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Abbreviations : 5-bromo-2-deoxy-uridine (BrdU); clodronate-encapsulated liposomes (clo-lip); cluster of differentiation (CD); dexamethasone (DEX); diphtheria toxin (DT); Diphtheria Toxin Receptor (DTR); latex beads (LX); macrophages (MPs); monocyte/MPs (MO/MPs); nitric oxide (NO); secretory leukocyte protease inhibitor (SLPI); tibialis anterior (TA)

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ABSTRACT

Macrophages are important for skeletal muscle regeneration in vivo and may exert beneficial effects on myogenic cell growth through mitogenic and anti-apoptotic activities in vitro. However, macrophages are highly versatile and may exert various, and even opposite, functions depending on their activation state. Here, we studied monocyte/macrophage phenotypes and functions during skeletal muscle repair. Selective labeling of circulating monocytes by latex beads in CX3CR1$^{GFP/+}$ mice showed that injured muscle recruited only CX3CR1^{lo}/Ly-6C^{+} monocytes from blood that exhibited a non-dividing, F4/80^{lo}, proinflammatory profile. Then, within muscle, these cells switched their phenotype to become proliferating anti-inflammatory CX3CR1^{hi}/Ly-6C^{-} cells that further differentiated into F4/80^{hi} macrophages. In vitro, phagocytosis of muscle cell debris induced a switch of proinflammatory macrophages toward an anti-inflammatory phenotype releasing TGFβ1. In cocultures, inflammatory macrophages stimulated myogenic cell proliferation whereas anti-inflammatory macrophages exhibited differentiating activity, assessed by both myogenin expression and fusion into myotubes. Finally, depletion of circulating monocytes in CD11b-DTR mice at time of injury totally prevented muscle regeneration, whereas depletion of intramuscular F4/80^{hi} macrophages at later stages reduced diameter of regenerating fibers. In conclusion, injured skeletal muscle recruits monocytes exhibiting inflammatory profile that operate phagocytosis and rapidly convert into anti-inflammatory macrophages that stimulate myogenesis and fiber growth.

INTRODUCTION

Stromal cells are involved in organogenic processes by controlling parenchymal cell functions such as migration, proliferation, differentiation and programmed cell death (1). Among stromal cells, macrophages (MPs) play a major role in maintenance of tissue homeostasis (2). In addition to phagocytosis and antigen presentation, these cells play a supportive function in various tissues (2,3). However, MPs are renowned for their heterogeneity as reflected by the various specialized functions they adopt in different anatomical locations (2,4). Many of these activities appear to be opposing in nature: proinflammatory vs. anti-inflammatory, immunogenic vs. tolerogenic, and tissue-destructive vs. tissue repair activities (4).

Different activation states have been described in vitro for MPs, each being associated with a particular phenotype and function (2,5). Classical activation, obtained after LPS/IFNγ treatment, induces production of proinflammatory cytokines and reactive oxygen species. Alternative activation, obtained after IL-4 treatment, increases expression of the mannose receptor (CD206). Deactivation by IL-10 and glucocorticoids induces production of IL-10, TGFβ1 and expression of the scavenger receptor CD163. However, it is not known to what extent these distinct activation states exist in vivo and whether MP fate is determined for ever or whether it remains constantly malleable (2), although several in vitro studies have shown MP adaptation to changing microenvironment (4,6-8).

An additional degree of complexity is reached with the existence of several subsets of circulating monocytes. In both human and mouse, two main monocyte subsets are present in blood: the main $CD14^{hi}$ $CD16^{-}$ ($CX3CR1^{lo}$/Ly-$6C^{hi}$ in mouse) monocyte subset that invades tissues during acute inflammation, and the $CD14^{lo}$ $CD16^{+}$ ($CX3CR1^{hi}$/Ly-$6C^{lo}$ in mouse) monocyte subset, that is not -or in a less extent- recruited during inflammation (9-13) but is expanded in chronic diseases such as HIV infection, sepsis, tuberculosis or asthma (14). Both fate and function of this population remain unclear since, in mouse, it is present in

normal spleen and may correspond to MPs residing in normal tissues (9). However, there is growing evidence that they may result from maturation of Ly-6C^{hi} monocytes (11,15,16).

MPs are involved in skeletal muscle repair. Muscle damage induces massive MP infiltration at the injury site (17,18). Partial depletion of monocyte/MPs (MO/MPs) impairs muscle regeneration while reconstitution of bone marrow restores regeneration (19-23). Initially limited to phagocytosis of necrotic fibers, the pro-regenerative role of MPs was shown to involve a direct stromal support function (24,25). We have previously shown that monocyte-derived MPs enhance myogenic cell growth (26). MPs release mitogenic growth factors for myogenic precursor cells (mpcs) and establish cell-cell interactions that protect mpcs from apoptosis (26,27). However, little is known about both MO/MP subsets and phenotypes at work during muscle regeneration. Studies in rat have shown that ED1+ MPs are associated with muscle necrosis, whereas ED2+ (CD163) MPs, regarded as “resident MPs”, invade muscle once necrosis has been removed and are associated with regenerative myofibers (17,28). Although the nature of ED1 and ED2 MPs is not characterized, these results suggest that different MP subpopulations are associated with different stages of muscle repair.

In this study we used: in vivo tracing methods to analyze which monocyte subset was recruited after muscle injury; ex-vivo phenotyping analyses to characterize MO/MP profile during muscle regeneration; in vitro coculture experiments to identify which MP phenotype was associated with myogenesis; and sequential in vivo MO/MP depletion studies to evaluate the respective roles of both circulating and intramuscular MO/MPs in skeletal muscle repair.

RESULTS

Two MO/MP subsets are sequentially present during muscle regeneration. MO/MPs collected from skeletal muscle of CX3CR1$^{GFP/+}$ mouse (9,15) were analyzed according to their GFP and Ly-6C expression during muscle regeneration. Normal muscle contained little CX3CR1^{hi}/Ly-6C^{-} MO/MPs (about 200 cells/mg of muscle). Notexin was injected in tibialis anterior (TA) muscle to induce the necrosis/regeneration process. As soon as 90 min after injury, CX3CR1^{lo}/Ly-6C^{+} MO/MPs were detected in muscle and rapidly invaded the tissue to reach a maximum point at 24 h post injury (25% of CD45^{+} cells). Then, CX3CR1^{lo}/Ly-6C^{+} MO/MP population quickly declined with time while CX3CR1^{hi}/Ly-6C^{-} MO/MPs appeared and continuously increased to reach a plateau at d 7 accounting for 35% of CD45^{+} cells extracted from muscle at this time point (Fig. 1A).

MO/MP percentage among CD45+ cells is a relative quantification that does not reflect the amount of MO/MPs actually present in muscle. Thus we calculated from these data the total number of extracted GFP^{+} cells per mg of muscle. Results indicated that the extent of the two MO/MP waves was different (Fig. 1B), and this was confirmed by histological examination (Fig. 1C). During the first 2 days, as injured myofibers became necrotic, 2000 to 3500 GFP^{+} cells were counted per mg of muscle. At 4 and 7 days, when myogenic cells proliferated and further differentiated (Fig. 1C), 5 to 6 fold more GFP^{+} MO/MPs were found in muscle (12000 to 16000 cells per mg) (Fig. 1B). Ten days after injury, when centrally nucleated fibers were visible throughout the injured area (Fig. 1C), the amount of GFP^{+} cells had dramatically declined to reach less than 2000 GFP^{+} cells per mg of tissue (Fig. 1B).

CX3CR1^{lo}/Ly-6C^{+} and CX3CR1^{hi}/Ly-6C^{-} MO/MP populations display distinct proliferative and inflammatory profiles. Between d 1 and d 4 post injury, the number of Ly-6C^{-} MO/MPs increased 20-fold while the number of Ly-6C^{+} MO/MPs increased 2-fold (Fig. 1B). We analyzed proliferation of both subsets that were isolated from regenerating muscle by cell sorting (Fig. 2A). Ki67 labeling showed that Ly-6C^{+} MO/MPs never proliferated

whereas Ly-6C$^-$ MO/MPs partially entered into cell cycle from d 2, then the number of proliferating cells doubled at d 4 and further decreased by 2-fold at d 7 (Fig. 2B).

To compare the inflammatory profile of the two MO/MP populations, we analyzed cytokine expression of isolated Ly-6C$^-$ and Ly-6C$^+$ MO/MPs at d 4 post injury by RT-PCR. Results showed that Ly-6C$^+$ MO/MPs expressed IL-1β and, to a lesser extent, TNFα transcripts more strongly than Ly-6C$^-$ MO/MPs (Fig. 2C). Conversely, Ly-6C$^-$ MO/MPs expressed more strongly TGFβ1 and IL-10 than Ly-6C$^+$ MO/MPs (Fig. 2C), indicating that Ly-6C$^+$ MO/MPs had an inflammatory profile and Ly-6C$^-$ MO/MPs had an anti-inflammatory profile.

Injured muscle only recruits CX3CR1^{lo}/Ly-6C$^+$ MO/MPs that subsequently switch into anti-inflammatory CX3CR1^{hi}/Ly-6C$^-$ MO/MPs and differentiate into MPs. We labeled each circulating subset with fluorescent latex beads (LX) before injury, as previously described (12,15). Labeling of monocytes does not alter their migratory capacities (12). After i.v. LX injection, circulating LX$^+$ cells were CX3CR1^{hi}/Ly-6C$^-$ monocytes (Fig. 3A upper panel, "blood"); LX$^+$ cells were never detected into muscle after injury (Fig. 3A upper panel, "muscle"), indicating that Ly-6C$^-$ MO/MPs appearing in muscle at d 2 were not recruited from blood. After liposome-encapsulated clodronate (clo-lip) injection followed by LX injection, circulating LX$^+$ cells were CX3CR1^{lo}/Ly-6C$^+$ monocytes, as previously described (15). 36 h after notexin injection, LX$^+$ cells were still Ly-6C$^+$ monocytes (95%) in the blood (Fig. 3A lower panel, "blood") while LX$^+$ cells present within muscle were both Ly-6C$^+$ and Ly-6C$^-$ (38 ± 2 and 61 ± 3 %, respectively, on 3 experiments) (Fig. 3A right lower, "muscle"). These data indicate that muscle Ly-6C$^-$ cells derived from early recruited Ly-6C$^+$ MO/MPs.

