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Transplanted Mesoangioblasts Require Macrophage IL-10 for Survival in a Mouse Model of Muscle Injury

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The aim of this study was to verify whether macrophages influence the fate of transplanted mesoangioblasts—vessel-associated myogenic precursors—in a model of sterile toxin-induced skeletal muscle injury. We have observed that in the absence of macrophages, transplanted mesoangioblasts do not yield novel fibers. Macrophages retrieved from skeletal muscles at various times after injury display features that resemble those of immunoregulatory macrophages. Indeed, they secrete IL-10 and express CD206 and CD163 membrane receptors and high amounts of arginase I. We have reconstituted the muscle-associated macrophage population by injecting polarized macrophages before mesoangioblast injection: alternatively activated, immunoregulatory macrophages only support mesoangioblast survival and function. This action depends on the secretion of IL-10 in the tissue. Our results reveal an unanticipated role for tissue macrophages in mesoangioblast function. Consequently, the treatment of muscle disorders with mesoangioblasts should take into consideration coexisting inflammatory pathways, whose activation may prove crucial for its success. The Journal of Immunology, 2012, 188: 6267–6277.

Inflammation is a hallmark of muscle damage: leukocytes are recruited and persist while repair and regeneration occur (1). Macrophages are the best characterized inflammatory cells in the injured muscle (2, 3), and in vivo studies have shown that they play an active role in the tissue repair process (3–17). Macrophages dispose of apoptotic myofiber remnants and of debris, produce signals involved in matrix remodeling and neovessel formation, and regulate the activation, proliferation, and differentiation of muscle stem cells.

Macrophages represent a heterogeneous population with different signatures, reflecting their various functions within the environment (18–20). Macrophages exposed in vitro to bacterial products and to IFN-γ or TNF-α differentiate toward inflammatory cells and are referred to as “classically activated” or M1 macrophages. In contrast, macrophages exposed to IL-4 and IL-13 acquire features of M2 or “alternatively activated” macrophages, and macrophages exposed to cellular debris and IL-10 share some but not all features of M2 macrophages (M2-like macrophages) and acquire immunoregulatory functions. This classification is predicated on polarization studies in vitro: as such, it represents a “useful oversimplification” (20) and does not necessarily reflect phenotypes found in vivo.

Satellite cells are resident “stem-like” cells in the skeletal muscle responsible for muscle growth and regeneration in postnatal life (21–25). In response to muscle injuries, quiescent satellite cells undergo activation, proliferate, and fuse with each other or with damaged fibers (26). Other sources of myogenic precursors, mostly of mesodermal origin, have been identified (27–36) including mesoangioblasts from the embryonic dorsal aorta and their counterparts associated with microvascular walls in the adult skeletal muscle, pericytes (37), and have been shown to contribute to muscle regeneration (36, 38–42). Upon transplantation in regenerating injured skeletal muscle, mesoangioblasts participate in tissue growth and regeneration and fuse with resident satellite cells (43). They have been used for therapy in dystrophic mice and dogs (44, 45).

Local cues limit the efficacy of experimental therapeutic protocols for muscular dystrophies: the limited knowledge of the mechanisms that control transplanted cell fate and function in vivo make the identification of the local adverse events difficult. Mesoangioblasts for example are sensitive to the trophic action of stromal cells (46), suggesting that nonmuscle cells in normal conditions regulate their survival and differentiation.

In this study, we have investigated whether macrophages, which are physiologically recruited into damaged muscle, regulate the differentiation of myogenic precursors into terminally differentiated myofibers and have used mesoangioblasts as a model system to study the interaction between the two cell populations. We have identified the signature of macrophages specifically involved in the differentiation of progenitor cells, and, taking advantage of both in vivo and in vitro ad hoc coculture systems, we have established for the first time, to our knowledge, that IL-10–IL-10R signaling is a critical regulator of the outcome of the interaction. Altogether, these data point to macrophages as crucial regulators of the fate of transplanted myogenic precursors in the regenerating skeletal muscle.

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The online version of this article contains supplemental material.

Abbreviations used in this article: CLP, liposomes containing clodronate; CTX, cardiotoxin; GFP, green fluorescent protein; CLP–GFP, liposomes containing clodronate and GFP-expressing mesoangioblasts; HS, horse serum; IFN-γ–MIF, IFN-γ macrophage; IGF-1, insulin-like growth factor-1; IL-10–M6, IL-10 macrophage; LDH, lactate dehydrogenase.
**Materials and Methods**

**Cell culture and media**

Adult mesangioblasts were isolated from tibialis anterior muscles of C57BL/6 female mice according to Ref. 47 and cloned by limiting dilution at 0.3 cell/well. A single clone was transduced with a lentiviral vector expressing enhanced GFP under a constitutive promoter (pRLSsin.PPT. CMV.GFP) generated and prepared as previously described (48). Transduced cells with the higher GFP expression were then verified by FACSort sorting (49). The identity of the cells (GFP-expressing mesangioblasts; GFP

**GFP+MAB** was then confirmed by PCR and FACS analysis: sorted cells homogeneously expressed mesangioblasts markers, CD34, SCa1, alpha7 integrin, NG2, platelet-derived growth factor receptor, and aSMA, and failed to express the endothelial marker, VE-cadherin, or the satellite cells marker, Pax7. They differentiate into myotubes under low serum conditions; treatment with bFGF commits them to differentiate into satellite cells; treatment with BMP2 commits them to differentiate into osteocytes, as expected (49). Satellite cells were isolated as described (50).

