

## Effects of Macrophage Conditioned-Medium on Murine and Human Muscle Cells: Analysis of Proliferation, Differentiation, and Fusion

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### Abstract

Skeletal muscle is a highly plastic tissue, which is able to regenerate after an injury. Effective and complete regeneration requires interactions between myogenic precursor cells and several cell types such as macrophages. Bone marrow derived macrophages in mouse and monocyte-derived macrophages in human are useful tools to obtain macrophage populations that may be specifically activated/polarized in vitro (e.g., pro-inflammatory, anti-inflammatory, and alternatively activated macrophages). In vitro, human or murine primary myogenic cells recapitulate the adult myogenesis program through proliferation, myogenic differentiation, and fusion. Macrophages being highly secreting cells, they act on various biological processes including adult myogenesis. Here, we present protocols to analyze in vitro the effect of macrophage-secreted factors on muscle cell proliferation or differentiation in both mouse and human.

**Key words** Macrophages, Muscle precursor cells, Proliferation, Differentiation, Myogenesis, Human, Mouse, Conditioned media

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## 1 Introduction

Macrophages are immune cells essential to skeletal muscle regeneration [1]. During this process, they adopt distinct and sequential phenotypes [1–3]. Soon after injury, circulating blood monocytes infiltrate the damaged muscle and differentiate into inflammatory macrophages. These macrophages stimulate the proliferation of muscle precursor cells (MPCs) through the secretion of pro-inflammatory molecules such as TNF $\alpha$ , IL-6, and IL-1 $\beta$ . Upon phagocytosis of muscle debris, inflammatory macrophages skew their phenotype to an anti-inflammatory profile, which sustains the differentiation and fusion of MPCs and the growth of myofibers through the secretion of anti-inflammatory effectors (e.g., IL-10, TGF $\beta$ ) [1–2]. Thus, depending on their

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phenotype, macrophages support and act on the sequential phases of muscle regeneration. This sequence of inflammatory then anti-inflammatory macrophages has been also evidenced in human regenerating muscle, although both inflammatory types of macrophages may be present in the same regenerating muscle areas at the same time [2]. In vitro, mouse Bone Marrow Derived Macrophages (BMDM) [4] and monocyte-derived macrophages in human [1] have been shown to be useful to characterize macrophage functions in well-defined inflammatory conditions. As macrophages are highly secreting cells, culture of MPCs with conditioned media (CM) of macrophages is an efficient way to analyze the effects of macrophage populations on the different steps of myogenesis (proliferation, differentiation, and fusion). Here are presented the conditions that have been already used to stimulate macrophages in distinct inflammatory phenotypes: pro-inflammatory (stimulation with IFN $\gamma$  and /or LPS), anti-inflammatory (IL10 and/or glucocorticoids), and alternatively activated macrophages (IL-4). Of course, a variety of stimuli may be tested to trigger specific inflammatory state in macrophages [5].

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## 2 Materials

### 2.1 Culture of Murine Macrophages and MPCs

1. 2-well permanox Lab-Tek® (Nunc #177429, *see Note 1*).
2. 12- and 24-well plates.
3. Matrigel® low growth factor (Corning #356231).
4. Horse serum, heat inactivated at 56 °C for 30 min.
5. Ultrosor™ G serum substitute (#15950-017, Pall Corporation) (*see Note 2*).
6. Serum-free macrophage medium: DMEM Glutamax, 4.5 g/l glucose, 1 $\times$  pyruvate (#31966-021) containing 100 U/ml Penicillin/streptomycin (P/S).
7. Macrophage medium: DMEM Glutamax, 4.5 g/l Glucose, 1 $\times$  Pyruvate (#31966-021), containing 10 % Heat-inactivated Fetal Bovine Serum (FBS) and 100 U/ml P/S.
8. MPC growth medium: DMEM/F-12 Glutamax (#31331-028), 20 % FBS, 100 U/ml P/S. Filter through Stericup-GP 0.22  $\mu$ m and add 2 % Ultrosor G (*see Note 2*).
9. Cytokines (R&D system) are dissolved in PBS 1X Ca<sup>2+</sup> and Mg<sup>2+</sup> free (PBS): IFN $\gamma$  (#485-MI, stock solution at 50  $\mu$ g/ml), IL-4 (#404-MI, stock solution at 5  $\mu$ g/ml), IL-10 (#417-ML, stock solution at 5  $\mu$ g/ml).
10. Murine MPCs are isolated from *gastrocnemius* and *tibialis anterior* and cultured as previously described in [6].
11. Murine macrophages are isolated from bone marrow and prepared as previously described in [7].