Several markers have been associated with MP phenotype transition at time of resolution of inflammation. Among them, secretory leukocyte protease inhibitor (SLPI) is induced in MPs by proinflammatory signals to trigger down-regulation of proinflammatory cues (29-32). RT-PCR analysis showed that Ly-6C$^+$ MO/MPs strongly expressed SLPI compared to Ly-6C$^-$

MO/MPs (62% increase of band intensity on 3 experiments) (Fig. 3B) 4 days after injury. Inversely, PPARγ is expressed by MPs that down-regulate inflammation (33-35). Our results showed that PPARγ mRNA was strongly expressed by Ly-6C$^-$ compared to Ly-6C$^+$ MO/MPs (49% increase of band intensity on 3 experiments) (Fig. 3B).

Forward and side scatter of the two MO/MP subsets isolated at d 7 post injury showed that Ly-6C$^-$ MO/MPs had higher side scatter, and were larger than Ly-6C$^+$ MO/MPs (Fig. 3C), in accordance with a maturation of the Ly-6C$^-$ subset. Analysis of differentiation markers showed that Ly-6C$^-$ MO/MPs expressed CD11c and higher levels of F4/80 (Fig. 3C). Nearly all (> 90%) F4/80$^+$ cells were CD11c$^+$ and I-A^{b+}, and no positivity was observed for the dendritic cell marker DEC-205 (data not shown). These results show that on the contrary to Ly-6C$^+$ MO/MPs, Ly-6C$^-$ MO/MPs exhibited high levels of MP differentiation markers.

Altogether, these results suggest that after muscle injury, inflammatory Ly-6C$^+$ monocytes are recruited from blood, convert into anti-inflammatory Ly-6C$^-$ MO/MPs that proliferate in situ and further differentiate into mature MPs.

Phagocytosis of muscle cell debris participates to MP phenotype transition. To study interactions between MPs and myogenic cells possibly involved in MP phenotype transition, we used in vitro human cell cocultures. Indeed, in vitro pro and anti-inflammatory MP activation states described in humans most likely correspond to Ly-6C$^+$ and Ly-6C$^-$ MO/MP profiles found in regenerating mouse muscle. Consistently, LPS/IFNγ treatment of human MPs induced TNFα ($P<0.05$) and IL-1β ($P<0.05$) secretion (Fig. 4A) whereas dexamethasone (DEX)/IL-10 treatment induced IL-10 ($P<0.05$) secretion (Fig. 4A), as previously shown (36,37). Since differentiating muscle cells secrete IL-4 (38), a key inducer of alternative activation of MPs (5), we tested IL-4 treatment, that induced TGFβ1 secretion (Fig. 4A) as previously shown (39).

As a major role of MPs is to operate phagocytosis of dead cells, we evaluated the effect of phagocytosis of muscle cell debris on MP inflammatory profile. Upon phagocytosis of

necrotic mpcs, LPS/IFNγ treated MPs both decreased their TNFα secretion (P<0.05) and increased their TGFβ1 secretion (P<0.03) (Fig.4B). We attempted to inhibit phagocytosis of proinflammatory MPs by colchicine (40), cytochalasin D (41) and recombinant Annexin V (42). Colchicine failed to inhibit phagocytosis and MPs changed their cytokine secretion upon mpc debris phagocytosis, as in untreated cells (Fig. 4C). Conversely, phagocytosis was inhibited in the presence of cytochalasin D or recombinant Annexin V; in these conditions, TNFα and TGFβ1 secretion did not change in presence of mpc debris (Fig. 4C). These data indicate that phagocytosis of mpc debris induced a switch of MP phenotype toward an anti-inflammatory profile, as previously shown for other cell types (43-46).

We also evaluated whether interactions with living mpcs may influence MP phenotype. Neither addition of mpcs to previously activated MPs nor addition of mpcs prior to MP activation did significantly alter MP phenotype (data not shown). These data show that mpcs neither modified MP activation state nor prevented acquisition of any given MP phenotype.

Inflammatory MPs increase mpc growth whereas anti-inflammatory MPs stimulate their differentiation. In coculture experiments we analyzed mpc behavior depending on MP activation state. We have previously shown that untreated MPs stimulate mpc growth and proliferation (26). As shown in Fig. 5, mpc differentiation (Fig. 5C) and fusion (Fig. 5D) were also increased by untreated MPs (P≤0.05). LPS/IFNγ-treated MPs were more potent to enhance mpc growth (63%, P<0.05) (Fig. 5A), and accordingly, they enhanced mpc proliferation, as shown by increased 5-bromo-2-deoxy-uridine (BrdU) incorporation (25%, P<0.05) (Fig. 5B). Inversely, both myogenin expression (Fig. 5C) and myotube formation (Fig. 5D) were strongly reduced (58% and 68% respectively, P<0.05), indicating an inhibition of differentiation by inflammatory MPs. IL-4-treated MPs slightly reduced mpc proliferation (14%, P<0.05) (Fig. 5B) while they stimulated myogenin expression (28%, P<0.05) (Fig. 5C) and stimulated myotube formation (158%, P<0.05) (Fig. 5D) compared to untreated MPs. DEX/IL-10-treated MPs did not stimulate growth (Fig. 5A) and proliferation (Fig. 5B)

compared to untreated MPs, but presented a strong pro-differentiating activity, as assessed by increased myogenin expression (52%, $P<0.05$) (Fig. 5C) and increased myotube formation (136%, $P<0.01$) (Fig. 5D). These results strongly suggest that activation state of MPs may monitor the myogenic process.

MO/MPs presence is mandatory for skeletal muscle regeneration. We took advantage of the CD11b-diphtheria toxin receptor (DTR) mouse to study the respective roles of MO/MP subsets during muscle regeneration in vivo, i.m. injection of clo-lip being toxic for myogenic cells (unpublished data). In CD11b-DTR mice, monocytes - and possibly tissue MPs depending on the way of injection - are depleted after diphtheria toxin (DT) injection (47). After a single i.v. DT injection, circulating monocytes loss occurred from 6 h to reach a maximum at 12 h, that lasted 12 h more, with subsequent restoration and normalization at 48 h (Fig. 6A). At maximum of depletion, 75-80% of cells in the monocyte gate had disappeared (Fig. 6A) as did F4/80+, CD11b+ and Ly6C+ cells (data not shown). Time course of post injury muscle regeneration in untreated CD11b-DTR mice was similar to that of C57BL/6 mouse shown in figure 1 until d 4, but centrally nucleated myofibers appeared earlier in CD11b-DTR than in C57BL/6 (d 7 vs. d 10) (Fig. 6B). When DT was injected simultaneously to (Fig. 6C), or 12 h before (Fig. 6D) muscle injury, necrotic fibers were removed more slowly, and regeneration was impaired with appearance of adipose cells (Fig. 6C,D, Fig. 7). After double DT injection both 12 h before and simultaneously to notexin injection (that induced a monocyte depletion lasting 0-24 h post injury), almost no MO/MP infiltration was observed (Fig. 6E). None of the hallmarks of regeneration were detected and persistence of necrotic fibers was observed until d 7 (Fig. 6E, Fig. 7), although circulating monocytes reappeared in the circulation from d 2 post injury (data not shown). When DT was injected 4 days after notexin, no difference was observed compared to control (Fig. 6B, Fig. 7), indicating that circulating monocytes were no longer recruited into muscle at this time. Notably, i.v. injected DT did not significantly affect intramuscular MO/MPs, as shown by

unchanged expression of both F4/80 and CD11b by $CD45^+$ cells isolated from muscle (Fig. 6F, insert).

We attempted to target intramuscular antiinflammatory MO/MPs, at a time when phagocytosis of necrotic myofibers is finished (Fig. 6B, Fig. 7A). To assess MP depletion in muscle, flow cytometric analysis of $CD45^+$ cells was performed 24 h after a single i.m. DT injection. The total number of $F4/80^+$ cells present in muscle was diminished by 25% after DT injection, especially that of $F4/80^{hi}$ cells, by 75% (Fig. 8A) ($P<0.05$), indicating that these differentiated $FSC^{hi/}F4/80^{hi}$ MPs, corresponding to Ly-$6C^-$ MPs, were targeted by DT. However, total number of $CD45^+$ cells increased by 2 fold in DT vs. PBS injected muscle (Fig. 8A), indicating that i.m. DT injection induced a secondary inflammation accompanied by the recruitment of $F4/80^-$ leukocytes (likely neutrophiles and $F4/80^{lo/-}$ monocytes). Consistently, histological examination 4-5 days later of whole TA muscle showed an area in which new cycles of regeneration were visible as assessed by the presence of necrotic and basophilic fibers (Fig. 8B, arrow). This area was restricted to the site of needle puncture, and was therefore excluded from the analysis to avoid counting of small regenerating myofibers coming from the secondary lesion (Fig. 8B). Diameter of centrally nucleated regenerating myofibers was evaluated in all the rest of TA and was found reduced by 41% in DT- vs. PBS-injected muscle (30.8 vs. 18.2 µm, respectively) (Fig. 8C) ($p<0.001$).

DISCUSSION

In the present study, we have shown that: i) injured skeletal muscle selectively recruits Ly-6C$^+$ circulating monocytes which exhibits an inflammatory profile; ii) within muscle, Ly-6C$^+$ monocytes switch their phenotype to acquire an anti-inflammatory profile, phagocytosis of muscle cell debris being most likely involved in this transition; iii) as necrosis disappears from the injured area, anti-inflammatory Ly-6C$^-$ MO/MPs proliferate and further differentiate into MPs; iv) in vitro, inflammatory MPs enhance myogenic cell proliferation and repress their differentiation, whereas anti-inflammatory MPs stimulate myogenesis and fusion; v) consistently, in vivo depletion of circulating monocytes at time of injury totally prevents muscle regeneration, whereas later depletion of intramuscular MPs decreases diameter of regenerating fibers.

