and myogenic cells also express these molecules (79-81). CD206 and
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5 CD163 are considered as markers for anti-inflammatory subsets (see Suppl. Fig.1). Careful
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7 examination of the immunostainings showed that several levels of fluorescence brightness were
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9 observed for these markers (data not shown), in accordance with their increased expression by anti-
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11 inflammatory MPs (82, 83). The second lesson from this series of experiments is conceptual. Our
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13 results clearly show that, according to the 6 markers used here to identify MPs *in vivo*, several
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15 subsets of MPs are present in the same areas at the same time. These results argue against the
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17 overly simplistic view that a biological event is associated with one type of MPs. Also found in the
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19 same regenerating areas were cycling myogenic cells (CD56^{pos} or Pax7^{pos} ki67^{pos}) together with
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21 differentiating cells (myogenin^{pos}). Therefore, although general conclusions regarding the time course
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23 of some major biological events can be drawn, one must keep in mind that these events spatially and
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25 temporally overlap.
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29 We present here the first time course of analysis of inflammation during human skeletal muscle
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31 regeneration. Our results confirm that both in normal muscle and once regeneration is completed,
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33 almost no MP is present in the muscle parenchyma. Regenerating areas are associated with MPs, the
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35 vast majority (over 80%) being associated with MPs expressing pro and anti-inflammatory markers.
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37 However, despite this complex situation, our analysis depicts a time course of inflammation during
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39 human skeletal muscle repair that resembles what has been observed in mouse (11, 84). When
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41 analysing regenerating areas on the basis of whether they did or did not contain myogenin^{pos} cells, a
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43 segregation in MP distribution was observed. Regenerating areas that contained only myogenin^{neg}
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45 cells were preferentially associated with MPs expressing pro-inflammatory markers. Conversely,
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47 regenerating areas containing at least one myogenin^{pos} cell were preferentially associated with MPs
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49 expressing anti-inflammatory markers. The evidence of such localisation indicates privileged
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51 interactions between the two cell types and argues for a synchronous regulation of adult myogenesis
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53 and inflammation during muscle repair. The distinction between M2a MPs and M2c MPs during
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55 muscle regeneration is not yet clear. It is likely that the so-called "anti-inflammatory" subset(s)
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3 include(s) some MPs that stimulate matrix remodelling and deposition (thus resembling M2a MPs).
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5 Perdiguero et al. (84) showed that 3 MP subsets are successively present during muscle repair:
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7 inflammatory MPs, anti-inflammatory MPs, and a deactivated MP subset associated with the last
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9 steps of muscle repair. "Canonical" M2a MPs have been identified under very specific conditions, *i.e.*
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11 parasitic challenge; it is therefore not known whether this type of MPs may be also - even transiently
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13 - present during normal tissue repair.
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16 17 18 **CONCLUSION**

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20 Combining our *in vivo* and *in vitro* data indicates that human skeletal muscle regeneration is
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22 sequentially associated with several MP subsets. Furthermore, we provide evidence of new functions
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24 of MPs in the coordinated regulation of MPC fate during muscle repair. *In vivo* analyses in a model of
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26 skeletal muscle regeneration in the healthy adult revealed that regenerating areas at the time of
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28 MPC proliferation are associated with M1 MPs, while *in vitro* experiments accordingly showed that
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30 M1 MPs migrate towards MPCs, stimulate their proliferation and prevent premature differentiation
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32 through the secretion of IL-6, IL1- β , high TNF α , VEGF and IL-13. *In vivo* immunostainings showed that
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34 later, at the time of terminal differentiation, regenerating areas are associated with M2c MPs and/or
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36 M2a MPs. In line with this, *in vitro* experiments showed that M2a MPs and M2c MPs attract MPCs,
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38 and promote *in vitro* myogenesis by stimulating their commitment into myocytes and the formation
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40 of mature myotubes, through the secretion of TGF β and low TNF α . Thus, MPs orchestrate an
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42 effective skeletal muscle repair process by finely tuning the sequential steps of adult myogenesis.
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44 These results strengthen the emerging concept that inflammation regulates stem cell homeostasis.