## 2.2 Immunolabeling of Murine MPCs

1. 4 % Paraformaldehyde (PFA) (Boster Biological Technology #AR1068).
2. Anti-Ki67 antibody made in rabbit (Abcam #ab15580, use at 5 µg/ml).
3. Anti-desmin antibody made in rabbit (Abcam #ab6322, use at 0.085 µg/ml).
4. Donkey anti-rabbit Cy3 secondary antibodies (Jackson Immuno Research Inc. #711-165-152) (*see Note 3*).
5. Hoescht 33342 (Sigma Aldrich #B2261, dilute in PBS at 2 mM for stock, use at 2 nM).
6. Mounting medium (Interchim#FP-483331).
7. Coverslips 18 × 18 mm.

## 2.3 Culture of Human Macrophages and MPCs

1. 12-well plates.
2. T75 culture flasks.
3. Glass coverslips 18 mm diameter (Marienfeld #0111580) that have been sterilized.
4. MPC growth medium: Ham-F12 Glutamax (#31331) containing 15 % FBS, 100 U/ml P/S (#15140).
5. MPC differentiation medium: Ham-F12Glutamax (#31331) containing 5 % FBS, 100 U/ml P/S.
6. Macrophage medium: Advanced RPMI 1640 (#12633) containing 1× Glutamine, 100 U/ml P/S, 10 mM Hepes, 100× MEM vitamins, 0.5 mM 2-mercaptoethanol, 15 % FBS.
7. Low serum macrophage medium: Advanced RPMI 1640 containing 1× Glutamine, 100 U/ml P/S, 10 mM Hepes, 100× MEM vitamins, 0.5 mM 2-mercaptoethanol, 0.5 % FBS.
8. Lipopolysaccharide (LPS) (#L4130 Sigma Aldrich, stock solution at 1 mg/ml in culture medium)
9. Dexamethasone (Dex) (D4902 Sigma Aldrich, stock solution at 20 µg/ml, add 1 ml of 100 % ethanol *per* mg, and then add 49 ml of culture medium *per* ml of ethanol).
10. Cytokines (R&D system): IFN $\gamma$  (#285-IF/CF, stock solution at 0.1 mg/ml in deionized water), IL-4 (#204-IL/CF, stock solution at 0.1 mg/ml in PBS, IL-10 (#217-IL/CF, stock solution at 50 µg/ml are dissolved in PBS).
11. Cell Proliferation ELISA, BrdU (colorimetric) (Roche Diagnostics GmbH #11647229001).
12. Human MPCs are isolated from human muscle biopsies and cultured as previously described [8].
13. Human macrophages are isolated from human blood and cultured as previously described [8].