It has been shown that the CX3CR1^{lo}/Ly-6C$^+$ monocyte population is recruited in injured or inflamed tissues (9,13,48). Our experiments using LX labeled circulating monocytes showed that CX3CR1^{lo}/Ly-6C$^+$ monocytes were selectively recruited rapidly after muscle injury. Mouse CX3CR1^{lo}/Ly-6C$^+$ monocytes and their human analogs bear several chemokine receptors, including CCR2, CCR1, CCR4, CCR7, CXCR1 and CXCR2 (9). The corresponding chemokines are expressed by muscle tissue during the first days post injury (22,49,50) (cytokine expression profile is available at http://pepr.cnmcresearch.org). MCP-1 has been particularly involved in monocyte recruitment by injured muscle (19,20). We did not evidence recruitment of CX3CR1^{hi}/Ly-6C$^-$ monocytes from blood to muscle. Moreover, transient depletion of circulating monocytes during the first 0-24 h after muscle injury showed no subsequent MP infiltration despite reconstitution of the pool of circulating monocytes. This suggests that CX3CR1^{lo}/Ly-6C$^+$ MO/MP recruitment takes place early after injury, within the 2/3 first d post injury.

Our experiments showed that initially LX labeled CX3CR1^{lo}/Ly-6C$^+$ monocytes converted into CX3CR1^{hi}/Ly-6C$^-$ MO/MPs from d 2 after injury, indicating a phenotype transition of MO/MPs within muscle. Consistently, MP differentiation markers were weakly expressed by

CX3CR1^{lo}/Ly-6C^{+} MO/MPs and markedly expressed by CX3CR1^{hi}/Ly-6C^{-} MO/MPs (see below). We propose that CX3CR1^{lo}/Ly-6C^{+} cells, that exhibit a inflammatory phenotype (expressing TNFα and IL-1β) progressively loose Ly-6C expression while converting into anti-inflammatory MO/MPs (expressing TGFβ1 and IL-10). These data are in keeping with the cytokine mRNA analysis of post injured muscle extract (51) (available at http://pepr.cnmcresearch.org): TNFα and IL-1β expression peaks at d 2-3 post injury; IL-10 and TGFβ1 expression increases from d 2 post injury and is maintained throughout regeneration. MP phenotype conversion observed in skeletal muscle further supports previous in vitro evidence that MPs are capable of rapid adaptation to changing environments (4,6-8) and in vivo demonstration that MPs alter their phenotype according to their tissue environment (52).

MPs participate to both amplification of inflammation at time of injury and down-regulation of the inflammatory response to avoid excess tissue damage (31,53,54). MP stop-signals associated with resolution of inflammation include: i) induction of negative regulators of inflammation in MPs by environmental cues, and ii) non phlogistic phagocytosis. As shown herein, both likely operate during skeletal muscle regeneration. SLPI is considered as a brake on the response of MPs to inflammation (29,31). Its expression is induced by LPS and IL-6 and leads to inhibition of nitric oxide (NO) and TNFα production (30). In vivo, the highest SLPI synthesis occurs during the proinflammatory phase and thus anticipates increase of TGFβ1 and IL-10 production (32). Accordingly, we have shown that CX3CR1^{lo}/Ly-6C^{+} MO/MPs expressed more SLPI than CX3CR1^{hi}/Ly-6C^{-} cells. Another marker associated with the resolution of inflammation by MPs is PPAR-γ. Fatty acids and prostaglandin metabolites bind to PPAR-γ in activated MPs, which triggers inhibition of both inducible NO synthase and release of proinflammatory cytokines (33,35), thus conferring anti-inflammatory properties to PPAR-γ (34). Here we showed that antiinflammatory CX3CR1^{hi}/Ly-6C^{-} MO/MPs strongly expressed PPAR-γ. Non phlogistic phagocytosis by MPs is another signal for switching to healing (31). Binding and phagocytosis of apoptotic cells inhibit secretion of proinflammatory

mediators and stimulate secretion of TGFβ1 and IL-10 by inflammatory MPs (43-46). Induction of an anti-inflammatory process by phagocytosis of necrotic cells depends on the cell type ingested (42,43,55). We showed that both unstimulated and inflammatory MPs adopted an anti-inflammatory profile upon phagocytosis of necrotic muscle cell debris and that inhibition of MP phagocytosis prevented this phenotype switch. Altogether, these results strongly suggest that recruited inflammatory CX3CR1^{lo}/Ly-6C^{+} MO/MPs convert into anti-inflammatory CX3CR1^{hi}/Ly-6C^{-} MO/MPs upon both ingestion of muscle cell debris and expression of stop signals extinguishing inflammation.

Our results showed that once switched, CX3CR1^{hi}/Ly-6C^{-} MO/MPs actively proliferated, therefore contributing to the large amounts of MO/MPs observed in muscle at d 4 to d 7 post injury. In vitro, MP proliferation is inhibited by inflammatory mediators (LPS, IFNγ, NO, IL-6) and is stimulated by TGFβ1 (56-58). Thus, proliferation of recruited MO/MPs may be due to changes in their environment, although as yet unknown mechanisms related to the change of phenotype cannot be excluded. Later during muscle regeneration (d 7), CX3CR1^{hi}/Ly-6C^{-} MO/MP proliferation slowed down together with the fully differentiation into MPs, as shown by their strong F4/80 expression (59). Almost all F4/80^{+} cells were also positive for CD11c and MHCII, as previously observed after inflammation in various organs (60,61), and were DEC-205^{-}. In the blood, Ly-6C^{-} monocytes, that come from Ly-6C^{+} cells, are also CD11c^{+} (12). Therefore, appearance of CD11c^{+} cells in the muscle may be a recapitulation of conversion from Ly-6C^{+}/CD11c^{-} to Ly-6C^{-}/CD11c^{+} monocytes within the tissue. Thus, proliferating CX3CR1^{hi}/Ly-6C^{-} MO/MPs and then differentiating CX3CR1^{hi}/Ly-6C^{-} MPs are associated with the regenerative phase of muscle repair.

Depending on the context, MPs may have supportive or deleterious effects on cells: in chronic diseases, including those affecting skeletal muscle, MPs are deleterious (62,63) while they support tissue repair in muscle and other various tissues including liver, brain, peripheral nerve and epithelium (19-22,64-67). If one except regulation of inflammation, studies documenting a direct role of MPs on cell behavior are scant and include intestinal

progenitor proliferation (65), erythroblast proliferation and maturation (68), oligodendrocytic differentiation and myelination (69). In vitro studies have shown that MP activation state may direct neural progenitor differentiation towards either neurogenesis or oligodendrogenesis (70). Our results substantiate the view that MP function may be related to an activation state. In coculture experiments, inflammatory MPs -the counterpart of inflammatory CX3CR1^{lo}/Ly-6C^{+} cells observed in vivo- stimulated mpc proliferation and inhibited their differentiation. Inversely, IL-4 treated and anti-inflammatory MPs exhibited a strong differentiating activity on mpc, assessed by both stimulation of myogenic program and increase of fusion into multinucleated cells. Molecular mechanisms involved in these processes are currently under investigation. It is likely that cytokines released by activated MPs influence myogenic cell behavior: TNFα is mitogenic for myoblasts and inhibits their differentiation (71,72); IL-1β impairs myogenic differentiation through IGF-1 (73); in vitro effects of TGFβ1 are more controversial, although in vivo neutralization of TGFβ1 in regenerating muscle was shown to reduce the diameter of regenerating myofibers (74). Beyond cytokines, COX2 and its metabolites may also play a role, as they were shown to be fusogenic and necessary for good muscle repair (75-77).

Previous in vivo studies using different injury models have shown that partial reduction of MO/MP entry into injured muscle hinders muscle regeneration, as shown by a delay in the appearance of regenerating myofibers and the persistence of intermuscular adipocytes (19-22). We observed similar features upon partial inhibition of monocyte recruitment in toxic-induced muscle regeneration. Moreover, when nearly no MO/MP was allowed to enter damaged muscle, myofibers remained in the necrotic state, pointing out the indispensable role of MPs in muscle repair. Interestingly, despite normalized level of circulating monocytes from d 2 post injury, no delayed recruitment was observed suggesting that signals governing monocyte recruitment are only transiently expressed by the damaged tissue, as it was recently documented in ultraviolet-injured skin (48). Accordingly, depletion of circulating monocytes at d 4 post injury had no effect on muscle regeneration. On the other hand,

depletion of intramuscular anti-inflammatory F4/80^{hi} MO/MPs at time of regeneration, i.e. once phagocytosis of necrotic myofibers is achieved, reduced the diameter of the centrally nucleated regenerating myofibers, indicating that these MO/MPs were involved in fiber growth, in accordance with our in vitro data.

In conclusion, our data evidence a phenotype transition operated by recruited MO/MPs during resolution of inflammation and tissue repair, that is associated to changes in their functions. We propose sequential involvements of these two phenotypically distinct MP populations during skeletal muscle repair as follows: within the first 24-48 h after injury, skeletal muscle recruits inflammatory circulating monocytes, that stimulate myogenic cell proliferation and prevent their differentiation. While they are exposed to inflammatory environment and operate phagocytosis of muscle cell debris, inflammatory MO/MPs convert into anti-inflammatory MO/MPs. As phagocytosis of necrotic myofibers is finished, these anti-inflammatory MO/MPs actively proliferate and further differentiate into anti-inflammatory MPs, that sustain myogenic differentiation and myofiber growth, in addition to their protective effect on differentiating myotubes (27) and their effect on fiber membrane repair (78).

MATERIALS AND METHODS

Animals. C57BL/6, CX3CR1$^{gfp/+}$ (9) and CD11b-DTR (47) mice were bred and used according to french legislation. Experiments were run at 4-8 weeks of age.

Muscle injury and muscle preparation. Notexin (10 µl, 25 µg/ml in PBS, Latoxan, Valence, France) was injected in the TA. For histological analysis, muscles were prepared as previously described (27). Quantitative analysis of muscle regeneration was performed on the whole injured area: about 7 fields (x20 objective) were analyzed in each mouse, representing 300-400 fibers per mouse. Myofiber diameter was evaluated after collagen IV immunolabeling (see below) on about 7 fields (x20 objective) in each mouse. The small diameter of only centrally nucleated myofibers was evaluated in late regenerating muscle (non hachured area in Fig. 8B) with Axiovision 4.6 software (Carl Zeiss SAS, LePecq, France), representing 250-350 fibers per mouse. In PBS-injected mice, the punctured fascicule was omitted from analysis.