**Polarized macrophages** were propagated as described (51). Briefly, after 7 d of culture in αMEM (Life Technologies, Invitrogen) containing 10% FCS in the presence of 100 ng/ml recombinant murine M-CSF (R&D Systems), bone marrow-derived macrophages from C57BL/6 female mice were differentiated into IFN-γ macrophages (IFN-γ-Mφ) or into IL-10 macrophages (IL-10-Mφ) when cultured for a additional 2 d with 50 ng/ml recombinant murine IFN-γ (Peprotech) or for an additional 4 d with 10 ng/ml recombinant murine IL-10 (R&D Systems), respectively. Macrophage polarization was verified by flow cytometry after staining with 10 ng/ml recombinant murine IL-10 (R&D Systems), a rat anti-mouse CD206 mAb (clone MR5D3; Serotec, SW; BD Biosciences), a PE-conjugated anti-CD86 mAb (clone GL1; BD Biosciences), a PerCp-conjugated anti-CD45 mAb (clone 30-F11; BD Biosciences), and a goat anti-rat Alexa Fluor 594-conjugated Ab (Invitrogen). Nuclei were counterstained with Hoechst 33342. Images were acquired using an In Cell Analyzer 1000 (GE Healthcare) system, which automatically randomly selects fields of view from representative transverse muscle sections. In parallel, we have quantified the efficacy of cell transplantation as described in Ref. 54. Analysis was performed in perform transverse sections from the whole muscle length. For every image taken, the fluorescence intensity variation at the site of the damage was measured with the ImageJ imaging software (National Institutes of Health, Bethesda, MD; http://rsbweb.nih.gov/ij/index.html). Immunohistochemistry was performed on muscle sections fixed with 4% paraformaldehyde and treated with 0.3% H2O2. Sections were treated with an avidin–biotin blocking kit (Vector Laboratories) according to the manufacturer’s instructions. To evaluate macrophage infiltration, tissue sections were stained with anti-rat mouse CD68 mAb (clone FA-11; AbD Serotec) or rat anti-mouse CD206 mAb (clone MR5D3; Serotec). Primary Abs were revealed using biotin-conjugated anti-rat IgG (eBioscience, San Diego, CA) or biotin-conjugated anti-rabbit IgG (eBioscience) and R.T.U. HRP streptavidin (Vector Laboratories), detected using Vector NovaRED substrate kit (Vector Laboratories). Slides were counterstained with hematoxylin and examined with a Nikon Eclipse 55i microscope (Nikon). Images were captured with a Digital Sight DS-5 M digital camera (Nikon). Parallel slides without primary Abs were processed identically and used as negative controls.

**Myogenic progenitor differentiation and survival assay**

Mesoangioblasts were plated on 0.5% Matrigel (BD Biosciences) pre-treated wells in DMEM (Life Technologies, Invitrogen) plus 20% FCS (Lonza) at a density of 2 x 10⁵. After 16 h, mesangioblasts were transferred in DMEM plus 2% horse serum (HS; Life Technologies, Invitrogen). In selected experiments, to evaluate possible interference with myogenic differentiation, C12L was added to mesangioblasts. In mesangioblast–macrophages coculture experiments, the negative control was represented by mesangioblasts cultured in DMEM plus 20% FCS and the positive control by mesangioblasts cultured in DMEM plus 2% HS plus 10 ng/ml IL-10 plus 50 ng/ml insulin-like growth factor-1 (IGF-1) (both from Sigma). Because macrophages grow in αMEM plus 10% FCS, we performed an additional control, culturing mesangioblasts in a mixture of DMEM plus 2% HS and αMEM plus 10% FCS. IFN-γ–Mφ or IL-10–Mφ were extensively washed to remove recombinant cytokines, resuspended in αMEM plus 10% FCS, and added to mesangioblasts. Alternatively, conditioned media used to differentiate IFN-γ–Mφ or IL-10–Mφ were added to mesangioblasts. To evaluate cytoxicity capacity per se to induce differentiation, recombinant IL-10 (10 ng/ml) or IFN-γ (50 ng/ml) was added to mesangioblasts. After 4 d of culture, cells were fixed with paraformaldehyde 4% and processed for immunofluorescence.