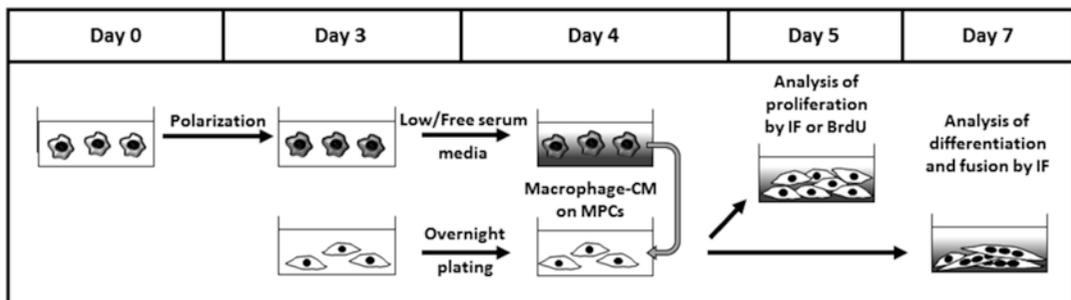
## 2.4 Immunolabeling of Human MPCs

1. 4 % PFA (Boster Biological Technology #AR1068).
2. Anti-Myogenin antibody made in mouse (BD Pharmingen #556358, use at 10 µg/ml).
3. Anti-desmin antibody made in rabbit (Abcam #32362, use at 60 µg/ml).
4. Donkey anti-rabbit Cy3 1/200 secondary antibodies (Jackson ImmunoResearch Inc. #711-165-152), biotinylated anti-mouse antibodies (Vector Laboratories #BA-2000), and Streptavidin DTAF (Beckman Coulter #PN IM0307) (*see Note 3*).
5. Hoescht 33342 (Sigma Aldrich #B2261, dilute in PBS at 2 mM for stock, use at 2 nM).
6. Mounting medium (Interchim#FP-483331).
7. Microscopic slides.

## 3 Methods

### 3.1 Effect of Macrophage-CM on MPC Proliferation in Mouse (Fig. 1)

1. Day 0, macrophages are seeded at 71,000 cells/cm<sup>2</sup> in three wells for each condition (24-well plate) in macrophage medium (500 µl/well).
2. 6 h after plating, macrophages (that should have adhered) are activated with cytokines: IFN $\gamma$  (50 ng/ml), IL-10 (10 ng/ml), IL-4 (10 ng/ml) in macrophage medium to obtain pro-inflammatory, anti-inflammatory, and alternatively activated macrophages, respectively.
3. Day 3, cells are washed three times with PBS 1 $\times$  (500 µl/well) and once with 500 µl serum-free DMEM medium (*see Note 4*).
4. 250 µl of serum-free macrophage medium is added *per* well (three empty wells are used for the control) and the cells are incubated for 24 h for the preparation of macrophage-CM.



**Fig. 1** Method to analyze the effects of macrophage-CM on MPCs. Day 0: macrophages are polarized with specific activators. Day 3: conditioned medium from macrophages is prepared through incubation of the cells for 24 h in serum-free or low-serum medium. MPCs are seeded. Day 4: macrophage-CM is recovered and added to MPC cultures. Day 5: Proliferation is analyzed by immunofluorescence or BrdU incorporation. Day 7: Differentiation/fusion is analyzed by immunofluorescence

5. Day 3 (*see Note 5*), MPCs are seeded at 10,000 cells/cm<sup>2</sup> on matrix-coated Lab-Tek<sup>®</sup>(Nunc #177429, *see Note 6*) in MPC growth medium.
6. Day 4, macrophage-CM is recovered. Triplicate-wells are pooled in 15 ml conical centrifuge tubes, FBS is added at 2.5 % (*see Note 7*) and macrophage-CM is centrifuged for 10 min at 800 × *g*, to eliminate any cell debris.
7. MPC cultures are carefully washed (*see Note 8*) three times with PBS 1× and 700 µl of macrophage-CM *per* well is added.
8. Day 5 and 24 h after addition of macrophage-CM, perform the Ki67 labeling (*see Note 9* and Subheading 3.3 for the protocol).

### **3.2 Effect of Macrophage-CM on MPC Differentiation in Mouse (Fig. 1)**

1. Day 0, macrophages are seeded at 71,000 cells/cm<sup>2</sup> in two wells for each condition (12-well plate) in macrophage medium (1 ml/well).
2. After 6 h of plating, macrophages are activated with cytokines: IFN $\gamma$  (50 ng/ml), IL-10 (10 ng/ml), IL-4 (10 ng/ml) in macrophage medium, to obtain pro-inflammatory, anti-inflammatory, and alternatively activated macrophages, respectively.
3. Day 3, cells are washed three times with PBS 1× (1 ml/well) and once with 1 ml serum-free macrophage medium (*see Note 4*).
4. 500 µl of serum-free macrophage medium is added *per* well (two empty wells are used for the control) and the cells are incubated for 24 h for the preparation of macrophage-CM.
5. Day 3 (*see Note 5*), MPCs are seeded at 30,000 cells/cm<sup>2</sup> on matrix-coated Lab-Tek<sup>®</sup>(Nunc #177429, *see Note 6*) in MPC growth medium.
6. Day 4, macrophage-CM is recovered. Duplicate-wells are pooled in 15 ml conical centrifuge tubes, 2 % horse serum is added (*see Note 10*), and macrophage-CM is centrifuged for 10 min at 800 × *g*, to eliminate any debris.
7. MPC cultures are carefully washed (*see Note 8*) three times with PBS 1× and 700 µl of macrophage-CM *per* well is added.
8. Day 7, check that in the untreated condition MPCs are differentiated (*see Note 11*), then perform desmin labeling (*see Note 9* and Subheading 3.3 for the protocol).

