

RESEARCH ARTICLE

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# Monocyte depletion increases local proliferation of macrophage subsets after skeletal muscle injury

Claude H Côté<sup>1,2</sup>, Patrice Bouchard<sup>1</sup>, Nico van Rooijen<sup>3</sup>, David Marsolais<sup>4,5</sup> and Elise Duchesne<sup>1,6\*</sup>

## Abstract

**Background:** Sequential accumulation of M1 and M2 macrophages is critical for skeletal muscle recovery after an acute injury. While M1 accumulation is believed to rely on monocyte infiltration, the mechanisms of M2 accumulation remain controversial, but could involve an infiltrating precursor. Yet, strong depletion of monocytes only partially impairs skeletal muscle healing, supporting the existence of alternative mechanisms to palliate the loss of infiltrating macrophage progenitors. The aims of this study are thus to investigate if proliferation occurs in macrophage subsets within injured skeletal muscles; and to determine if monocyte depletion leads to increased proliferation of macrophages after injury.

**Methods:** Injury was induced by bupivacaine injection in the tibialis anterior muscle of rats. Blood monocytes were depleted by daily intravenous injections of liposome-encapsulated clodronate, starting 24 h prior to injury. In separate experiments, irradiation of hind limb was also performed to prevent resident cell proliferation. Upon euthanasia, blood and muscles were collected for flow cytometric analyses of macrophage/monocyte subsets.

**Results:** Clodronate induced a 80%-90% depletion of monocyte but only led to 57% and 41% decrease of M1 and M2 macrophage accumulation, respectively, 2 d following injury. Conversely, the number of M1 macrophages in monocyte-depleted rats was 2.4-fold higher than in non-depleted rats 4 d after injury. This was associated with a 16-fold increase in the number of proliferative M1 macrophages, which was reduced by 46% in irradiated animals. Proliferation of M2 macrophages was increased tenfold by clodronate treatment 4 d post injury. The accumulation of M2 macrophages was partially impaired by irradiation, regardless of monocyte depletion.

**Conclusions:** M1 and M2 subsets proliferate after skeletal muscle injury and their proliferation is enhanced under condition of monocyte depletion. Our study supports the conclusion that both infiltrating and resident precursors could contribute to M1 or M2 macrophage accumulation in muscle injury.

**Keywords:** M1, M2, Bupivacaine, Clodronate, Flow cytometry, ED1, ED2, Ki67, Irradiation, Healing

## Background

Macrophages are classically known for their pro-inflammatory roles in innate immunity and more recently for their active contribution to the resolution of inflammation and tissue repair [1-6]. This versatility is reflected by their ability to adopt distinct phenotypes depending on the microenvironment [7-9]. Macrophages can be divided in two main subsets according to their mode of activation and specific functions. In the context of skeletal muscle

tissue injury, “classically activated” [10] M1 macrophages are found during the inflammatory phase and are associated with phagocytosis, while “alternatively activated” [10] M2 macrophages accumulate at the site of injury once necrotic tissue has been removed and participate to the repair and remodeling processes [7-9]. In rats, former nomenclature ED1<sup>+</sup> and ED2<sup>+</sup> refers to M1 (CD68<sup>+</sup> CD163<sup>-</sup>) and M2 (CD68<sup>+</sup> CD163<sup>+</sup>) macrophage subsets, respectively. For the sake of clarity we will thereafter refer to M1 and M2 macrophages. Macrophages insuring homeostasis of uninjured muscles are believed to be resident cells and would present a M2 phenotype [7,9]. Macrophage involvement is clearly a prerequisite for skeletal muscle repair and their deregulation can determine

\* Correspondence: elise1\_duchesne@uqac.ca

<sup>1</sup>Centre de Recherche du CHUL (CHUQ), 2705 Boulevard Laurier, RC-9800 Québec, Québec, Canada

<sup>6</sup>Unité de Physiothérapie, Département des Sciences de la Santé, Université du Québec à Chicoutimi, Chicoutimi, Québec, Canada

Full list of author information is available at the end of the article

the outcome of skeletal muscle healing. The current dogma is that macrophages must freely accumulate in the injured muscle to ensure an adequate healing [1-3,11-13]. However, depletion of macrophages has only led to partial alteration of skeletal muscle recovery after injury [3], [1], supporting the existence of alternative mechanisms to ensure the functions of macrophages. Given the critical involvement of macrophage subsets in skeletal muscle healing, a better understanding of the mechanisms governing their accumulation may reveal new points of regulation for intervention.