Isolation of MO/MPs from muscle. Fascia of the TA was removed. Muscles were dissociated in DMEM containing collagenase B 0.2% (Roche Diagnostics GmbH, Mannheim, Germany) and trypsine-EDTA 0.2% at 37°C for 45 min twice, filtered and counted. CD45^{+} cells were isolated using magnetic sorting (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and stained with PE- or PC5- conjugated anti-Gr1 Ab (reacts with Ly-6C and Ly-6G, but only Ly-6C is expressed by monocytes) (eBiosciences, San Diego, CA). Cells were sorted using an Epics Elite cell sorter (Beckman-Coulter, Fullerton, CA). Populations presenting over 90% of purity were used. In some experiments, PE-conjugated CD11c, I-A^{b} (BD Biosciences) and F4/80 (AbD Serotec, Oxford, UK) Ab were used. Analysis was performed with a FACSCalibur cytometer (BD Biosciences).

Labeling of blood monocytes. Labeling of circulating monocytes was performed exactly as described (15) with plain microspheres (Fluoresbrite polychromatic red 0.5-µm microspheres 2.5% solids; Polysciences Inc., Warrington, PA) and clo-lip, that were prepared as previously described (79). Clodronate was a kind gift of Roche Diagnostics GmbH.

Depletion of circulating monocytes and intramuscular MPs. DT was i.v. injected into CD11b-DTR mice (12 ng/g). Blood was retroorbitally harvested at various times post-injection and cells were labelled with anti-CD11b, anti-Gr1, F4/80 Ab and analyzed by flow cytometry. Due to high interindividual variations (3 to 12% of PBMC), the number of circulating monocytes in control mice was normalized to 5% of PBMC for each series (mean calculated > 25 mice). To deplete infiltrated MO/MP, DT was i.m. injected (25 ng/g in less than 10 µl) at d 5 and 6 post injury. Controls included i.v. injection of PBS.

Immunolabeling. Muscle slides were incubated with anti-collagen IV Ab (1/50, Chemicon International, Inc.,Temecula, CA) revealed with Cy5-conjugated anti-rabbit Ab. Murine sorted cells were centrifugated on slides and labeled with anti-Ki67 Ab (1 µg/ml) (Abcam plc, Cambridge, UK), revealed with FITC-conjugated anti-rabbit Ab. Cultured human mpcs were incubated with anti-desmin Ab (60 µg/ml, Abcam) revealed by a Cy3-conjugated anti-rabbit Ab and with anti-myogenin Ab (10 µg/ml, BD Biosciences) revealed by a biotinylated anti-mouse (Vector Laboratories Inc, Burlingame, CA) and by DTAF-streptavidin (Beckman Coulter). Controls included incubation with whole rabbit or mouse IgGs. Other secondary antibodies and IgGs were from Jackson ImmunoResearch Laboratories, Inc. West Grove, PA.

RT-PCR. Total RNA were prepared from sorted cells using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany). 0.5 µg of total RNA was reverse transcripted using Superscript II reverse transcriptase and then amplified with a platinum Taq DNA polymerase (Invitrogen Ltd, Paisley, UK) and specific primers (sens and antisens, respectively): β2microglobulin: 5'-CAGTTCCACCCG-CCTCAC-3', 5'-CACATGTCTCGATCCCAG-3'; TNFα: 5'-TTCCAGATTCTTCCCTGAGGT-3', 5'-TAAGCAAAAGAGGAGGCAACA-3'; IL-1β: 5'-TGACGTTCCCATTAGACAACTG-3'; 5'-CCGTCTTTCATTACACAGGACA-3'; TGFβ1: 5'-GAGACGGAATACAGGGCTTTC-3', 5'-TCTCTGTGGAGCTGAAGCAAT-3'; IL-10: 5'-ACCAGCTGGACAACATACTGC-3', 5'-TCACTCTTCACCTGCTCCACT-3'; SLPI: 5'-CCTTAAGCTTGAGAAGCCACA-3', 5'- AGCACTTGTATTTGCCGTCAC; PPARγ: 5'-

AAGAGCTGACCCAATGGTTG-3', 5'-GGATCCGGCAGTTAAGATCA-3'. Amplification was performed at 94, 60 and 72°C for 1 min each step for 30 cycles. Amplification products (10 µl) were subjected to electrophoresis on 2% agarose gel containing ethidium bromide for visualization. Quantification was performed by using Scion Image software.

Human mpc culture. Culture media components were from Invitrogen. Human mpcs were cultured from muscle samples as previously described (26).

Human MP cell culture. MPs were differentiated from monocytes isolated from human blood as previously described, at time of corrective surgery in conformity with french legislation (Code de la Santé Publique, livre II) (26) . Differentiated MPs were further cultured in either RPMI medium containing 15% FBS or advanced RPMI medium containing 0.5% FBS (supplemented with sodium pyruvate 1%, Hepes 10 mM, βmercaptoethanol 50 µM, non essential amino acids 1%, vitamins 100X 1%). MPs were treated, or not, for 48 h with either LPS (1 µg/ml, Sigma-Aldrich, St. Louis, MO) and IFNγ (10 ng/ml, PeproTech Inc., Rocky Hill, NJ), or IL-4 (10 ng/ml, PeproTech) or DEX (80 ng/ml, Sigma-Aldrich) and IL-10 (10 ng/ml, PeproTech).

Phagocytosis. Mpc necrosis was induced by H_2O treatment for 1 h at 37°C. 100% of cells were propidium iodide positive. Necrotic mpcs were seeded on MPs (5 dead mpcs for 1 MP) for 3 h at 37°C. MP cultures were washed 3 times to remove non-ingested material and further cultured in serum-free advanced RPMI supplemented medium during 24 h to make conditioned media. In some experiments, cells were treated, as previously described, with colchicine (10 µg/ml, Sigma) (40), cytochalasin D (1 µg/ml, Sigma) (41) or recombinant annexin V (40 µg/ml, BD Biosciences) (42). Phagocytosis was quantified in the same conditions after incubation with fluorescent microspheres (same as above). The number of LX^+ cells was quantified under an inverted microscope and expressed as percentage of total cells.

Co-cultures. Mpcs were plated on previously prepared MP cultures (3:1 [MP:mpc] ratio) in advanced RPMI supplemented medium, except in some experiments in which MPs and mpcs were seeded together before MP treatment was applied as described above.

Mpc behavior. Mpc growth was evaluated as described before (26). Mpc proliferation was estimated by BrdU incorporation (Roche Diagnostics). Mpc differentiation was evaluated by counting the number of myogenin$^+$ cells among desmin$^+$ cells. Mpc fusion was estimated by counting the number of nuclei per myotube.

Statistical analyses. All experiments were performed using at least 3 different cultures or animals in independent experiments. The student t test was used for statistical analyses. $P < 0.05$ was considered significant.

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REFERENCES

1. Lapidot, T., and I. Petit. 2002. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Exp.Hematol.* 30:973-981.
2. Gordon, S., and P. R. Taylor. 2005. Monocyte and macrophage heterogeneity. *Nat.Rev.Immunol.* 5:953-964.
3. Gordon, S. 1995. The macrophage. *Bioessays* 17:977-986.
4. Stout, R. D., and J. Suttles. 2004. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J.Leukoc.Biol.* 76:509-513.
5. Gordon, S. 2003. Alternative activation of macrophages. *Nat.Rev.Immunol.* 3:23-35.
6. Gratchev, A., J. Kzhyshkowska, K. Kothe, I. Muller-Molinet, S. Kannookadan, J. Utikal, and S. Goerdt. 2006. Mphi1 and Mphi2 can be re-polarized by Th2 or Th1 cytokines, respectively, and respond to exogenous danger signals. *Immunobiology.* 211:473-486.
7. Porcheray, F., S. Viaud, A. C. Rimaniol, C. Leone, B. Samah, N. Dereuddre-Bosquet, D. Dormont, and G. Gras. 2005. Macrophage activation switching: an asset for the resolution of inflammation. *Clin.Exp.Immunol.* 142:481-489.
8. Stout, R. D., C. Jiang, B. Matta, I. Tietzel, S. K. Watkins, and J. Suttles. 2005. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J.Immunol.* 175:342-349.
9. Geissmann, F., S. Jung, and D. R. Littman. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19:71-82.
10. Ancuta, P., L. Weiss, and N. Haeffner-Cavaillon. 2000. CD14+CD16++ cells derived in vitro from peripheral blood monocytes exhibit phenotypic and functional dendritic cell-like characteristics. *Eur.J.Immunol.* 30:1872-1883.
11. Sunderkotter, C., T. Nikolic, M. J. Dillon, N. van Rooijen, M. Stehling, D. A. Drevets, and P. J. Leenen. 2004. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J.Immunol.* 172:4410-4417.

12. Tacke, F., D. Alvarez, T. J. Kaplan, C. Jakubzick, R. Spanbroek, J. Llodra, A. Garin, J. Liu, M. Mack, N. van Rooijen, S. A. Lira, A. J. Habenicht, and G. J. Randolph. 2007. Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J.Clin.Invest* 117:185-194.

13. Swirski, F. K., P. Libby, E. Aikawa, P. Alcaide, F. W. Luscinskas, R. Weissleder, and M. J. Pittet. 2007. Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J.Clin.Invest* 117:195-205.

14. Fingerle, G., A. Pforte, B. Passlick, M. Blumenstein, M. Strobel, and H. W. Ziegler-Heitbrock. 1993. The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients. *Blood* 82:3170-3176.

15. Tacke, F., F. Ginhoux, C. Jakubzick, N. van Rooijen, M. Merad, and G. J. Randolph. 2006. Immature monocytes acquire antigens from other cells in the bone marrow and present them to T cells after maturing in the periphery. *J.Exp.Med.* 203:583-597.

16. Varol, C., L. Landsman, D. K. Fogg, L. Greenshtein, B. Gildor, R. Margalit, V. Kalchenko, F. Geissmann, and S. Jung. 2006. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J.Exp.Med.* 104:171-180.

17. McLennan, I. S. 1996. Degenerating and regenerating skeletal muscles contain several subpopulations of macrophages with distinct spatial and temporal distributions. *J.Anat.* 188:17-28.

18. Pimorady-Esfahani, A., M. D. Grounds, and P. G. McMenamin. 1997. Macrophages and dendritic cells in normal and regenerating murine skeletal muscle. *Muscle Nerve* 20:158-166.

19. Contreras-Shannon, V., O. Ochoa, S. M. Reyes-Reyna, D. Sun, J. E. Michalek, W. A. Kuziel, L. M. McManus, and P. K. Shireman. 2007. Fat Accumulation with Altered Inflammation and Regeneration in Skeletal Muscle of CCR2 -/- Mice Following Ischemic Injury.*Am. J. Physiol Cell Physiol.* 292:C953-C967.