### **3.3 Immunolabelings in Mouse**

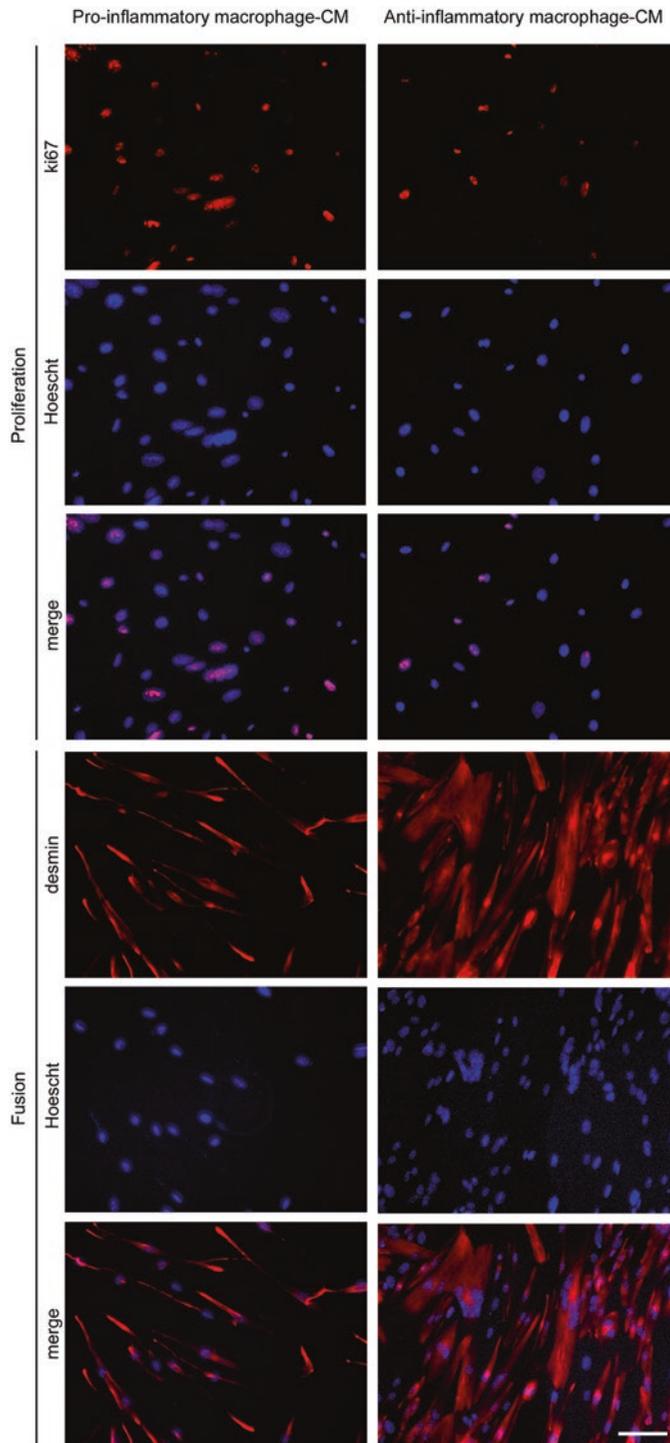
All steps are performed at room temperature (RT), unless otherwise indicated (*see Note 12*).