In selected experiments, satellite cells were cultured for 4 d in DMEM plus 20% FCS as negative control (nonpermissive), in DMEM plus 2% HS as positive control (permissive), or cocultured with IFN-γ–Mφ or IL-10–Mφ. Effects of IL-10 on mesangioblast differentiation was evaluated using IL-10–Mφ. As a control, an isotype unmatched in Novum mAb was used. mAbs (5 ng/ml) were added to the coculture between macrophages and mesangioblasts every day. Myotubes were identified by staining with mouse anti-myosin H chain Ab (MP20 hybridoma supernatant; Hybridoma Bank). An anti-mouse Alexa Fluor 488-conjugated Ab (Invitrogen) was used as a second-step reagent. Macrophages were labeled with a rat anti-CD11b mAb (clone M1/70; BD Biosciences), followed by a goat anti-rat Alexa Fluor 594-conjugated Ab (Invitrogen). Nuclei were counterstained with Hoechst 33342. Images were acquired using an In Cell Analyzer 1000 (GE Healthcare) system, which automatically randomly acquires 20 microscope fields in duplicate. Numbers of polynucleated myotubes were assessed examining MyHC⁺ cells containing two or more nuclei in each slice. Fluorescent measurement of lactate dehydrogenase (LDH) released from cells with a damaged membrane was performed into the supernatant of cultured mesangioblasts using a commercial kit (Promega, Madison, WI). Analysis was performed in basal condition of culture, after culture of mesangioblasts with polarized macrophages or their conditioned medium, or after culture of mesangioblasts with C12L.
FIGURE 1. Macrophages, recruited to the CTX-damaged muscle, promote mesoangioblast differentiation in vivo. C57BL/6 mice were treated i.m. with CTX 1 d before the transplantation of GFP-MAB (5 × 10^5). Serial systemic injections of CLL were performed to deplete macrophages. Mice were sacrificed 8 or 15 d after CTX injection. Tibialis anterior and quadriceps muscles were collected. (A) Number of CD45^+CD11b^+F4/80^+ cells isolated from digested quadriceps muscles at day 8. (B) Fraction of myofibers expressing GFP as a result of differentiation of transplanted mesoangioblasts (y-axis, left) and GFP fluorescence intensity (arbitrary units, y-axis, right) both at day 15; see text for experimental details. Error bars indicate the mean ± SEM of 15 independent samples (n = 3 mice per five independent experimental cohorts). *p < 0.05, **p < 0.01, ***p < 0.001 (statistically different from control).

H&E staining of tibialis anterior muscles retrieved 8 and 15 d after (C, E) CTX injection; (G, I) CTX injection and GFP-MAB transplantation; (K, M) CTX and CLL repeated injections; and (O, Q) CTX injection, CLL repeated injections, and GFP-MAB transplantation. Scale bar, (Figure legend continues)
FIGURE 2. CD45+ cells isolated from damaged muscle display an IL-10–Mφ-like signature. (A) Number of CD45+ cells retrieved from digested muscles collected from CTX-treated mice at days 1, 3, 7, 10, and 15 (x-axis) was assessed. Results indicate the mean ± SEM of nine independent samples (n = 3 mice per three independent experimental cohorts). (B) IL-10, TNF-α, IFN-γ mRNA expression was assessed in CD45+ cells at the indicated times after injury (days, x-axis). AU, arbitrary units; mRNA levels were normalized to corresponding levels of three independent experimental cohorts. *p < 0.05, **p < 0.01, ***p < 0.001 (statistically different from control). (C) Expression of arginase I (Arg I) on CD45+ cells isolated from damaged muscle was assessed by Western blotting. Results are from a representative experiment out of three. In vitro polarized IFN-γ–Mφ and IL-10–Mφ were used as internal control. (D) Immunohistochemistry for expression of CD68 and CD206 macrophage markers on tibialis anterior muscle sections at days 3, 7, and 10 after CTX injection. Images are representative of nine independent samples (n = 3 mice per three independent experimental cohorts). Scale bars, 20 μm. (E and F) Quadriceps muscles collected from CTX-treated mice at days 3, 7, and 10, and were digested, and the cells retrieved were analyzed by flow cytometry for the expression of the CD163, CD206, and CD80 markers. The gating strategy is shown on (E). CD45+CD11b+ cells are identified, and cells with a compatible side scatter (SSC-A) and coexpressing F4/80–CD206, F4/80–CD163, or F4/80–CD80 within this cell cluster analyzed. Results of the multiparametric flow cytometry assessment at days 3, 7, and 10 after CTX injection are reported on the pie charts. Charts are representative of one sample out of nine (n = 3 mice per three independent experimental cohorts). CLL. Liposomes containing clodronate; CTX, cardiotoxin; GFP-MAB, GFP-expressing mesoangioblasts; UNT, untreated.

Quantitative real-time PCR analysis

Quantitative real-time PCR was performed on total muscle lystate or on CD45+ cells. In both conditions, analysis was performed on material retrieved from tibialis anterior and quadriceps pooled muscles. Muscle lysis or CD45+ cells isolation was performed after 1, 3, 7, 10, and 15 from CTX injection. Lysis of total muscles was also performed on muscles from untreated healthy mice. Samples were homogenized, and 1 μg of extracted RNA/sample was used for first-strand synthesis of cDNA as described (51). Each cDNA sample was amplified in duplicate on a real-time PCR system (7900HT Fast Real-Time PCR System; Applied Biosystems).

