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

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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 mesodermic 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.
Materials and Methods

Cell culture and media

Adult mesoangioblasts 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 (pRLSin.PPT. CMV::GFP) generated and prepared as previously described (48). Transduced cells with the higher GFP expression were then retrieved by FACS sorting (49). The identity of the cells (GFP-expressing mesoangioblasts; GFP-MAB) was then confirmed by PCR and FACS analysis: sorted cells homogeneously expressed mesoangioblasts markers, CD34, Sca1, alpha7 integrin, NG2, platelet-derived growth factor receptor, and oSMa, and failed to express the endothelial marker, VE-cadherin, or the satellite cell marker, Pax7. They differentiate into myotubes under low serum conditions, a PE-conjugated anti-F4/80 mAb (clone BM8; BioLegend), and a rabbit anti-mouse CD163 mAb (clone M-96; Santa Cruz Biotechnology, Santa Cruz, CA), a PE-conjugated anti-F4/80 mAb (clone BM8; BioLegend), and a PerCp-conjugated anti-CD45 mAb (clone 30-F11; BD Biosciences), a rat anti-mouse C D206 mAb (clone MR5D3; Serotec, Dusseldorf, Germany), a rabbit anti-mouse CD163 mAb (clone M-96; Santa Cruz Biotechnology, Santa Cruz, CA), a PE-conjugated anti-CD11b mAb (clone AF598; BD Biosciences), and an FITC-conjugated CD16 mAb (clone 2.4G2; BD Biosciences).

Muscle injury

Anesthetized 2-mo-old C57BL/6 female mice were treated with a single intramuscular injection of 50 μM cardio toxicity in saline (CTX; Naja mossambica mossambica; Sigma-Aldrich) in tibialis anterior and quadriceps muscles (25 μl for tibialis anterior muscles and 50 μl for quadriceps muscles) and sacrificed at different time points after injury. All procedures were performed in the animal facility of H. San Raffaele Scientific Institute (Milan, Italy) in accordance with European Union guidelines and with the approval of the institutional animal care and use committee of our institution.

Macrophage depletion

C57BL/6 2-mo-old female mice were injected i.v. with 200 μl liposomes containing clodronate (CLL) 1 d before and at days 2, 6, 9, and 12 after CTX injection. CLL were purchased from http://www.clodronateliposomes.org/ashwindigital.asp?docid=26 (52).

Intramuscular mesoangioblast transplantation

GFP+ mesoangioblasts (5 × 10^5) in 50 μl saline were transplanted into a single site of tibialis anterior and quadriceps muscles 1 d after CTX injection. When indicated, 2.5 × 10^5 in vitro-differentiated IFN-γ-Mø or IL-10–Mø were injected together with GFP-MAB. In selected experiments, mice were treated with anti–IL-10R mAb (clone 1B1.2) (0.5 mg/mouse) administered i.v. at days −1, 0, 7, and 8 from CTX injection.

Tissue digestion and retrieval of CD45+ cells

Minced muscles underwent four cycles of enzymatic digestion at 37°C for 10 min in the presence of 3.5 mg/ml dispase (Invitrogen) and 0.5 mg/ml collagenase type V (Sigma-Aldrich). CD45+ infiltrating cells were further purified from digested muscle cell suspension using CD45-conjugated magnetic beads (Miltenyi Biotec) as previously described (53).

Macrophage analysis

Single-cell suspension obtained from quadriceps muscles digestion was processed by flow cytometry. Cells were incubated with an allophycocyanin-conjugated anti-CD11b mAb (clone M1/70; BD Biosciences), a PerCp-conjugated anti-CD45 mAb (clone 30-F11; BD Biosciences), a PE-conjugated anti-F4/80 mAb (clone BM8; BioLegend), and then analyzed on a FACSCanto flow cytometer (BD Biosciences). Analysis was performed with the FlowJo software (Tree Star).