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FIGURE LEGENDS**Figure 1. MPs exert their effect on *in vitro* myogenesis through the delivery of soluble factors.**

MPCs were cocultured with M1 MPs, M2a MPs or M2c MPs (black bars) or were cultured with or without conditioned medium from the same activated MPs (white bars) and grown for 3 days. MPCs were immunolabelled for desmin and myogenin and the number of desmin^{pos} myogenin^{pos} (A) and of myotubes (B) was calculated as a percentage of desmin^{pos} cells. Results are means \pm s.e.m. of 3 independent experiments. (C) Examples of desmin (red) and myogenin (green) labellings in MPCs cocultured with MPs or treated with MP-conditioned-media for 3 days. Blue = Hoechst. Bar=50 μ m. *: p<0.05 vs. M1 MPs, #: p<0.05 vs. None.

Figure 2. Effects of MPs on MPC commitment into myocytes. MPCs were cultured at low density with or without conditioned medium from M1 MPs, M2a MPs or M2c MPs for 3 days. (A) MPCs were immunolabelled for desmin (red) and myogenin (green) (blue=Hoechst). Bar=50 μ m. (B) The number of myogenin^{pos} nuclei is expressed as a percentage of total nuclei. Results are means \pm s.e.m of 3 independent experiments. *: p<0.05 vs. None and vs. M1 MPs.

Figure 3. Effects of MPs on MPC motility and migration. (A,B): MPCs were cultured with or without conditioned medium from M1 MPs, M2a MPs or M2c MPs and observed for 16 h. Individual cell trackings were followed with Image J software and the velocity for each cell was calculated. (A) Mean velocity of whole MPC population is expressed in μ m/min. (B) Boxes represent the velocity of total cell population (min to max), with the median. Results are means \pm s.e.m of 3 independent experiments. *: p<0.05 vs. None and vs. M1 MPs. (C-E): MPCs and MPs (M1 MPs, M2a MPs or M2c MPs) were cultured in two-chamber devices (C, here as an example, MPCs and M2a MPs) so the two cell types faced each other, allowing migration towards each other over a 24 h period. Pictures were taken at 0 and 24 h. A virtual line was drawn on the picture at the cell front at time 0 and copied to the picture at time 24 h. Individual cell migration was calculated from this line. Individual cell migration of MPCs towards activated MPs (D) and of MPs towards MPCs (E) is expressed in μ m.

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3 Results are means \pm s.e.m of 4 independent experiments. *: $p < 0.05$ vs. MPCs in D and vs. M1 MPs in
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7 **Figure 4. Effects of MPs on MPC fusion.** Differentiated MPCs (myocytes) were cultured at high
8 density with or without conditioned medium from M1 MPs, M2a MPs or M2c MPs for 3 days. (A)
9 MPCs were immunolabelled for desmin (red) (blue=Hoechst). Bar=50 μ m. Myotubes were counted
10 and classified according to their number of nuclei. (B) Fusion index is expressed in %. (C) Each class of
11 myotubes is expressed as percentage of total myotubes. Results are means \pm s.e.m of 3 independent
12 experiments. *: $p < 0.05$ vs. None.
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20 **Figure 5. MPs exert their effect through a series of secreted effectors.** MPCs were cultured with
21 or without conditioned medium from with M1 MPs, M2a MPs or M2c MPs in the absence (black
22 bars) or the presence (white bars) of blocking antibodies directed against IL-6 (A), IL-1 β (B), VEGF (C),
23 TNF α (D, antibody was used at 1/1000 (white bars) and 1/100 (gray bars), TWEAK (E), IL-13 (F), and
24 TGF β (G). Three days later, MPCs were immunolabelled for desmin and the number of myotubes was
25 calculated as a percentage of desmin^{pos} cells. (H) MPCs were incubated without MP-conditioned
26 medium in the presence of the blocking antibodies. Results are means \pm s.e.m of at least 3
27 independent experiments. *: $p < 0.05$ vs. control (medium without antibodies, black bars).