The level of each mRNA was normalized to the corresponding level of β-actin for CD45+ cells and of GAPDH for whole muscle. The following primers were used: IL-10 (forward, 5′-ATTTGAAATTCC-CTGGGTGAGAAG-3′; reverse, 5′-GTTCTGAGGAACATCGATGAC-3′), IFN-γ (forward, 5′-CATTGAAACCTAGAAAGTCTG-3′; reverse, 5′-CTCATGAAATGCATCCTTTTTCG-3′), TNF-α (forward, 5′-TCCCA-GTTCTCTCCAAAGGGG-3′; reverse, 5′-GGTGAGAGCAGCTAGC-3′), GAPDH (forward, 5′-GCAAATTCAACGGCACAGTCAAG-3′; reverse, 5′-GGTGACACACACACACTG-3′), and IL-10–Mφ (forward, 5′-ATTTGAATTCC-CTGGGTGAGAAG-3′; reverse, 5′-GGTACAAACACTACCCACACTTG-3′). Supernatants, indicating the maximum LDH release, are represented by supernatants of mesoangioblasts killed by three cycles of freeze and thaw. In selected experiments, allophycocyanin-conjugated annexin V staining was performed on GFP-expressing mesoangioblasts cultured for 4 d with IFN-γ–Mφ or IL-10–Mφ or with their conditioned supernatants.

ELISA

Supernatants from polarized macrophages culture were assayed for IL-10, IGF-1, and TNF-α using DuoSet ELISA Development System (R&D Systems).
Western blot analysis

Both IFN-γ–Mφ and IL-10–Mφ or CD45+ cells retrieved from tibialis anterior and quadriceps muscles were processed for Western blot analysis as previously described (53). Membranes were incubated with the following Abs: anti-arginase I (Santa Cruz Biotechnology), anti-inducible NO synthase (Cell Signaling), and anti-β-actin (Sigma). Primary Abs were revealed with HRP-conjugated secondary Abs (Amersham Biosciences) and a chemiluminescence kit (ECL; Amersham Biosciences).

CLL effects on survival

Mesoangioblasts cultured in DMEM plus 20% FCS were treated with CLL for 6, 12, and 24 h and labeled with FITC-conjugated annexin V (BD Biosciences) to assess the externalization of phosphatidylserine and propidium iodide (BD Biosciences). Untreated mesoangioblasts represent an internal negative control, and mesoangioblasts killed by primary necrosis (dead, upon three cycles of freeze and thaw) provide an internal positive control. Samples were analyzed on a FACSCanto flow cytometer (BD Biosciences). Analysis was performed with the FlowJo software (Tree Star). Representative images of CLL-treated or untreated mesoangioblasts for 24 h were acquired by a Nikon Eclipse TS100 (Nikon). Images were captured with a Digital Sight DS-5 M digital camera (Nikon), and mesoangioblasts treated with H2O2 (10 mM) were used as internal positive controls (dead).

Statistics

Comparisons between different experimental groups were evaluated by ANOVA analysis with least significant difference and by Bonferroni as post hoc test. Differences between only two experimental groups were compared by non-parametric Mann–Whitney U test. ANOVA test for repeated measurements and Student t test for every time acquisition were used to identify significant differences in the increase of GFP fluorescence intensity. Statistical significance was accepted for comparisons where \( p < 0.05 \). Values presented are means ± SEM.

Results

Mesoangioblasts in regenerating muscles require macrophages to differentiate into myotubes

Tibialis anterior and quadriceps muscles of C57BL/6 mice were acutely damaged by CTX injection and GFP-expressing mesoangioblasts (5 × 10^5/muscle) transplanted in the regenerating tissue, a condition in which they are known to fuse and yield novel myofibers (e.g., see Refs. 43, 54, 55). Mice were sacrificed after 8 or 15 d. CD45^+CD11b^+F4/80^+ macrophages, as assessed by flow cytometry after enzymatic digestion of the tissue, effectively infiltrated the injured tissue whereas they were virtually absent in the uninjured muscle (56). In contrast, the number of CD45^−CD11b^−F4/80^− cells were increased two- to three-fold. The number of CD45^−CD11b^−F4/80^− cells increased to levels similar to CD45^+CD11b^+F4/80^+ macrophages after 15 d, but were not detected in the uninjured muscle (56).