The mechanisms of M1 and M2 macrophage accumulation after sterile injury remain elusive. In models like peritoneal infection, M1 or M2 macrophage accumulation results from the differentiation of distinct infiltrating M1 and M2 myeloid precursors [14]. In contrast, recent evidences support *in situ* proliferation of M1 and M2 macrophage subsets in T<sub>H</sub>2-mediated inflammation [14,15]. In the context of muscle injury, M1 macrophage accumulation is thought to result exclusively from the infiltration and differentiation of a precise monocyte subset into macrophages. On the other hand, the origin of the increased number of M2 macrophages is a matter of debate. A number of hypotheses are currently put forward including sequential mobilization of M1 and M2 macrophage circulating precursors [16], differentiation of monocyte-derived M1 macrophages into M2 macrophages following phagocytic activity after skeletal muscle injury [2] or M2 macrophage proliferation [15]. It appears that the mechanisms of M1 and M2 accumulation in this specific situation are complex, sometimes overlapping, and most likely determined by the context of the immune response.

The importance of sequential accumulation of M1 and M2 macrophages for optimal muscle healing is now well accepted. However, the cellular origin and respective contribution of proliferation vs. infiltration remain elusive following skeletal muscle injury. In addition, there is no information on how those mechanisms might be altered under anti-inflammatory conditions. The goal of this study was to determine if local proliferation could contribute to M1 and M2 macrophage accumulation following skeletal muscle injury, under normal or monocyte depletion conditions. Given that M1 macrophage accumulation in the context of sterile skeletal muscle injury is believed to rely exclusively on monocyte infiltration [2,16], and that M2 macrophage accumulation could be derived from M1 [2], our working hypothesis is that following sterile muscle injury in rat, blood monocyte depletion will impair M1 and M2 tissue macrophage accumulation.

## Methods

### Animals

Female Wistar rats weighing between 125 – 150 g were purchased from Charles River (St-Constant, QC, Canada)

and housed 3 per cage. They were maintained on a 12 h – 12 h light – dark cycle. Water and food were provided *ad libitum*. All care, handling and experimental procedures were approved by the Université Laval Research Center Animal Care (Permit Number: 2012–025) and Use Committee according to the guidelines of the Canadian Council on Animal Care.

### Muscle injury

At 0 d, the right tibialis anterior (TA) muscle was chemically injured with bupivacaine. Rats received 0.05 mg/kg buprenorphine (Temgesic®, Reckitt Benckiser Healthcare (UK) Ltd) intraperitoneally (i.p.) as an analgesic 15 min before surgery and were then anesthetized with 1.5 – 2% isoflurane (Abbott Laboratories, Montreal, QC, Canada) under a flow of 400 – 800 mL/min of oxygen. Fur of the anterior side of the right hind limb was shaved off and skin was disinfected with isopropyl alcohol. Then, 120 µL of bupivacaine hydrochloride 0.5% (Marcaine; Hospira, Lake Forest, IL, USA) was injected through the skin in 3 sites of 40 µL along the right TA using a syringe with a 29 G needle. A single dose of 0.05 mg/kg of buprenorphine was also administered i.p. 1 d after the injury.

### Monocyte depletion

Liposome-encapsulated dichloromethylene diphosphonate (Cl<sub>2</sub>MDP; clodronate) was used to deplete blood monocytes/macrophages [17]. Clodronate was a gift from Roche Diagnostics GmbH (Mannheim, Germany). Rats received 1 mL, at the concentration established by the provider, of the liposome-encapsulated clodronate suspension. Injections were made daily in the tail vein or in the internal jugular veins under anesthesia with isoflurane starting 24 h before injury until sacrifice at 1, 2, 3 or 4 d post-injury.

### Single leg irradiation

Liposome-encapsulated clodronate does not deplete resident macrophages in muscle. To study the contribution of resident macrophages, irradiation was performed since it prevents proliferation by inducing DNA damage, especially in immune cells [18,19]. 24 h before injury, rats were anesthetized by i.p. injection of a ketamine/xylazine cocktail (80 mg/kg ketamine and 10 mg/kg xylazine) and then installed under a 4 cm-thick lead shield plate with their right hind limb facing a hole allowing radiation to pass for local irradiation. The right leg received a single dose of 20 Gy of γ rays delivered at 1.1 Gy per minute using a Gammacell® 40 Exactor (Best Theratronics Ltd, Ottawa, ON, Canada). This dose prevents resident leukocyte replication without killing mature muscle cells [18,20-23].