20. Shireman, P. K., V. Contreras-Shannon, O. Ochoa, B. P. Karia, J. E. Michalek, and L. M. McManus. 2006. MCP-1 deficiency causes altered inflammation with impaired skeletal muscle regeneration.*J. Leukoc. Biol.* doi: 10.1189/jlb.0506356.

21. Summan, M., G. L. Warren, R. R. Mercer, R. Chapman, T. Hulderman, N. van Rooijen, and P. P. Simeonova. 2006. Macrophages and skeletal muscle regeneration: a clodronate-containing liposome depletion study. *Am.J.Physiol Regul.Integr.Comp Physiol.* 290:R1488-R1495.

22. Warren, G. L., T. Hulderman, D. Mishra, X. Gao, L. Millecchia, L. O'Farrell, W. A. Kuziel, and P. P. Simeonova. 2004. Chemokine receptor CCR2 involvement in skeletal muscle regeneration. *FASEB J.* 19:413-415.

23. Grounds, M. D. 1987. Phagocytosis of necrotic muscle in muscle isografts is influenced by the strain, age, and sex of host mice. *J.Pathol.* 153:71-82.

24. Robertson, T. A., M. A. L. Maley, M. D. Grounds, and J. M. Papadimitriou. 1993. The role of macrophages in skeletal muscle regeneration with particular reference to chemotaxis. *Exp.Cell Res.* 207:321-331.

25. Cantini, M., and U. Carraro. 1995. Macrophage-released factor stimulates selectively myogenic cells in primary muscle culture. *J.Neuropathol.Exp.Neurol.* 54:121-128.

26. Chazaud, B., C. Sonnet, P. Lafuste, G. Bassez, A. C. Rimaniol, F. Poron, F. J. Authier, P. A. Dreyfus, and R. K. Gherardi. 2003. Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth. *J.Cell Biol* 163:1133-1143.

27. Sonnet, C., P. Lafuste, L. Arnold, M. Brigitte, F. Poron, F. J. Authier, F. Chretien, R. K. Gherardi, and B. Chazaud. 2006. Human macrophages rescue myoblasts and myotubes from apoptosis through a set of adhesion molecular systems. *J.Cell Sci.* 119:2497-2507.

28. St Pierre, B. A., and J. G. Tidball. 1994. Differential response of macrophage subpopulations to soleus muscle reloading after rat hindlimb suspension. *J.Appl.Physiol.* 77:290-297.

29. Jin, F., C. F. Nathan, D. Radzioch, and A. Ding. 1998. Lipopolysaccharide-related stimuli induce expression of the secretory leukocyte protease inhibitor, a macrophage-derived lipopolysaccharide inhibitor. *Infect.Immun.* 66:2447-2452.

30. Jin, F. Y., C. Nathan, D. Radzioch, and A. Ding. 1997. Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide. *Cell* 88:417-426.

31. Nathan, C. 2002. Points of control in inflammation. *Nature* 420:846-852.

32. Odaka, C., T. Mizuochi, J. Yang, and A. Ding. 2003. Murine macrophages produce secretory leukocyte protease inhibitor during clearance of apoptotic cells: implications for resolution of the inflammatory response. *J.Immunol.* 171:1507-1514.

33. Ricote, M., A. C. Li, T. M. Willson, C. J. Kelly, and C. K. Glass. 1998. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* 391:79-82.

34. Moore, K. J., M. L. Fitzgerald, and M. W. Freeman. 2001. Peroxisome proliferator-activated receptors in macrophage biology: friend or foe? *Curr.Opin.Lipidol.* 12:519-527.

35. Alleva, D. G., E. B. Johnson, F. M. Lio, S. A. Boehme, P. J. Conlon, and P. D. Crowe. 2002. Regulation of murine macrophage proinflammatory and anti-inflammatory cytokines by ligands for peroxisome proliferator-activated receptor-gamma: counter-regulatory activity by IFN-gamma. *J.Leukoc.Biol.* 71:677-685.

36. Hogger, P., J. Dreier, A. Droste, F. Buck, and C. Sorg. 1998. Identification of the integral membrane protein RM3/1 on human monocytes as a glucocorticoid-inducible member of the scavenger receptor cysteine-rich family (CD163). *J.Immunol.* 161:1883-1890.

37. Stein, M., S. Keshav, N. Harris, and S. Gordon. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J.Exp.Med.* 176:287-292.

38. Horsley, V., K. M. Jansen, S. T. Mills, and G. K. Pavlath. 2003. IL-4 Acts as a Myoblast Recruitment Factor during Mammalian Muscle Growth. *Cell* 113:483-494.

39. Song, E., N. Ouyang, M. Horbelt, B. Antus, M. Wang, and M. S. Exton. 2000. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cell.Immunol.* 204:19-28.

40. Lucas, M., L. M. Stuart, A. Zhang, K. Hodivala-Dilke, M. Febbraio, R. Silverstein, J. Savill, and A. Lacy-Hulbert. 2006. Requirements for Apoptotic Cell Contact in Regulation of Macrophage Responses. *J.Immunol.* 177:4047-4054.

41. Cvetanovic, M., and D. S. Ucker. 2004. Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. *J.Immunol.* 172:880-889.

42. Brouckaert, G., M. Kalai, D. V. Krysko, X. Saelens, D. Vercammen, Ndlovu', G. Haegeman, K. D'Herde, and P. Vandenabeele. 2004. Phagocytosis of necrotic cells by macrophages is phosphatidylserine dependent and does not induce inflammatory cytokine production. *Mol.Biol.Cell* 15:1089-1100.

43. Fadok, V. A., D. L. Bratton, L. Guthrie, and P. M. Henson. 2001. Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. *J.Immunol.* 166:6847-6854.

44. Huynh, M. L., V. A. Fadok, and P. M. Henson. 2002. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J.Clin.Invest* 109:41-50.

45. Savill, J., and V. Fadok. 2000. Corpse clearance defines the meaning of cell death. *Nature* 407:784-788.

46. Xu, W., A. Roos, N. Schlagwein, A. M. Woltman, M. R. Daha, and C. van Kooten. 2006. IL-10-producing macrophages preferentially clear early apoptotic cells. *Blood.* 107:4930-4937.

47. Duffield, J. S., S. J. Forbes, C. M. Constandinou, S. Clay, M. Partolina, S. Vuthoori, S. Wu, R. Lang, and J. P. Iredale. 2005. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J.Clin.Invest* 115:56-65.

48. Ginhoux, F., F. Tacke, V. Angeli, M. Bogunovic, M. Loubeau, X. M. Dai, E. R. Stanley, G. J. Randolph, and M. Merad. 2006. Langerhans cells arise from monocytes in vivo. *Nat.Immunol.* 7:265-273.

49. De Rossi, M., P. Bernasconi, F. Baggi, R. de Waal Malefyt, and R. Mantegazza. 2000. Cytokines and chemokines are both expressed by human myoblasts: possible relevance for the immune pathogenesis of muscle inflammation. *Int.Immunol.* 12:1329-1335.

50. Hirata, A., S. Masuda, T. Tamura, K. Kai, K. Ojima, A. Fukase, K. Motoyoshi, K. Kamakura, Y. Miyagoe-Suzuki, and S. Takeda. 2003. Expression profiling of cytokines and related genes in regenerating skeletal muscle after cardiotoxin injection: a role for osteopontin. *Am.J.Pathol.* 163:203-215.

51. Zhao, P., S. Iezzi, E. Carver, D. Dressman, T. Gridley, V. Sartorelli, and E. P. Hoffman. 2002. Slug is a novel downstream target of MyoD. Temporal profiling in muscle regeneration. *J.Biol.Chem.* 277:30091-30101.

52. Lumeng, C. N., J. L. Bodzin, and A. R. Saltiel. 2007. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J.Clin.Invest* 117:175-184.

53. Fujiwara, N., and K. Kobayashi. 2005. Macrophages in inflammation. *Curr.Drug Targets.Inflamm.Allergy* 4:281-286.

54. Serhan, C. N., and J. Savill. 2005. Resolution of inflammation: the beginning programs the end. *Nat.Immunol.* 6:1191-1197.

55. Hirt, U. A., and M. Leist. 2003. Rapid, noninflammatory and PS-dependent phagocytic clearance of necrotic cells. *Cell Death.Differ.* 10:1156-1164.

56. Celada, A., and R. A. Maki. 1992. Transforming growth factor-beta enhances the M-CSF and GM-CSF-stimulated proliferation of macrophages. *J.Immunol.* 148:1102-1105.

57. Valledor, A. F., M. Comalada, J. Xaus, and A. Celada. 2000. The differential time-course of extracellular-regulated kinase activity correlates with the macrophage response toward proliferation or activation. *J.Biol.Chem.* 275:7403-7409.

58. Xaus, J., M. Cardo, A. F. Valledor, C. Soler, J. Lloberas, and A. Celada. 1999. Interferon gamma induces the expression of p21waf-1 and arrests macrophage cell cycle, preventing induction of apoptosis. *Immunity.* 11:103-113.

59. Gordon, S. 1999. Macrophage-restricted molecules: role in differentiation and activation. *Immunol.Lett.* 65:5-8.

60. Gonzalez-Juarrero, M., and I. M. Orme. 2001. Characterization of murine lung dendritic cells infected with Mycobacterium tuberculosis. *Infect.Immun.* 69:1127-1133.

61. Liu, G., X. P. Xia, S. L. Gong, and Y. Zhao. 2006. The macrophage heterogeneity: difference between mouse peritoneal exudate and splenic F4/80+ macrophages. *J.Cell Physiol* 209:341-352.

62. Carenini, S., M. Maurer, A. Werner, H. Blazyca, K. V. Toyka, C. D. Schmid, G. Raivich, and R. Martini. 2001. The role of macrophages in demyelinating peripheral nervous system of mice heterozygously deficient in p0. *J.Cell Biol* 152:301-308.

63. Wehling, M., M. J. Spencer, and J. G. Tidball. 2001. A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice. *J.Cell Biol* 155:123-131.

64. Luk, H. W., L. J. Noble, and Z. Werb. 2003. Macrophages contribute to the maintenance of stable regenerating neurites following peripheral nerve injury. *J.Neurosci.Res.* 73:644-658.