1. The above part of the Lab-Tek<sup>®</sup> is removed (walls and silicone joint).
2. Cells are washed three times for 5 min with PBS 1× in a Coplin jar.

3. Cells are fixed for 15 min with PFA 4 % in a Coplin jar.
4. Cells are washed three times for 5 min with PBS 1× in a Coplin jar.
5. Cells are permeabilized for 10 min with 0.5 % Tritonin PBS 1× in a Coplin jar.
6. Cells are washed three times for 5 min with PBS 1× in a Coplin jar.
7. 40 µl of primary antibodies diluted in PBS 1× (anti-Ki67 antibody for the proliferation assay or anti-desmin antibody for the differentiation assay) is dropped on each well and is covered with a piece of parafilm (*see Note 13*).
8. Cells are incubated with primary antibodies for 2 h at 37 °C in a humid chamber (*see Note 14*).
9. The parafilm is carefully removed and cells are washed three times for 5 min with PBS 1× in a Coplin jar.
10. 40 µl of secondary antibodies diluted in PBS 1× (anti-rabbit antibody and cover the wells with a piece of parafilm) is dropped on each well and is covered with a piece of parafilm (*see Note 13*).
11. Cells are incubated with secondary antibodies for 1 h at 37 °C in a humid chamber (*see Note 14*).
12. The parafilm is carefully removed and cells are washed three times for 5 min with PBS 1× in a Coplin jar.
13. Cells are quickly (10 s) incubated in a Coplin jar containing 2 nM Hoechst in PBS 1×.
14. Cells are washed once for 5 min with PBS 1× in a Coplin jar.
15. Cells are mounted in mounting medium with a coverslip and stored at +4 °C.
16. About 10 pictures of each well are recorded at 20× magnification with an epifluorescence microscope (*see Note 15* and Fig. 2).

### **3.4 Effect of Macrophage-CM on MPC Proliferation in Human (Fig. 1)**

1. Day 0, macrophages are seeded at 36,000 cells/cm<sup>2</sup> in three wells for each condition (24-well plate) in macrophage medium (500 µl/well).
2. After 6 h (minimum) of plating, macrophages are activated with cytokines and hormone for polarization: LPS (1 µg/ml) and IFN $\gamma$  (10 ng/ml), IL-10 (10 ng/ml) and Dex (80 ng/ml), and IL-4 (10 ng/ml), to obtain pro-, anti- and alternatively activated macrophages, respectively, in macrophage medium (500 µl/well).
3. Day 3, macrophages are washed three times with 500 µl PBS 1× (*see Note 4*).
4. 250 µl of low serum macrophage medium is added *per* well (three empty wells are used for the control) and cells are incubated for 24 h.



**Fig. 2** Immunolabeling of murine MPCs cultured with inflammatory or anti-inflammatory macrophage-CM in proliferation or fusion conditions. Proliferation (*upper panel*): Ki67 (*red*) and nuclei (*blue*) labeling of MPCs in proliferation condition with inflammatory macrophage-CM (*left*) or anti-inflammatory macrophage-CM (*right*). Fusion (*lower panel*): Desmin (*red*) and nuclei (*blue*) labeling of MPCs in differentiation condition with inflammatory macrophage-CM (*left*) or with anti-inflammatory macrophage-CM (*right*). Inflammatory macrophage-CM activates proliferation of MPCs while anti-inflammatory macrophage-CM activates fusion of MPCs. Bar = 50  $\mu$ m

5. MPCs are seeded at 12,000 cells/cm<sup>2</sup> in a 24-well plate in MPC growth medium (three wells *per* condition) (*see* **Note 16**).
6. Day 4, macrophage-CM is recovered: triplicate-wells are pooled in 15 ml conical centrifuge tubes and centrifuged for 10 min at 800 × *g*.
7. MPCs are washed three times MPCs with PBS 1× and 250 μl of macrophage-CM is added *per* well.
8. BrdU is added at 10 μM in each well.
9. Day 5, MPCs are fixed and BrdU is measured as recommended by manual supplier (colorimetric assay).