FIGURE 3. Polarized macrophages differentially modulate mesoangioblast differentiation in vitro. (A) Immunofluorescence staining for MyHC (green) and CD11b (red) expression in mesoangioblasts and polarized macrophages, respectively. Mesoangioblasts have been cultured for 4 d in DMEM plus 20% FCS as negative control (neg); in αMEM plus 10% FCS plus DMEM plus 2% HS (ctr sup) supplemented or not with recombinant IFN-γ (rec IFN-γ) or IL-10 (rec IL-10); in DMEM plus 2% HS plus IGF-1 plus IL-10 as positive control (pos); in conditioned media from IFN-γ–Mφ (IFN-γ–Mφ sup) or IL-10–Mφ (IL-10–Mφ sup); or cocultured for 4 d with IFN-γ–Mφ or IL-10–Mφ. Nuclei were counterstained with Hoechst (blue). Images are representative of five independent experiments. Original magnification ×20. (B) Quantitative analysis of the mesoangioblast differentiation experiment depicted in (A). The y-axis shows the number of MyHC^+ myotubes displayed, respectively, 2 (gray) or 3–5 nuclei per cell (black). Error bars indicate the mean ± SEM of five independent experiments performed in duplicate. IL-10–Mφ and their supernatants were equally effective at eliciting mesoangioblast differentiation (compare middle and bottom panels). (C) LDH activity in the supernatant of the mesoangioblast differentiation experiment depicted in (A). LDH from mesoangioblasts subjected to freeze and thaw (necrotic) represent the internal positive control. *\( p < 0.05 \), ***\( p < 0.001 \).
untreated muscle (Fig. 1A); consistent results were obtained by immunohistochemistry evaluating the expression of the CD68 macrophage marker (data not shown). Centrally nucleated regenerating fibers were visible throughout the injured areas (Fig. 1C, 1E, 1G, 1I). Mesoangioblasts were quantified at day 8 by flow cytometry in enzymatically digested muscle: they represented 1.15 ± 0.06% of all mononuclear cells (Fig. 1H). At this time, we failed to detect GFP+ fibers originating from injected mesoangioblasts (data not shown). At day 15, mesoangioblasts were no longer detectable among mononuclear cells retrieved from muscle (data not shown). In contrast, we detected GFP+ myofibers in the regenerating muscle (Fig. 1J), indicating that the environment had allowed and sustained mesoangioblasts differentiation/fusion in muscle fibers. In parallel, we depleted macrophages by systemic administration of CLL 1 d before the injury and at days 1, 6, 9, and 12 after injury. The treatment was effective: the number of CD45+CD11b+F4/80+ macrophages abated in CLL-treated mice (Fig. 1A), and regeneration was jeopardized, as revealed by the striking decrease in the number of centrally nucleated regenerating myofibers associated with the persistence of necrotic fibers (Fig. 1K, 1M, 1O, 1Q).

Transplanted mesoangioblasts were virtually absent at day 8, suggesting that in the absence of macrophages, they did not survive (*p < 0.05 in CTX plus GFP+MAB versus CLL plus CTX plus GFP+MAB) (Fig. 1P). In the absence of macrophages, we did not detect GFP+ myofibers at day 15 (Fig. 1R). The result was statistically significant (Fig. 1B). The action of CLL is unlikely to be due to a direct toxic effect on mesoangioblasts, as the treatment did not affect their survival and differentiation in vitro (Supplemental Fig. 1).

Altogether, these data suggest that the fate of transplanted mesoangioblasts in damaged muscles is largely regulated by and dependent on macrophages.

**Characteristics of muscle macrophages that help mesoangioblast survival**

To investigate the features of macrophages that support muscle healing, we have carried out the following on CD45+ mononucleated cells isolated at days 1, 3, 7, 10, and 15 after CTX injection: 1) quantitative PCR analysis of cytokine mRNA expression and 2) Western blotting analysis of arginase I protein expression. IL-10 mRNA was detectable at day 1 and peaked at day 3 after injury, progressively to decrease thereafter in parallel with the decrease of leukocyte infiltration (Fig. 2A, 2B). CD45+ mononucleated cells expressed high levels of TNF-α at day 1 after injury; levels dropped thereafter (Fig. 2B). Infiltrating macrophages expressed high amounts of arginase I, a bona fide marker of alternatively activated macrophages; this feature could be reproduced in vitro by exposing macrophage precursors to recombinant IL-10 but not by exposure to the stimulus triggering classical macrophage activation, IFN-γ (Fig. 2C).

Immunohistochemical analysis of infiltrating macrophages also indicates their alternative activation, as they expressed the mannose scavenger receptor, CD206 (Fig. 2D). We also analyzed CD45+ infiltrating leukocytes by multiparametric flow cytometry (Fig. 2D). The CD45+ population was highly enriched in macrophages (88.6 ± 0.76% of CD45+CD11b+F4/80+ cells, Fig. 2E). Three days after injury, most of them (84.9 ± 1.1% of F4/80+ macrophages) were transplanted in damaged muscles in combination with IFN-γ-M0 or IL-10–M0 (2.5 × 105). Mice were sacrificed 15 d after CTX injection. H&E staining (A, C) or immunofluorescence for GFP (green) and laminin (red) (B, D) on tibialis anterior muscle sections isolated from mice transplanted with a combination of mesoangioblasts and IFN-γ–M0 or IL-10–M0 (CLL+CTX+GFP+MAB+IFN-γ–M0 or CLL+CTX+GFP+MAB+IL-10–M0). Arrows indicate necrotic fibers. Nuclei were counterstained with Hoechst (blue). Images are representative of 12 independent samples (n = 3 mice per four independent cohorts). Scale bars, 20 μm (H&E), 10 μm (immunofluorescence). (E) Percentage of GFP+ myofibers per section analyzed in mice injected with CTX and transplanted with mesoangioblasts (CTX+GFP+MAB) or injected with CTX 1 d after systemic CLL administration and transplanted with a combination of mesoangioblasts and IFN-γ–M0 or IL-10–M0 (CLL+CTX+GFP+MAB+IFN-γ–M0 or CLL+CTX+GFP+MAB+IL-10–M0) is reported. Error bars indicate the mean ± SEM of 9 independent samples (n = 3 mice per three independent experimental cohorts). *p < 0.01. (F) Quantitative analysis of GFP+ fibers detection in mesoangioblasts/macrophages transplantation experiments. Error bars indicate the mean ± SEM. *p < 0.05.