65. Pull, S. L., J. M. Doherty, J. C. Mills, J. I. Gordon, and T. S. Stappenbeck. 2004. Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. *Proc.Natl.Acad.Sci.U.S.A* 102:99-104.

66. Rai, R. M., S. Loffreda, C. L. Karp, S. Q. Yang, H. Z. Lin, and A. M. Diehl. 1997. Kupffer cell depletion abolishes induction of interleukin-10 and permits sustained

overexpression of tumor necrosis factor alpha messenger RNA in the regenerating rat liver. *Hepatology* 25:889-895.

67. Rapalino, O., O. Lazarov-Spiegler, E. Agranov, G. J. Velan, E. Yoles, M. Fraidakis, A. Solomon, R. Gepstein, A. Katz, M. Belkin, M. Hadani, and M. Schwartz. 1998. Implantation of stimulated homologous macrophages results in partial recovery of paraplegic rats. *Nat.Med.* 4:814-821.
68. Sadahira, Y., and M. Mori. 1999. Role of the macrophage in erythropoiesis. *Pathol.Int.* 49:841-848.
69. Diemel, L. T., S. J. Jackson, and M. L. Cuzner. 2003. Role for TGF-beta1, FGF-2 and PDGF-AA in a myelination of CNS aggregate cultures enriched with macrophages. *J.Neurosci.Res.* 74:858-867.
70. Butovsky, O., Y. Ziv, A. Schwartz, G. Landa, A. E. Talpalar, S. Pluchino, G. Martino, and M. Schwartz. 2006. Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. *Mol.Cell Neurosci.* 31:149-160.
71. Langen, R. C., J. L. Van Der Velden, A. M. Schols, M. C. Kelders, E. F. Wouters, and Y. M. Janssen-Heininger. 2004. Tumor necrosis factor-alpha inhibits myogenic differentiation through MyoD protein destabilization. *FASEB J.* 18:227-237.
72. Li, Y. P. 2003. TNF-{alpha} is a mitogen in skeletal muscle. *Am.J.Physiol Cell Physiol* 285:C370-C376.
73. Broussard, S. R., R. H. McCusker, J. E. Novakofski, K. Strle, W. H. Shen, R. W. Johnson, R. Dantzer, and K. W. Kelley. 2004. IL-1beta impairs insulin-like growth factor i-induced differentiation and downstream activation signals of the insulin-like growth factor i receptor in myoblasts. *J.Immunol.* 172:7713-7720.
74. Lefaucheur, J. P., and A. Sebille. 1995. Muscle regeneration following injury can be modified in vivo by immune neutralization of basic fibroblast growth factor, transforming growth factor beta 1 or insulin-like growth factor I. *J.Neuroimmunol.* 57:85-91.

75. Bondesen, B. A., S. T. Mills, and G. K. Pavlath. 2006. The COX-2 pathway regulates growth of atrophied muscle via multiple mechanisms. *Am.J.Physiol Cell Physiol.* 290:C1651-C1659.

76. Horsley, V., and G. K. Pavlath. 2003. Prostaglandin F2(alpha) stimulates growth of skeletal muscle cells via an NFATC2-dependent pathway. *J.Cell Biol.* 161:111-118.

77. Shen, W., V. Prisk, Y. Li, W. Foster, and J. Huard. 2006. Inhibited skeletal muscle healing in cyclooxygenase-2 gene-deficient mice: the role of PGE2 and PGF2alpha. *J.Appl Physiol.* 101:1215-1221.

78. Tidball, J. G., and M. Wehling-Henricks. 2007. Macrophages promote muscle membrane repair and muscle fiber growth and regeneration during modified muscle loading in vivo.*J. Physiol.* 578:327-336.

79. van Rooijen, N., and A. Sanders. 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J.Immunol.Methods* 174:83-93.

FIGURE LEGENDS

Figure 1: Kinetics of MO/MP subsets during muscle regeneration in CX3CR1$^{GFP/+}$ mouse. (A) CD45$^+$ cells present into injured muscle were analyzed for Ly-6C and GFP (CX3CR1) expression by flow cytometry. Top: Results are expressed in percentage of CD45$^+$ cells isolated from muscle and are means ± SD of 3 experiments. Bottom: representative examples of FACS analysis at each time point. **(B)** Total number of MO/MPs was calculated from results obtained in (A) and the number of isolated CD45$^+$ cells plotted to muscle weight. Results are means ± SD of 3 experiments. **(C)** Representative hematoxylin-eosin staining of muscle sections at various times after notexin injection. Bar = 50 µm.

Figure 2: Phenotype of Ly-6C$^+$ and Ly-6C$^-$ MO/MPs during muscle regeneration. Ly-6C$^+$ and Ly-6C$^-$ MO/MP subsets were isolated by cell sorting at various times post injury. **(A)** Representative example of cell sorting of Ly-6C$^-$ (middle plot) and Ly-6C$^+$ (right plot) cells from whole MO/MPs (left plot) at d 4 post injury. **(B)** Ki67 immunostaining is expressed in percentage of isolated MO/MPs. **(C)** Expression of TNFα, IL-1β, TGFβ1 and IL-10 was analyzed by RT-PCR in isolated populations at d 4 post injury. Corresponding band intensities are given as means of 3 experiments.

Figure 3: Fate of Ly-6C$^+$ and Ly-6C$^-$ MO/MPs during muscle regeneration. (A) Circulating Ly-6C$^-$ (upper panel) and Ly-6C$^+$ (lower panel) monocytes were labeled with LX red microspheres and analyzed by flow cytometry. LX$^+$ CD45$^+$ GFP$^+$ cells (gate in "all cells" plots) were analyzed for Ly-6C expression ("LX$^+$ cells" plots) in both blood and muscle at d 3 (for Ly-6C$^-$ labeling) and d 2 (for Ly-6C$^+$ labeling) post injury. Results are representative of 3 experiments. **(B)** SLPI and PPARγ expression was analyzed by RT-PCR in Ly-6C$^+$ and Ly-6C$^-$ MO/MPs sorted at d 4 post injury. **(C)** Flow cytometry analysis of FSC/SSC characteristics, F4/80 and CD11c expression of Ly-6C$^+$ (blue label) and Ly-6C$^-$ (red label)

MO/MPs sorted at d 7 post injury (black dotted line: isotypic control). Results are representative of 3 experiments.

Figure 4: Phagocytosis and cytokine secretion by activated MPs. Cytokine secretion was evaluated by ELISA in MP-conditioned medium. **(A)** Cytokine secretion by untreated (NT), LPS/IFNγ, IL-4 and DEX/IL-10 treated MPs. **(B)** Cytokine secretion by untreated (NT) and LPS/IFNγ treated MPs after phagocytosis of muscle cell debris. **(C)** Left: phagocytosis of LX by LPS/IFNγ treated MPs incubated or not (none) with colchicine (colc) cytochalasin D (cyt D) or recombinant annexin V (ann.V). Right: cytokine secretion by LPS/IFNγ treated MPs after phagocytosis of muscle cell debris in the presence of the same effectors. Results are means ± SEM of 3 experiments.

Figure 5: Effects of activated MPs on mpc fate. Mpc were cocultured with untreated (NT), LPS/IFNγ, IL-4 and DEX/IL-10 treated MPs and further analyzed for their: **(A)** growth, **(B)** proliferation, **(C)** differentiation and **(D)** fusion. All parameters were analyzed at d 3 of coculture except for BrdU incorporation that was monitored during 24 h of coculture. Results are means ± SEM of 3 experiments. Bars bearing different symbols were significantly different.

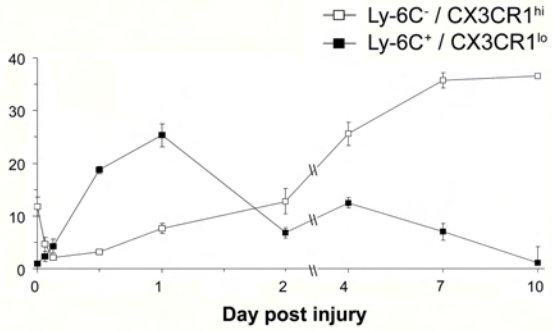
Figure 6: Effects of circulating monocyte depletion on skeletal muscle regeneration. (A) Left: kinetics of circulating monocyte depletion after one i.v. DT injection, results are means ± SD of at least 4 experiments. Right: examples of FACS analysis of blood before (-DT) and 18h after i.v. DT injection (+DT). **(B-F)** Notexin (Nx) was injected into TA of CD11b-DTR mice at d 0 and DT was injected i.v. at various times as indicated by the red arrows. Muscle histology was analyzed until d 7 after hematoxylin-eosin staining. Results are representative of at least 2 independent experiments. Bar = 50 µm. Insert in (F): $CD45^+$ cells

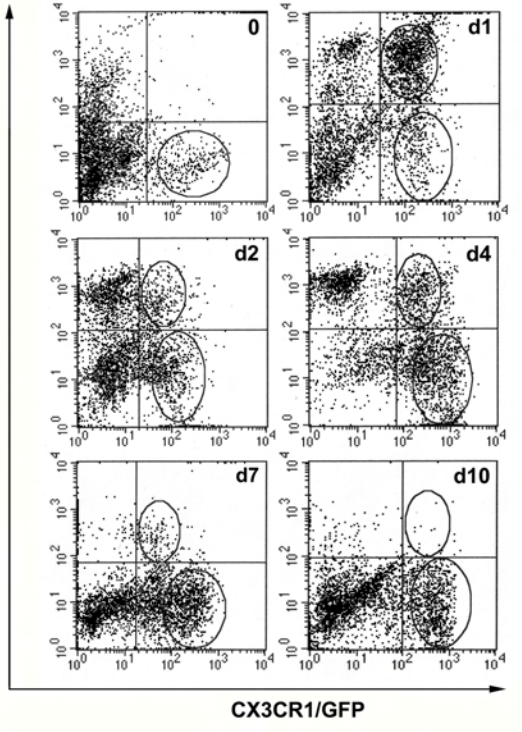
were isolated from muscle and analyzed for F4/80 and CD11b expression 24 h after an i.v. DT (blue line) or PBS (red line) injection at d 4 post injury (dotted line: isotypic control).