### **3.5 Effect of Macrophage-CM on MPC Differentiation in Human (Fig. 1)**

1. Day 0, macrophages are seeded at 36,000 cells/cm<sup>2</sup> in three wells for each condition (12-well plate) in macrophage medium (1 ml/well).
2. After 6 h (minimum) of plating, macrophages are activated with cytokines and hormone for polarization: LPS (1 μg/ml) and IFNγ (10 ng/ml), IL-10 (10 ng/ml) and Dex (80 ng/ml), and IL-4 (10 ng/ml), to obtain pro-, anti- and alternatively activated macrophages, respectively, in macrophage medium (500 μl/well).
3. Plate MPCs at 3000 cells/cm<sup>2</sup> in a 75 cm<sup>2</sup> culture flask in MPC differentiation medium (*see* **Note 17**).
4. Day 3, macrophages are washed three times with 1 ml of PBS 1× (*see* **Note 4**).
5. 500 μl of low serum macrophage medium is added *per* well (three empty wells are used for the control) and cells are incubated for 24 h.
6. Glass coverslips are deposited in the bottom of 12-well plates (three wells for each condition), and 500 μl of MPC differentiation media is added (the air under the coverslip is removed by pressing the coverslip with a tip or a pipette so that the coverslip sticks in the bottom of the well).
7. MPCs (which are now myocytes) are trypsinated: cells are washed with PBS 1× then incubated with 1.5 ml of Trypsin-EDTA. Cell detachment is monitored under a microscope and should occur within few minutes. Cells are recovered in MPC differentiation media, centrifuged for 10 min at 250 × *g*, and seeded at 500 cells/cm<sup>2</sup> in MPC differentiation media on the coverslips in the 12-well plates.
8. Day 4, macrophage-CM is recovered. Triplicate-wells are pooled in 15 ml conical centrifuge tubes and centrifuged for 10 min at 800 × *g* to remove cell debris.
9. MPCs are washed three times with PBS 1× and 500 μl of macrophage-CM is added *per* well.

10. Day 7, MPCs are washed three times with PBS 1×. Myogenin-desmin immunolabeling is directly performed on coverslips. Alternatively, coverslips can be dried at RT and stored at  $-20^{\circ}\text{C}$  for further use (*see* Subheading 3.6 for the protocol).

### **3.6 Myogenin-Desmin Immunolabeling on Human MPCs**

Steps are performed at RT unless otherwise indicated (*see* **Note 12**). Washes of coverslips are done in staining blocs (Vaccine dishes #2020, Glaswarenfabrik Karl Hecht GmbH & Co KG). Coverslips are handled with thin forceps, on the edges of the coverslips (*see* **Note 18**).

1. If the coverslips have been frozen, they must be rehydrated for 5 min in PBS 1×.
2. Cells are fixed with PFA 4 % for 15 min.
3. Cells are washed three times with PBS 1× for 5 min.
4. Cells are permeabilized with 0.5 % triton in PBS 1× for 10 min.
5. Cells are washed three times with PBS 1× for 5 min.
6. A big piece of parafilm is secured in the bottom of an incubation box. 50  $\mu\text{l}$  of primary antibodies diluted in PBS 1× (anti-myogenin and anti-desmin antibodies) is dropped on the parafilm. The coverslip is laid on the antibody drop (cells facing the antibody) and incubated for 2 h at  $37^{\circ}\text{C}$  in a humid chamber (*see* **Note 14**).
7. Cells are washed three times with PBS 1× for 5 min.
8. Using the same procedure as for primary antibodies, cells are incubated with the secondary antibodies diluted in PBS 1× (anti-mouse biotinylated and anti-rabbit secondary antibodies) for 1 h at  $37^{\circ}\text{C}$  in a humid chamber (*see* **Note 14**).
9. Cells are washed three times with PBS 1× for 5 min.
10. Using the same procedure as for the antibodies, the cells are incubated with Streptavidin-DTAF for 20 min at  $37^{\circ}\text{C}$  in a humid chamber (*see* **Note 14**).
11. Cells are washed four times with PBS 1× for 5 min.
12. Each coverslip is quickly (10 s) incubated with 2 nM Hoechst in PBS 1× (either the coverslip is laid on the top of 50  $\mu\text{l}$  of Hoechst on a piece of parafilm, or the coverslip handled with a forceps is soaked for 10 s in a jar containing Hoechst).
13. Cells are washed once with PBS 1× for 5 min.
14. Coverslips are mounted with 50  $\mu\text{l}$  of mounting medium on glass slides and stored at  $+4^{\circ}\text{C}$ .
15. About 10 pictures of each condition are recorded at 20× magnification with an epifluorescence microscope (*see* **Note 19**).