![Image](http://www.jimmunol.org/Downloadable.pdf)
phages) expressed both the CD163 and CD206 markers of alternative activation. This fraction was even higher at later time points (90.9 ± 3.5% at day 7 and 89.6 ± 0.2% at day 10, Fig. 2F).

**Requirement of alternative activated macrophages for mesoangioblast differentiation in vitro and in vivo**

Our results suggest that macrophages that support mesoangioblast survival and differentiation/fusion in vivo have characteristics of alternatively activated macrophages (18, 20). To verify this hypothesis, we propagated differentially polarized macrophages in vitro by challenging bone marrow-derived progenitors with recombinant cytokines, IFN-γ or IL-10. The functional differentiation of macrophages is depicted in Supplemental Fig. 2. IFN-γ–Mφ expressed higher levels of MHC class II (I-A^d^) and of the costimulatory molecule CD86 (Supplemental Fig. 2B) and secreted TNF-α (Supplemental Fig. 2C). IL-10–Mφ expressed higher levels of the scavenger receptors, CD163 and CD206 (Supplemental Fig. 2A), expressed arginase I (Supplemental Fig. 2C), the inducible isoform of NO synthase (Supplemental Fig. 2B) and secreted TNF-α, produced IL-10 (Supplemental Fig. 2D). IL-10–Mφ expressed higher levels of MHC class II (I-Ab) and of the costimulatory molecule CD86 (Supplemental Fig. 2B) and secreted TNF-α (Supplemental Fig. 2C). IL-10–Mφ expressed higher levels of MHC class II (I-Ab) and of the costimulatory molecule CD86 (Supplemental Fig. 2B) and secreted TNF-α (Supplemental Fig. 2C). IL-10–Mφ expressed higher levels of the scavenger receptors, CD163 and CD206 (Supplemental Fig. 2A), expressed arginase I (Supplemental Fig. 2C), the inducible isoform of NO synthase (Supplemental Fig. 2B) and secreted TNF-α, produced IL-10 (Supplemental Fig. 2D). IL-10–Mφ, but not IFN-γ–Mφ, produced high amounts of IGF-1 (Supplemental Fig. 2E), a growth factor known as a central regulator of muscle regeneration (56, 57).

We then cultured for 4 d mesoangioblasts with syngeneic IL-10–Mφ or IFN-γ–Mφ, or with the soluble factors that polarized macrophages had released in their supernatants. Differentiation of mesoangioblasts was assessed, verifying by immunofluorescence staining for GFP (green) and laminin (red) expression (Fig. 3A) or exposure of anionic phospholipids using fluorochrome-labeled annexin V (Supplemental Fig. 3A). The influence of macrophage polarization on muscle stem cells is not restricted to mesoangioblasts. Indeed, IFN-γ–Mφ limited in a permissive medium in vitro satellite cells differentiation into myotubes (Supplemental Fig. 3B, 3C).

We then evaluated in vivo the effect of macrophage polarization on the behavior of transplanted mesoangioblasts. To this aim, we depleted endogenous macrophages using the systemic administration of CLL and reconstituted the muscle macrophage population 1 d after injury by the local injection of IFN-γ–Mφ or IL-10–Mφ. Mesoangioblast differentiation into myofibers was evaluated 15 d after injury. IL-10–Mφ effectively supported mesoangioblast differentiation (Fig. 4C, 4D). Regeneration was...
less effective in muscles injected with IFN-γ–Mφ, and the number of centrally nucleated myofibers dropped, and virtually no GFP⁺ regenerating fiber derived from mesoangioblasts could be detected (Fig. 4A, 4B). Necrotic fibers were substantially more prominent, possibly also as a result of the well-characterized lytic activity of IFN-γ–Mφ (Fig. 4A, arrows). Fig. 4E and 4F reports the quantification of mesoangioblasts differentiation/fusion in myofibers.

**IL-10 is a key signal in the myogenic differentiation of mesoangioblasts in vitro and in vivo**

Immunoregulatory macrophages are massive producers of IL-10, a cytokine known to control immune homeostasis and tissue integrity. We have investigated whether the cytokine contributes to the effect of macrophages on mesoangioblasts. IL-10 mRNA expression increased in CTX-injected muscles with a peak at 3 d after injury. Macrophage depletion by CLL significantly restricted IL-10 mRNA expression (Fig. 5A). We therefore injected anti–IL-10R mAb into the muscle 1 and 2 d before muscle injury and monitored at various times in the regenerating muscle the differentiation of transplanted mesoangioblasts (Fig. 5B–G). Blockade of IL-10R prevented the differentiation into myofibers of transplanted mesoangioblasts (Fig. 5D, 5E). The effect was selective, as anti–IL-10R mAb apparently did not influence other features of the inflammatory scenario (Fig. 5D–G), including the number of CD45⁺CD11b⁺ macrophages retrieved from damaged muscles (Fig. 5H) or the extent of tissue regeneration (Fig. 5B with respect to 5D). The quantification of this result is reported in Fig. 5J. When mesoangioblasts were exposed in vitro to IL-10–M, anti–IL-10R mAb treatment significantly inhibited differentiation in myotubes (Fig. 6). Cell viability was routinely monitored by measuring LDH in the culture supernatants and was unaffected (data not shown).