Histochemistry and immunofluorescence

Serial sections (10 μm thick) of isopentane-frozen muscles were used for H&E staining. For immunofluorescence, muscles were fixed in 4% paraformaldehyde, immersed sequentially in 10–20–30% sucrose, and frozen in isopentane cooled with liquid nitrogen. Sections were blocked with BSA 5%, then 0.1% in PBS–0.1% (v/v) Tween 20. Mouse anti-galectin-3 (Alexa Fluor 488-conjugated anti-GFP Ab (Santa Cruz Biotechnology) and with a rabbit anti-laminin Ab (Sigma-Aldrich) followed by Alexa Fluor 594-conjugated goat anti-rabbit Ab (Invitrogen). Sections were counterstained with Hoechst 33342 (Molecular Probes) and analyzed using a PerkinElmer Confocal UltraVIEW scanning microscope. The percentage of GFP+ myofibers was evaluated by counting GFP+ myofibers on the total number of fibers observed in five randomly selected 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 serial 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://rsweb.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-rabbit 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 × 10^5. After 16 h, mesoangioblasts were transferred in DMEM plus 2% horse serum (HS; Life Technologies, Invitrogen). In selected experiments, to evaluate possible interference with myogenic differentiation, CCL was added to mesoangioblasts. In mesoangioblast–macrophages coculture experiments, the negative control was represented by mesoangioblasts cultured in DMEM plus 20% FCS and the positive control by mesoangioblasts 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 OMEM plus 10% FCS, we performed an additional control, culturing mesoangioblasts in the supernatant of cultured mesoangioblasts with CCL. MesOAM plus 2% HS and OMEM plus 10% FCS. IFN-γ–Mø or IL-10–Mø were extensively washed to remove recombinant cytokines, resuspended in CCL plus 10% FCS, and added to mesoangioblasts. Alternatively, conditioned media used to differentiate IFN-γ–Mø or IL-10–Mø were added to mesoangioblasts. To evaluate cyto kinase capacity per se to induce differentiation, recombinant IL-10 (10 ng/ml) or IGF-1 (50 ng/ml) was added to mesoangioblasts. 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–Mø or IL-10–Mø. Effects of IL-10 on mesoangioblast differentiation was evaluated using recombinant IL-10R mAb. As a control, an isotype-matched irrelevant mAb was used. mAbs (5 ng/ml) were added to the coculture between macrophages and mesoangioblasts every day. Myotubes were identified by staining with mouse anti-myosin H chain Ab (MF20 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 evaluating MyHC+ cells containing two or more nuclei in each section. Fluorescent measurement of lactate dehydrogenase (LDH) released from cells with a damaged membrane was performed into the supernatant of cultured mesoangioblasts using a commercial kit (Promega, Madison, WI). Analysis was performed in basal condition of culture, after culture of mesoangioblasts with polarized macrophages or their conditioned medium, or after culture of mesoangioblasts with CCL.
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 ($5 \times 10^5$). 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.
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 (independent experiments (independent experimental cohorts). *CLL, Liposomes containing clodronate; CTX, cardiotoxin; GFP+MAB, GFP-expressing mesoangioblasts; UNT, untreated.

Side scatter (SSC-A) and coexpressing F4/80-CD206, F4/80-CD163, or F4/80-CD80 within this cell cluster analyzed. (20 μm. Days 3, 7, and 10 after CTX injection. Images are representative of nine independent samples (10–M 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, 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. (F) 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). CIL. Liposomes containing clodronate; CTX, cardiotoxin; GFP+MAB, GFP-expressing mesoangioblasts; UNT, untreated.

Positive controls, indicating the maximum LDH release, are represented by supernatants of mesoangioblasts killed by three cycles of freeze and thaw. In selected experiments, aliphycocyanin-conjugated annexin V staining was performed on GFP-expressing mesoangioblasts cocultured for 4 d with IFN-γ–Mφ or IL-10–Mφ or with their conditioned supernatants.

Quantitative real-time PCR analysis
Quantitative real-time PCR was performed on total muscle lysate of or on CD45+ cells. In both conditions, analysis was performed on material retrieved from tibialis anterior and quadriceps pooled muscles. Muscle lysate or CD45+ cells isolation was performed after 1, 3, 7, 10, and 15 from CTX injection. Supernatants from polarized macrophages culture were assayed for IL-10, IFN-γ, and TNF-α using DuoSet ELISA Development System (R&D Systems). 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′-ATTGGAATCC-CTGGGTGAGAAG-3′; reverse, 5′-CACAGGGGAGAAATCGATGACA-3′), IFN-γ (forward, 5′-CATTGAAGCCTAGAAAGTCTG-3′; reverse, 5′-CTCATGAATGCATCCTTTTTCG-3′), TNF-α (forward, 5′-TCCCA-GTTTCTCCTCAAGGGA-3′; reverse, 5′-GTTGAGGACGACTGATGG-3′), GAPDH (forward, 5′-GCAAATTCACGCGGACAACAGTCAG-3′; reverse, 5′-GCTACAACACTACCCACACTTG-3′), and β-actin (forward, 5′-TGCTGTCTCCTGATGCTC-3′; reverse, 5′-GATGCAGCAGCATTCC-3′).