Figure 7: Quantitative analysis of muscle regeneration after circulating monocyte depletion. CD11b-DTR mice were treated as in figure 6 and in each case, the number of: **(A)** necrotic myofibers, **(B)** phagocyted myofibers, **(C)** regenerating basic myofibers and **(D)** centrally nucleated regenerating myofibers was evaluated. **(closed squares)**: control corresponding to Fig. 6B; **(open diamonds)**: simultaneous DT and notexine injection corresponding to Fig. 6C; **(open triangles)**: DT injection 12 h before notexine injection corresponding to Fig. 6D; **(open squares)**: double DT injection corresponding to Fig. 6E; **(closed circles)**: DT injection 4 days after notexine injection corresponding to Fig. 6F. Results are expressed in percent of total counted myofibers and are means of at least 2 experiments.

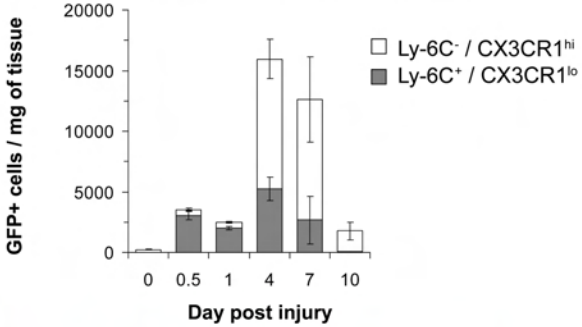
Figure 8: Effects of intramuscular DT injection on skeletal muscle regeneration. Notexin (Nx) was injected into TA of CD11b-DTR mice at d 0 and DT was injected in the same muscle at d 5 (A) and 5 and 6 (B,C). **(A)** 24 h after a single i.m. DT injection, CD45+ cells were isolated from muscle and analyzed for F4/80 expression. Red circles enclose $FSC^{hi}/F4/80^{hi}$ cells. **(B)** Reconstituted whole view of TA muscle at d 10 post Notexin injection (hematoxylin-eosine staining), presenting hallmarks of secondary regeneration restricted to the site of needle puncture (arrow). Hachured area on right picture represents the area excluded from analysis. Bar = 200 µm. **(C)** PBS- and DT-injected muscles were analyzed at d 10 post injury for myofiber diameter evaluation (hematoxylin-eosine staining). Quantified results are given for 3 independent experiments, each point corresponding to a field and red bars representing means. Bar = 50 µm.

A - Kinetics of MO/MP subsets during muscle regeneration

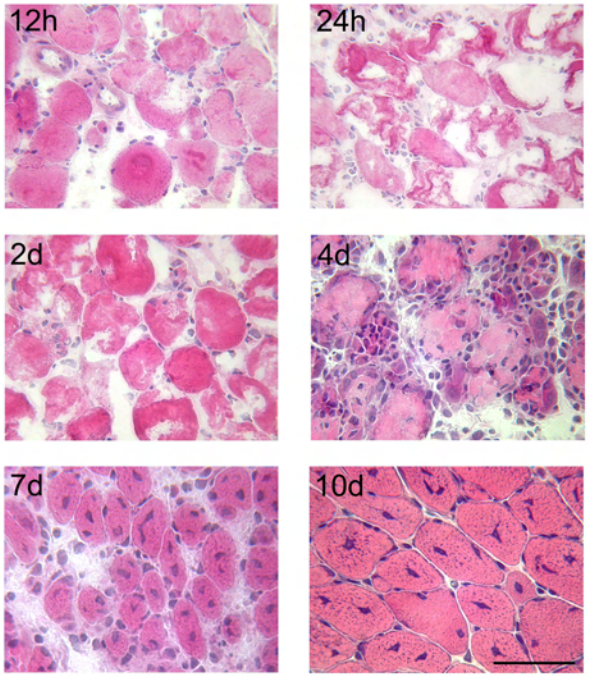




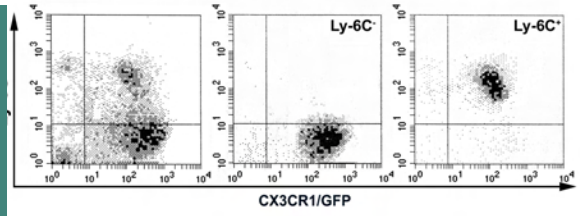
B - Quantification of MO/MP subsets in the infiltrate



C - Histological analysis of muscle regeneration



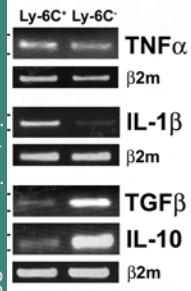
A - Sorting of MO/MP subsets



B - Ki67 labeling of Ly-6C+ and Ly-6C- MO/MP subsets

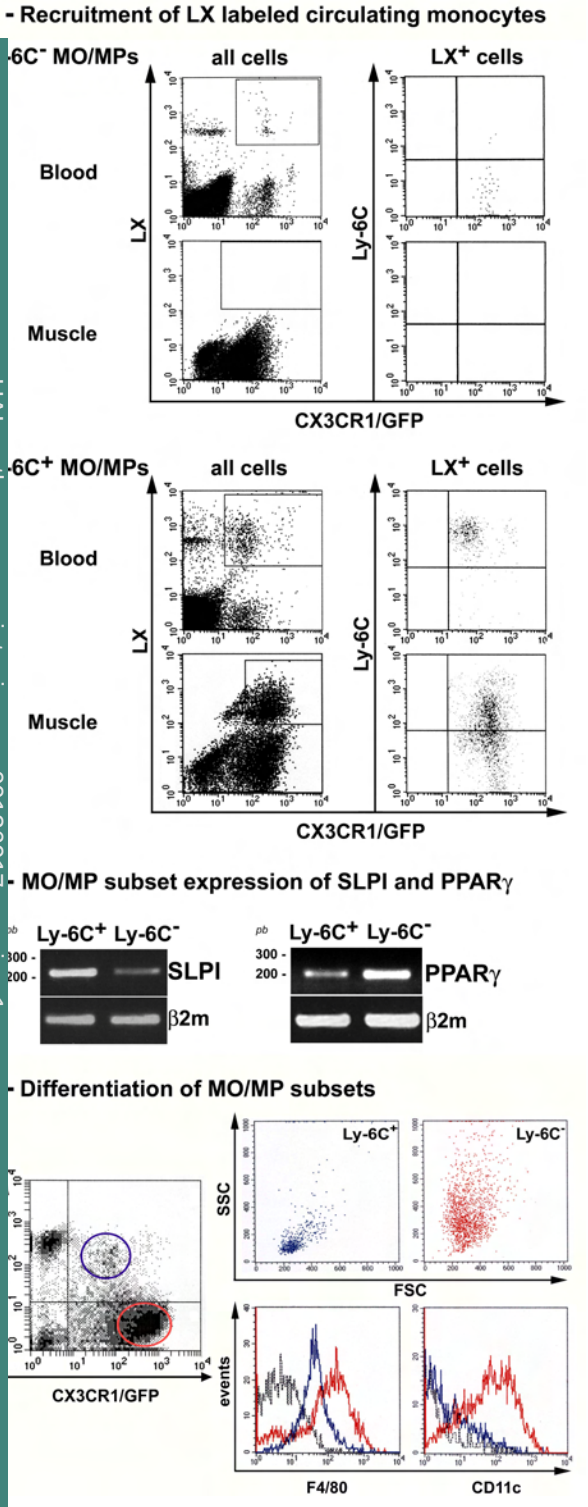
d post injury	15h	48h	4d	7d
X3CR1^{hi} Ly-6C^{-}	0 ± 0	13.5 ± 2.9	27 ± 4.9	12.8 ± 4.9
X3CR1^{lo} Ly-6C^{+}	0 ± 0	0 ± 0	0 ± 0	0 ± 0

C - Cytokine expression of Ly-6C+ and Ly-6C- MO/MP subsets

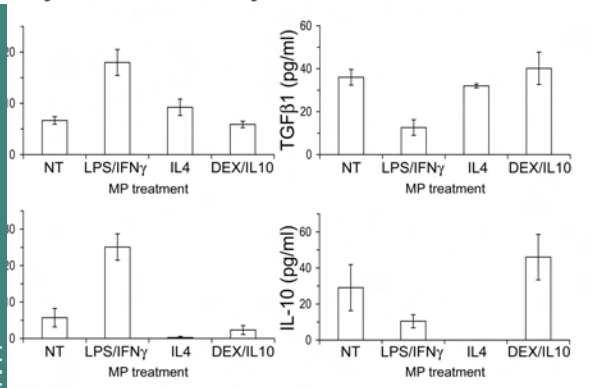


band intensity

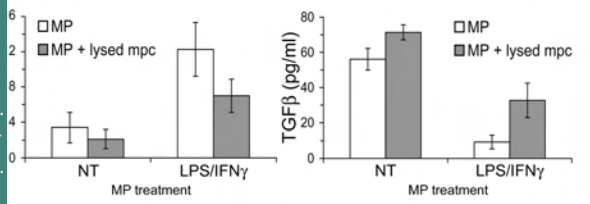
	Ly-6C^{+}	Ly-6C^{-}
TNFα	100	82
IL-1β	100	60
TGFβ	100	193
IL-10	100	230



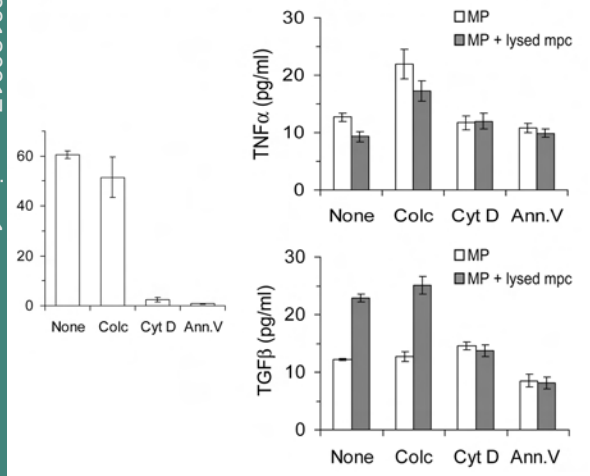
A - Cytokine secretion by MPs after activation