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38 **Figure 6. Kinetics of myogenic and MP markers during human skeletal muscle regeneration.**
39 Serial sections from control (d0) and electrostimulated human muscle at day 7 and day 30 after
40 electrostimulation were stained for various myogenic (CD56, myogenin) and MP (CD68, CD206,
41 CD163) markers. ki67 labels cycling cells, laminin labels the basal membrane, COX2, iNOS and Arg1
42 expression is associated with various states of MP activation. Arrows indicate regenerating areas that
43 do not contain myogenin^{pos} cells; arrowheads indicate regenerating areas that contain at least one
44 myogenin^{pos} cell. Bar=50 μ m. Pictures are representative of 3 independent experiments.
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52 **Figure 7. Association of differentially activated MPs with regenerating areas during skeletal**
53 **muscle regeneration in human.** Serial sections of human muscle on day 7 after electrostimulation
54 were stained for various myogenic (Pax7, CD56, myogenin) and MP (CD68, CD206, CD163) markers.
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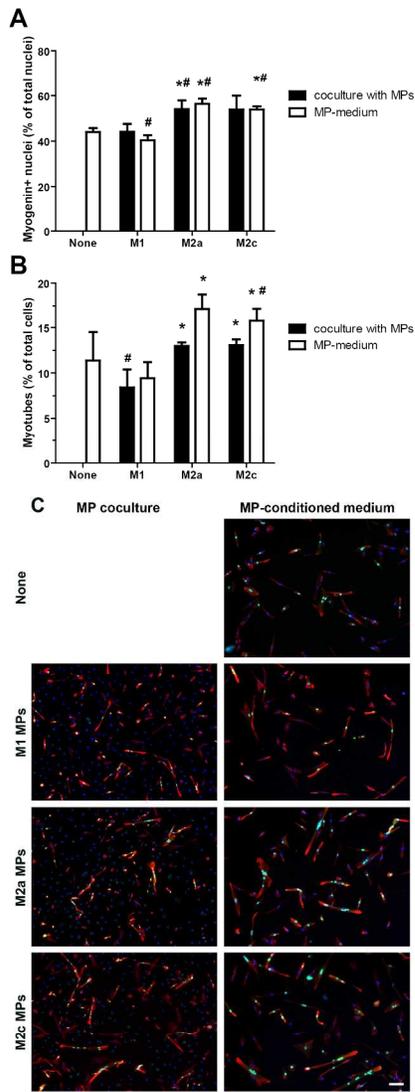
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3 ki67 labels cycling cells; laminin labels the basal membrane; COX2, iNOS and Arg1 expression is
4 associated with various states of MP activation. Arrows indicate regenerating areas that do not
5 contain myogenin^{pos} cells; arrowheads indicate regenerating areas that contain at least one
6 myogenin^{pos} cell. Bottom panel: confocal analysis was performed to assess colocalisation of COX2,
7 iNOS and Arg1 in CD68^{pos} cells. Bar=50 μ m. Histograms on the right panel display the percentage of
8 iNOS^{pos} (A), COX2^{pos} (B), Arg1^{pos} (C) CD68^{pos} cells in regenerating areas containing (w myog+) or not
9 containing (w/o myog+) myogenin^{pos} cells. D and E show the number of CD206^{pos} (D) and CD163^{pos} (E)
10 cells in regenerating areas containing (w myog+) or not containing (w/o myog+) myogenin^{pos} cells.
11 Results are means \pm s.e.m of 3 independent experiments.
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23 **Supplemental Figure 1. Flow cytometry analysis of MPCs and MPs.** (A) Primary human MPCs
24 were cultured and sorted before passage 1 with anti-CD56 magnetic beads and analysed by flow
25 cytometry for CD56 expression (B). Human MPs were activated as M1 MPs, M2a MPs or M2c MPs
26 and analysed for their expression of pan-macrophagic markers (CD14, HLA-DR) and markers
27 associated with the pro-inflammatory state (CD80), alternative activation state (CD206) and anti-
28 inflammatory state (CD163). Results are means \pm s.e.m of 3 independent experiments.