**Discussion**

In this study, we demonstrate that macrophages infiltrating damaged muscle dictate the fate of transplanted mesoangioblasts: macrophages sustain their viability and allow their differentiation and fusion. IL-10 produced in injured muscles represents a key signal.

Macrophage infiltration is a hallmark of the injury of skeletal muscle (2, 3), and these cells have been clearly shown to be involved in the natural history of primary inflammatory myopathies and genetic diseases of the tissue (Duchenne and Becker muscular dystrophies) (58, 59). Several studies have characterized the macrophage role in the muscle, demonstrating that their presence is not an epiphenomenon: in contrast, they actively participate to muscle healing (3–14, 16, 17, 60). Macrophages probably play several roles in the tissue, including clearance of apoptotic cells and debris and local generation of signals that sustain remodeling of the matrix and angiogenesis. Macrophages in the epimysial and perimysial connective tissue perceive the muscle injury and locally attract monocytes, thereby secreting signals that control their switch to tissue healing macrophages (17) (see also Refs. 5, 9, 61–64). At the early stages after acute injuries, they secrete inflammatory signals, including TNF-α and MCP-1/CCL2, and dispose of fiber remnants. At the later stages, macrophages sustain fiber reconstitution. Signals associated with the clearance of cell debris apparently sustain the functional polarization of macrophages in the injured/regenerating muscles (8).

The molecular pathways by which macrophages sustain muscle regeneration are still unclear (7, 8, 65). Notably, macrophages secrete both signals such as TNF-α that worsen muscle wasting via activation of the FOXO transcription factor (66, 67) and molecules that have an opposite function, such as IGF-1, a central regulator of muscle regeneration (7, 56, 57), or IL-10 (59, 68, 69). The activation of distinct pathways in muscle cells depending on macrophage activation leading to growth or differentiation had been described and characterized in elegant earlier studies (12, 70–77).

The function of macrophages in the regenerating muscle is nonredundant: indeed, regeneration is severely jeopardized when they are pharmacologically targeted (see earlier) or when their attraction and recruitment in the tissue is prevented, as it occurs in mice genetically defective in the chemokine-CC-motif receptor 2 (CCR2⁺/⁻ mice), which is activated by MCP-1/CCL2 (6). Macrophages are required for myogenic precursors to fuse, a critical check point for effective myofiber regeneration (12) and to releases IGF-1 (15). In support of a critical role of the latter cytokine, i.m. injection of IGF-1 in CCR2⁺/⁻ mice at least partially compensates the defective local recruitment of macrophages (15).

Macrophages that infiltrate injured muscle during regeneration display an alternatively activated phenotype, compatible with that of IL-10–elicited immunoregulatory cells (20, 78). The analytical flow cytometry approach we have used allows us to verify that from day 3 after damage, most infiltrating macrophages share these features. However, a minor population (<20% of infiltrating CD45⁺CD11b⁺ leukocytes) is apparently differently polarized at earlier times after injury, a characteristic that may contribute to a more effective clearance of cell remnants (8).

IL-10 is actively produced by immunoregulatory macrophages (20). In our model system, macrophages represent an absolute requirement for IL-10 production in the tissue: their depletion by means of CLL severely restricts the expression of the cytokine. This result makes it unlikely that other cells represent a relevant endogenous source of IL-10, including the most attractive candidates, such as T lymphocytes (79), even if we cannot rule out the possibility that they contribute to the cytokine production only when appropriately licensed by macrophages.
IL-10 is a pleiotropic signal (80) with important, nonredundant roles in preserving tissue integrity in hazardous conditions, such as infection by microbial pathogens. Moreover, IL-10 regulates excessive or otherwise potentially deleterious immune effector functions. In support, Villalta and collaborators (59) have recently demonstrated a pivotal role of IL-10 expression in mdx mice, where the cytokine reduces muscle wasting: the effect on the muscle stem cell compartment was more important in the regenerative phase of muscle damage and apparently associated with an alternative polarization of muscle macrophages. Our results suggest that IL-10 is directly implicated in shielding transplanted stem cells from noxious microenvironmental signals, further adding to the array of protective actions of this "anti-inflammatory" molecule (81–83).

Stem cell survival represents a limiting step for the regeneration of various tissues (84–87), particularly of skeletal muscle (88, 89). Our results suggest that macrophages, a key element in muscle regeneration (8, 12, 13, 16, 60, 61) known to deliver antiapoptotic and survival signals in other tissues (90–96), orchestrate the survival and eventually the differentiation of transplanted mesoangioblasts. This suggests that selective modulation of the function of endogenous macrophages could represent an additional strategy to increase the efficacy of stem cell transplantation. The notion that specific cues in the environment guarantee the persistence of transplanted mesoangioblasts in an injured and regenerating tissue should be taken into account at a time when much effort is being devoted to the design of clinical trials for cellular therapies in muscle disorders. Results of these trials may be hampered by a nonpermissive environment in which macrophages able to support stem cell survival and function are absent or do not secrete the correct array of signals. We have studied in detail the interaction between macrophages and mesoangioblasts: similar constraints could possibly apply to other stem cells, either delivered systemically or locally, and may contribute to the variable results that cellular treatments in the field have yielded to date. This possibility is being actively investigated.