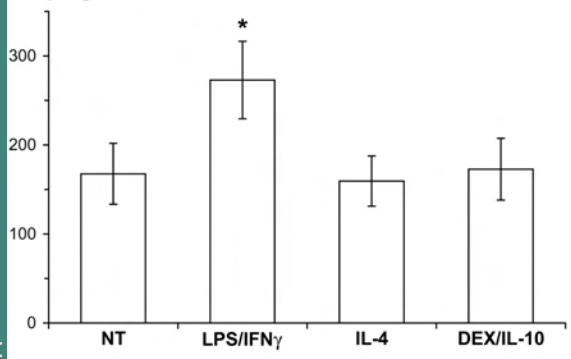
- Cytokine secretion by MPs after phagocytosis of muscle cell debris



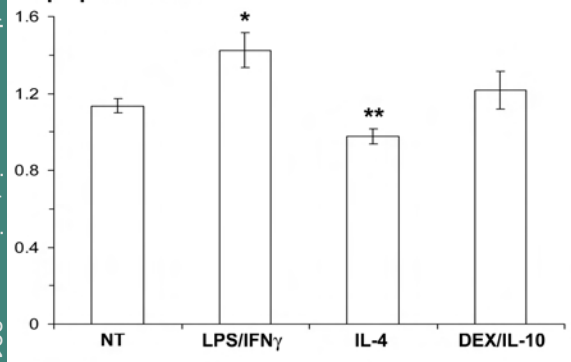
- Inhibition of phagocytosis in inflammatory MPs



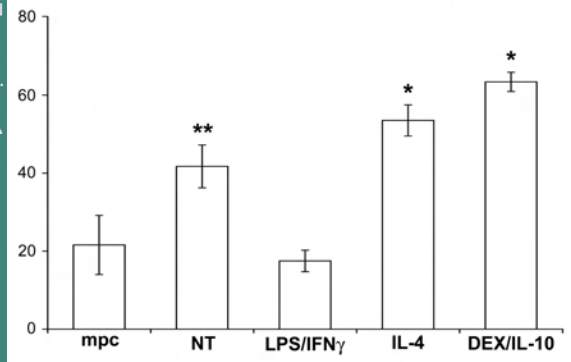
A - Mpc growth



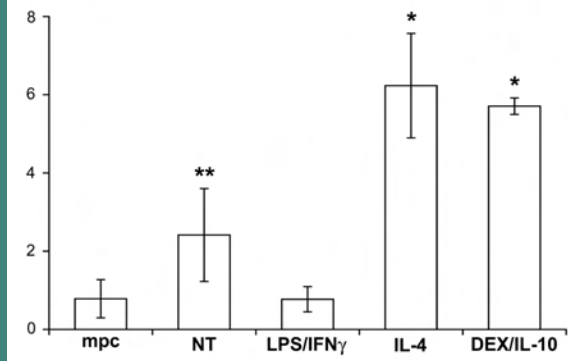
- Mpc proliferation



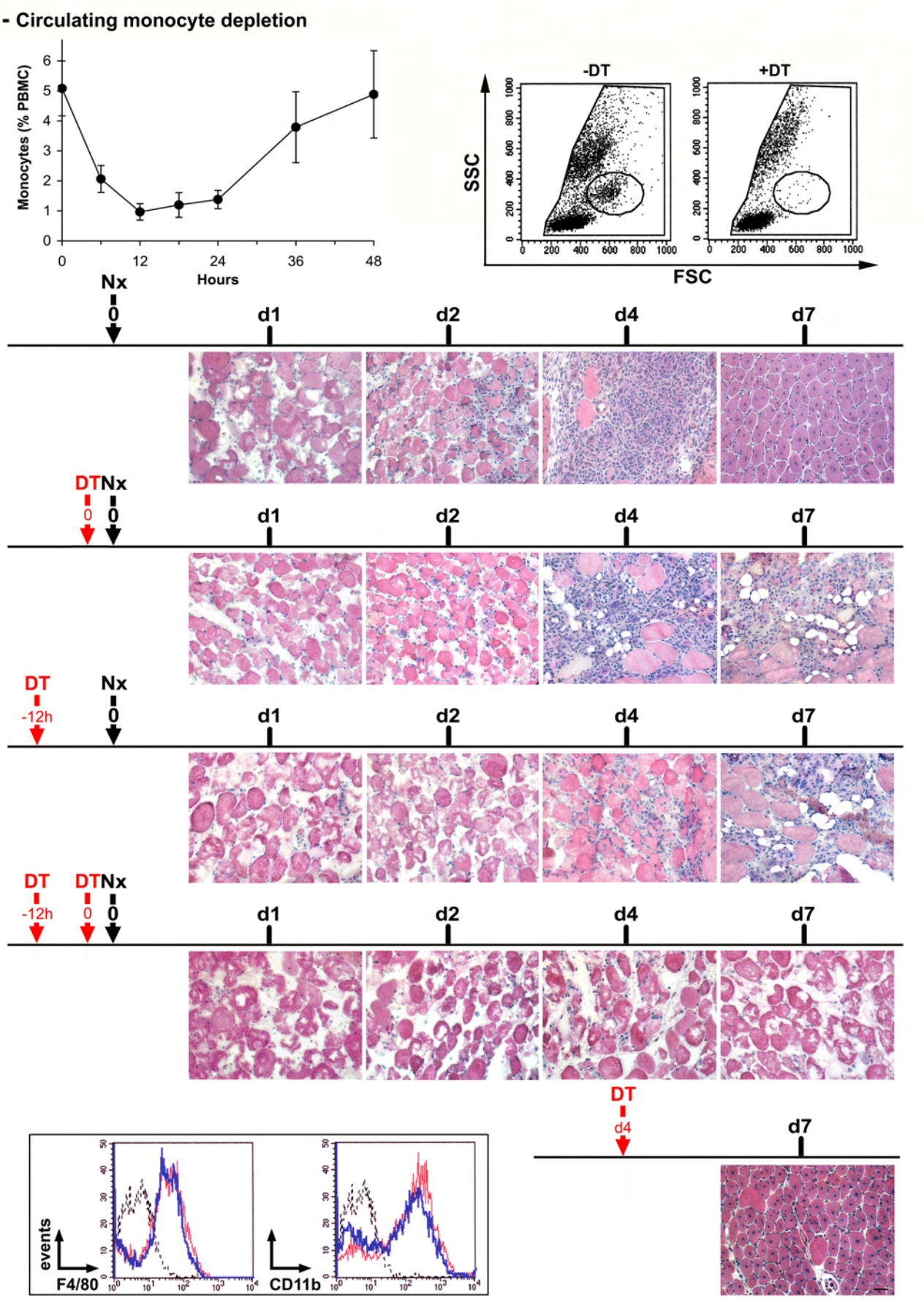
- Mpc differentiation

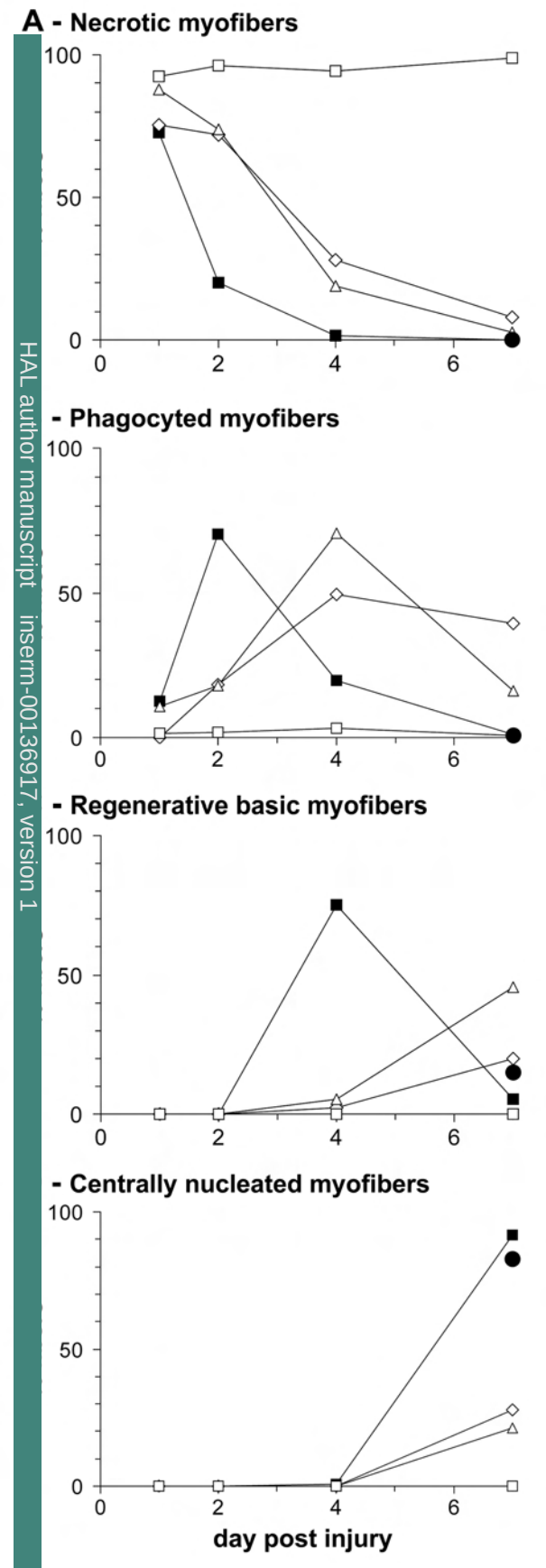


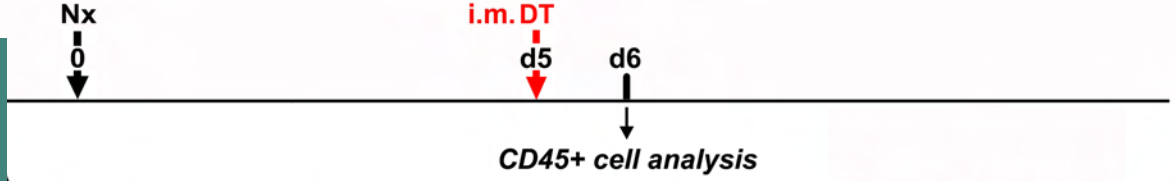
- Mpc fusion



MP treatment







Nb cells/mg muscle	PBS-injected TA	DT-injected TA
$CD45^{+}$	46400 ± 9100	88600 ± 19300
$F4/80^{+}$	38100 ± 9800	28300 ± 7700
$F4/80^{hi}$	17200 ± 3100	4200 ± 900