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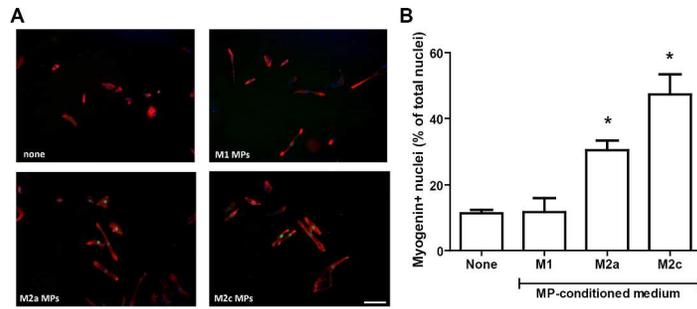
35 **Supplemental Figure 2. Model of *in vivo* human muscle regeneration.** Vastus lateralis muscle was
36 subjected to voluntary lengthening contractions and electrostimulation. The contralateral leg was
37 biopsied at day 0. Then the damaged leg was biopsied at day 7 and 30 after damage induction.
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59 **Supplemental Figure 3. *In vivo* costaining of CD68 and CD206.** Sections from human muscle day 7
60 after electrostimulation were stained for CD68 (green) and CD206 (red) markers. Most of the
CD206^{pos} cells were also labelled for CD68, indicating that they were macrophages. Few cells outside
the regenerating area were positive for CD206 alone and were likely myogenic or satellite cells