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Disclosures
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References
Local expression of IGf-1 accelerates muscle regeneration by rapidly modulating inflammatory cytokines and chemokines. FASEB J 21: 1393–1402.


Supplementary Figure 1. CLL per se does not influence mesoangioblast behavior in vitro. (A) Mesoangioblasts either untreated (Unt), challenged with clodronate-containing liposomes (CLL) for 6, 12 and 24 hours or killed by freeze and thaw (Dead) were analyzed. Representative dot plots report mesoangioblasts stained with FITC-labeled Annexin-V and propidium iodide (PI). One representative experiment out of five is shown. In 1 out of 5 experiments, clodronate treatement has been performed in duplicate. (B) Results of each experiment have been plotted as percentage of Annexin V-/PI*, Annexin V+/PI+ or Annexin-V+/PI+ positive cell. Untreated (Unt): open circles. CLL-treated for 6 hours: filled circles. For 12 hours: filled squares. For 24 hours, filled triangles. Killed by freeze and thaw (Dead): grey circles. Each symbol represents an independent evaluation. For each time point, CLL treatment was compared versus untreated control cells by one-way ANOVA followed by Bonferroni multiple comparison’s test: no significant difference was observed. Dead cells were compared versus all conditions, ***P<0.001. (C) Representative images of untreated cells (Unt), cells treated for 24 hours with CLL (CLL), or necrotic cells (Dead) are shown. (D) LDH activity in the supernatant of cells treated as reported in legend A confirms the lack of CLL toxicity after 24 hours of treatment. Results are representative of two independent experiments performed in triplicate. *** P<0.001. (E) Mesoangioblasts were cultured for four days in media that allow (Permissive, Perm) or not (Non-permissive, Non-perm) myogenic differentiation in the presence (CLL) or the absence (UNT) of CLL. MyHC staining (green) was assessed by confocal microscopy to monitor myotubes formation. Nuclei were counterstained with Hoechst (blue). (F) Differentiated mesoangioblasts were quantified as number of MyHC+ myotubes containing respectively 2 (grey) or 3 to 5 nuclei (black). Results represent the means±s.e.m. of two independent experiments performed in duplicate.
Supplementary Figure 2. Characterization of in vitro polarized macrophages. (A) Representative flow cytometry profiles of macrophages expressing integrins (CD11b, F4/80), MHC molecules (I-A\(^b\), H2-K\(^b\)), costimulatory molecule (CD86), scavenger receptors (CD206, CD163), colony stimulating factor-1 receptor (CD115) and Fc receptor (CD16). (B) Fluorescence associated to the expression of cell surface receptors (open histograms); filled histograms represent the background fluorescence in the presence of isotype-matched control antibodies or secondary step reagents only. Results in panels A and B are representative of 5 independent experiments. (C) Western blot analysis of the expression of iNOS and Arginase I enzyme in IFN-\(\gamma\)-M\(_\Phi\) and IL-10-M\(_\Phi\). (D) TNF\(\alpha\), (E) IL-10 and (F) IGF-1 secretion was evaluated by ELISA in IFN-\(\gamma\)-M\(_\Phi\) and IL-10-M\(_\Phi\) supernatants. Concentrations are expressed as means ± s.e.m., \(n=3\), ***P<0.0005, **P<0.001, *P<0.005
Supplementary Figure 3. Polarized macrophages do not induce mesoangioblast apoptosis and differently modulate satellite cell differentiation in vitro. 

(A) GFP-expressing mesoangioblasts (GFP, y axis) have been cultured in the presence of IFN-γ (IFN-γ-Mφ) or of IL-10 (IL-10-Mφ) macrophages or with their media (IFN-γ-Mφ sup, IL-10-Mφ sup) for 24 or 48h. APC-labeled Annexin-V was used to assess phosphatidylserine exposure (x axis). Untreated mesoangioblasts represent the internal negative control (Alive). Mesoangioblasts killed by freeze and thaw represent the internal positive control (Dead). Dot plots are representative of two independent experiments performed in duplicate. 

(B) Satellite cells were cultured for four days in media that allow (Permissive, Perm) or not (Non-permissive, Non-perm) myogenic differentiation with IFN-γ- and IL-10-Mφ. MyHC (green) was assessed by confocal microscopy to monitor myotubes formation. Macrophages were identified by CD11b expression (red). Nuclei were counterstained with Hoechst (blue). Images are representative of three independent experiments. 

(C) Differentiated satellite cells were quantified as the number of MyHC⁺ myotubes containing respectively 2 (grey) or 3 to 5 nuclei (black). Error bars indicate the mean ± s.e.m. of three independent experiments run in duplicate. Statistically different from control, *** p<0.001.