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## 4 Notes

1. Lab-Tek® are made in glass, permanox, or Poly D-lysine coated. Murine MPCs adhere only on permanox, although it induces some background in immunofluorescence. 2-well Lab-Tek® (each well is 4.3 cm<sup>2</sup>) are used to minimize impacts of the edges, which favor MPC fusion. Thus, areas near the edges should be excluded of the analysis.
2. 20 ml of sterile H<sub>2</sub>O are added to one bottle of Ultrosor G. After 15–20 min at RT, shaking the bottle ensures complete dissolution. Solution is filtered through 0.22 µm (Millipore #SLGP033RS) and kept aliquoted at –20 °C.
3. Antibodies and streptavidin are diluted in 400 µl of H<sub>2</sub>O and 400 µl of glycerol and stored at –20 °C. They are used at 1/200 in PBS 1×.
4. Washes are here important to remove any trace of the reagents used for macrophage activation.
5. The best time to plate MPCs is the evening. Cells adhere during the night and thus do not stay too long in growth medium (thus not too high number of cells resulting in proliferation).
6. For Lab-Tek® coating, Matrigel® is diluted in cold serum-free DMEM/F-12 at 1/10. 1 ml is added per well and incubated for 30 min at 37 °C. Excess of Matrigel® is discarded and the well is washed three times with PBS 1× to remove Matrigel® aggregates.
7. Mouse MPCs do not proliferate well under very low FBS concentrations in the medium. On the other way, doing the coculture experiment in 10 % FBS would hide the effect of factors contained in macrophage-CM. 2.5 % has been shown to be the minimum concentration of FBS for an effective MPC proliferation.
8. Cells detach easily, carefully aspirate PBS.
9. Freezing the Lab-Tek® prevents good immunolabeling.
10. Low concentration (2 %) of horse serum, coupled with high cell density, induces rapid murine MPC differentiation and fusion.
11. The untreated MPCs should show signs of differentiation, i.e., the presence of myotubes. If not, the experiment is not valid.
12. The preparation must never dry during the whole procedure.
13. The drop of diluted antibodies is deposited in a corner of the well and a piece of parafilm is slowly laid. Parafilm size should be over that of the well. Ideally, one parafilm is used to cover the entire Lab-Tek®, i.e., the same labeling is realized in the two wells. Use separate smaller pieces of parafilm and avoid

mixing of antibodies in neighboring wells if doing different labelings in each well.

14. To avoid drying of the preparation during the incubation, wet papers are placed in the incubating box.
15. For the analysis of proliferation, the number of Ki67<sup>+</sup> nuclei is calculated as a percentage of the total number of nuclei. For the differentiation assay, fusion is evaluated as the percentage of nuclei into myotubes among the total number of nuclei (Fig. 2).
16. If macrophage-CM is recovered in the morning of day 4, MPCs should be seeded in the evening of day 3. If macrophage-CM is recovered in the afternoon, MPCs should be seeded in the early morning of day 4 (at least 6 h of plating).
17. Human MPCs are seeded at low concentration of FBS to induce differentiation and at low density to avoid their fusion.
18. The user must keep in mind during the whole procedure on which side of the coverslip the cells are. She/he should establish an own rule, e.g., “the cells are on the up side for the washings.”
19. Desmin staining discriminates myogenic cells from non-myogenic cells (that may have raised in the culture with time). Only desmin positive cells and desmin-myogenin positive cells should be taken into account. Differentiation is evaluated as the percentage of the number of double myogenin-desmin<sup>+</sup> cells among the total number of desmin<sup>+</sup> cells.

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