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

20 μm. (D, H, L, and P) Flow cytometry analysis of quadriceps muscles digested 8 d after CTX injection: x-axis, GFP fluorescence; y-axis, side scatter (SSC-H). Insets report the percentage of GFP+MAB gated on total cell population. Results are from a representative experiment out of nine (n = 3 mice per three independent experimental cohorts). (F, J, N, and R) Immunofluorescence analysis of GFP expression (green) and laminin (red) in myofibers of tibialis anterior muscles retrieved 15 d after CTX injection. Nuclei are counterstained with Hoechst (blue). Scale bar, 10 μm. Images are representative of three independent experiments (n = 9 mice, three mice per three independent experimental cohorts were studied in CTX-treated group; n = 15 mice, five mice per three independent experimental cohorts in all other conditions).
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

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**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 displaying, 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 identify GFP+ fibers originating from injected mesoangioblasts (data not shown). At day 15, mesoangioblasts were no longer detectable among mononucleated 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). IFN-γ mRNA was barely expressed (Fig. 2B). Infiltrating macrophages expressed high amounts of arginase 1, 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 control stem cell fate in the muscle. To investigate the features of macrophages that help mesoangioblast survival. We carried out the following: 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. CD45+ mononucleated cells expressed high levels of TNF-α at day 1 after injury; levels dropped thereafter. IFN-γ mRNA was barely expressed. Infiltrating macrophages expressed high amounts of arginase 1, a bona fide marker of alternatively activated macrophages. We also analyzed CD45+ infiltrating leukocytes by multiparametric flow cytometry. The CD45+ population was highly enriched in macrophages (88.6 ± 0.76% of CD45+CD11b+F4/80+ cells). Three days after injury, most of them (84.9 ± 1.1% of F4/80+ macrophages).
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-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. 2B), and 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 the expression of MyHC, a marker of skeletal muscle terminal differentiation. As shown in Fig. 3A, mesoangioblasts cultured in the presence of IL-10–Mφ or of their supernatants yielded terminally differentiated multinucleated myotubes. Importantly, recombinant IL-10 per se, used at concentrations higher than those detectable by ELISA in supernatants of IL-10–Mφ, triggered limited mesoangioblasts differentiation. In contrast, mesangioblasts failed to differentiate in the presence of IFN-γ–Mφ, of their supernatants, or of recombinant IFN-γ. The quantification of these results is reported in Fig. 3B. Cell viability was unaffected, as routinely monitored by measuring LDH in the culture supernatants (Fig. 3C) 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

![FIGURE 5. IL-10R blockade inhibits mesangioblast differentiation in vivo. (A) IL-10 expression in macrophage-depleted muscles after CTX injection. C57BL/6 mice were treated or not with Cll to deplete macrophages. Mice were then sacrificed 1, 3, 8, 10, or 15 d after CTX injection. IL-10 expression was assessed by real-time PCR in quadriceps muscles. Error bars indicate the mean ± SEM, n = 3. **p < 0.005, ***p < 0.001. (B–J) C57BL/6 mice were treated systemically with anti–IL-10R mAb or with an irrelevant isotype-matched mAb 1 and 2 d before and 7 and 8 d after CTX injection. At day 1, GFP+MAB were transplanted. At day 15, mice were sacrificed. H&E staining (B, D, F) and immunofluorescence staining for GFP (green) and laminin (red) expression (C, E, G) in tibialis anterior muscles. Nuclei were counterstained with Hoechst (blue). Images are representative of eight animals. Scale bars, 20 μm (H&E), 10 μm (immunofluorescence). (H) The y-axis shows the number of CD45-Cd11b+F4/80− cells isolated from CTX-injected muscles (CTX), from CTX-injected muscles transplanted with GFP+MAB (CTX+ GFP+MAB), in the presence of anti–IL-10R or of irrelevant isotype-matched mAb. Error bars indicate the mean ± SEM, n = 8. (I) The y-axis shows the percentage of GFP+ myofibers per field of view. Error bars indicate the mean ± SEM, n = 8. *p < 0.05. (J) The y-axis shows GFP-associated fluorescence intensity (arbitrary units). Error bars indicate the mean ± SEM, n = 8. **p < 0.001 (statistically different from control). FoV, Field of view; aIL-10R, anti–IL-10R mAb; IrrAb, irrelevant isotype-matched mAb; Unt, untreated.

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less effective in muscles injected with IFN-γ–Mβ, 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–Mβ (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–G). 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. 5I, 5J. When mesoangioblasts were exposed in vitro to IL-10–Mβ, 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, Villaalta 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-inflammato
tory” 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 inves
tigated.

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Disclosures

The authors have no financial conflicts of interest.

References

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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 treatment 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 mean±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<0.05
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.