(arrows). Bar=50 μ m.

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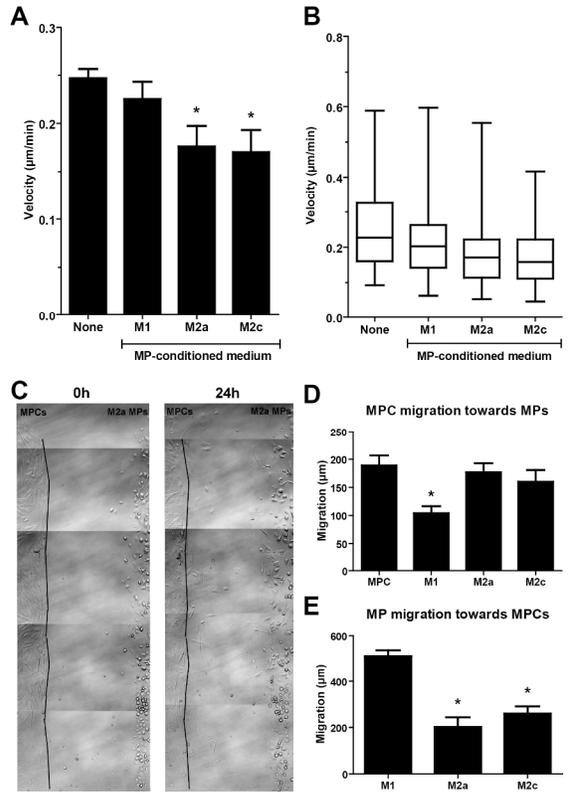


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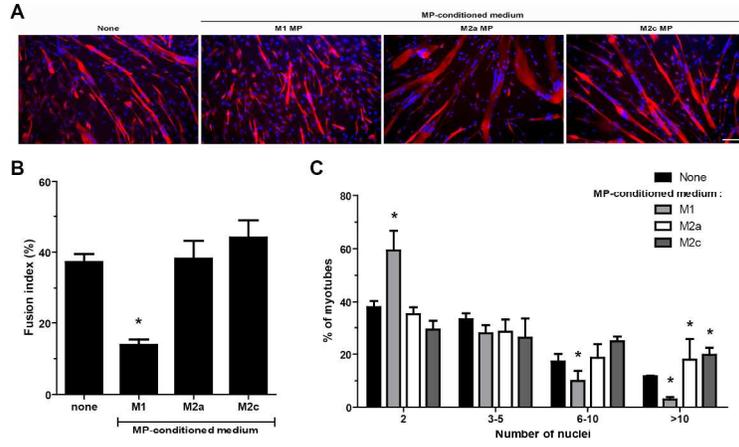


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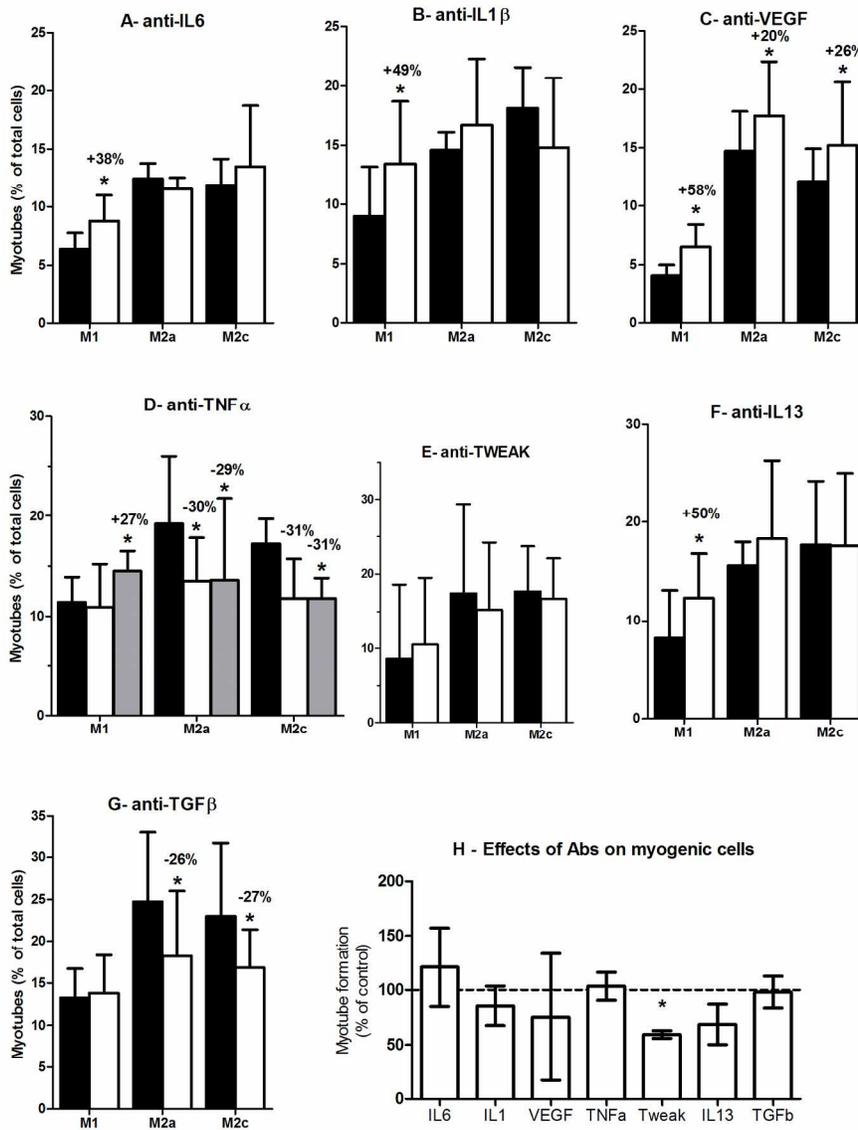


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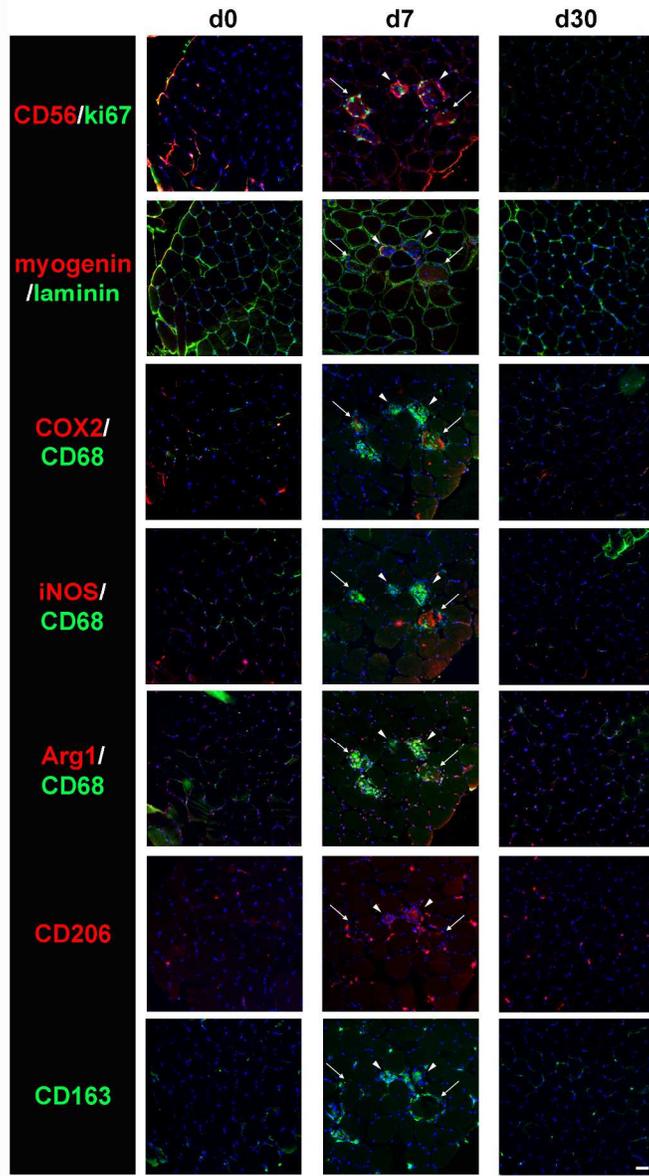
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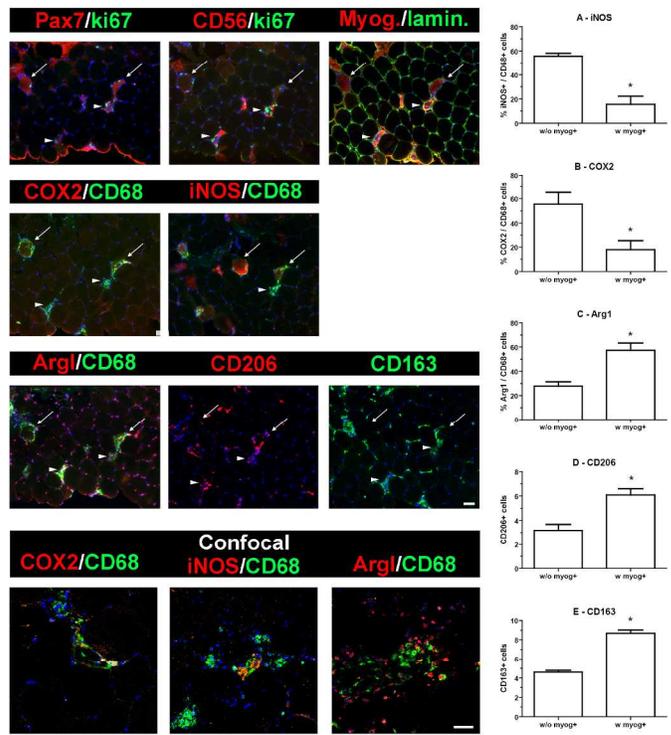
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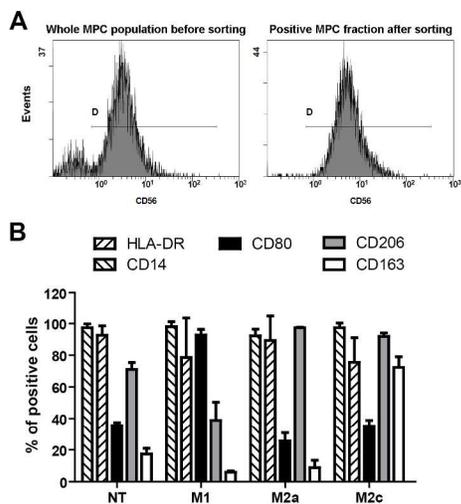
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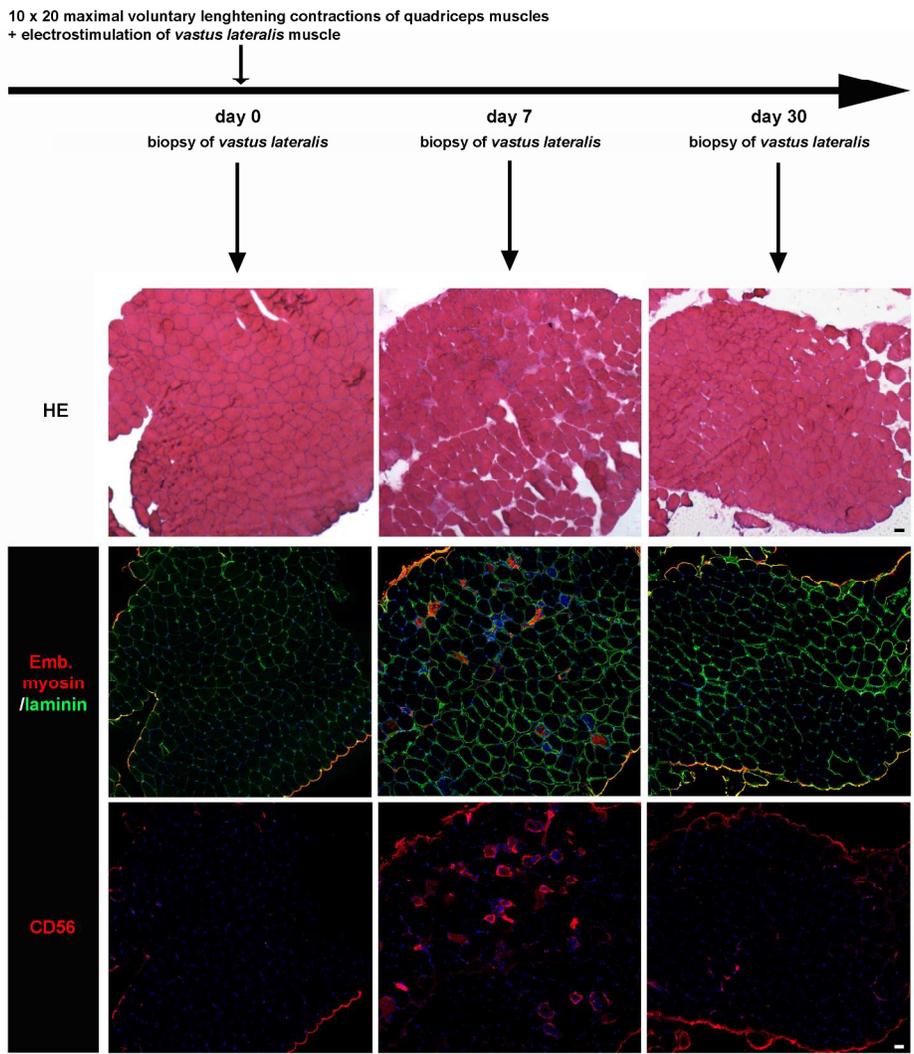
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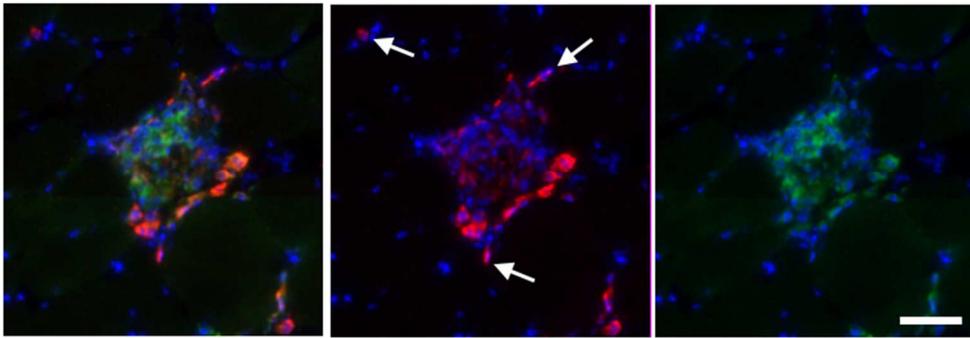


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