### Isolation of peripheral blood mononuclear cells (PBMCs)

5 mL of blood was obtained by cardiac puncture under anesthesia with isoflurane and collected in BD Vacutainer®

blood collection tubes containing EDTA (BD, Franklin Lakes, NJ, USA). PBMCs were isolated with a density-gradient centrifugation on Histopaque 1083 (Sigma-Aldrich, St. Louis, MO, USA). Blood was deposited on 3 mL of Histopaque 1083 in a 15 mL conical tube and centrifuged for 30 min at  $400 \times g$  at room temperature. The upper layer was discarded and the opaque interface containing mononuclear cells (buffy coat) was aspirated with a Pasteur pipette and transferred to a new 15 mL centrifuge tube. Cells were washed twice with PBS and contaminating erythrocytes were lysed with a 4 min incubation with 1 mL of sterile erythrocyte lysis buffer containing 155 mM  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{KHCO}_3$  and 0.342 mM EDTA. 10 mL of PBS was then added to stop the lysis. After washing twice with PBS, cells were resuspended in 0.5 mL PBS and counted with a hemocytometer using trypan blue exclusion.

#### Preparation of single cell suspension from TA muscle

Rats were euthanized by cervical dislocation under anesthesia with isoflurane. TA muscles were dissected and rinsed in PBS. Minced muscles were incubated for 3 h at  $37^\circ\text{C}$  with 5 mL of Roswell Park Memorial Institute media 1640 (RPMI 1640) (HyClone, Logan, UT, USA) containing 3 mg/mL collagenase D (Roche Diagnostics, Laval, QC, Canada). Collagenase-digested samples were then homogenized by trituration using a 1000  $\mu\text{L}$  micropipette. 10 mL PBS-EDTA (26 mM EDTA in PBS) was added to this suspension and centrifuged at  $500 \times g$  for 5 min at  $4^\circ\text{C}$ . After red blood cell lysis, the pellet was resuspended in 30% isotonic Percoll (GE healthcare, Waukesha, WI, USA) and centrifuged at  $500 \times g$  for 15 min at  $4^\circ\text{C}$ . The supernatant was discarded and the pellet resuspended in 1 mL PBS-EDTA. Cells were counted with a hemocytometer using trypan blue exclusion.

#### Flow cytometric analyses

Sequential extracellular and intracellular stainings were performed as described earlier [24]. Single cell suspensions were washed in staining buffer (PBS containing 2% FBS). Cells were then resuspended in staining buffer containing Fc Block (anti-CD32) (BD Biosciences, Mississauga, ON, Canada). A cocktail of antibodies containing Pacific Blue-coupled anti-CD11b (Serotec, Kidlington, UK), biotin-coupled anti-CD3 (Serotec, Kidlington, UK), biotin-coupled anti-CD45RA (Biolegend, San Diego, CA, USA), Alexa Fluor<sup>®</sup> 647-coupled anti-CD163 (Serotec, Kidlington, UK) or Alexa Fluor<sup>®</sup> 647 coupled-IgG1 isotype control (Serotec, Kidlington, UK) was added to the cell suspension and incubated for 20 min on ice. Cells were washed in staining buffer and incubated with Alexa Fluor<sup>®</sup> 700-coupled streptavidin (Invitrogen Life Technologies, Burlington, Canada) for 20 min on ice. Cells were washed

in staining buffer and fixed in PBS containing 2% paraformaldehyde for 20 min on ice, and then permeabilized in staining buffer containing 0.2% saponin for staining with anti RPE-coupled anti-CD68 (Serotec, Kidlington, UK) and Pe-Cy7-coupled anti-Ki-67 (BD Biosciences, Mississauga, ON, Canada) or RPE-coupled IgG1 isotype control (Serotec, Kidlington, UK) and Pe-Cy7-coupled k IgG1 isotype control (BD Biosciences, Mississauga, ON, Canada). Intracellular staining was performed for 30 min on ice. Single color controls were performed using CompBead Plus (BD Biosciences, Mississauga, ON, Canada). Data was acquired with a FACS Diva-driven FACS Aria II (Becton Dickinson) and analyzed with FlowJo (Tree Star Inc. Ashland, OR, USA). Isotype controls or omission of primary antibodies (when appropriate) were used to set gates. To determine numbers of specific cell subsets (from blood or skeletal muscles), the percentages obtained from flow cytometric analyses were multiplied by the absolute cell numbers obtained from the hemocytometer count.

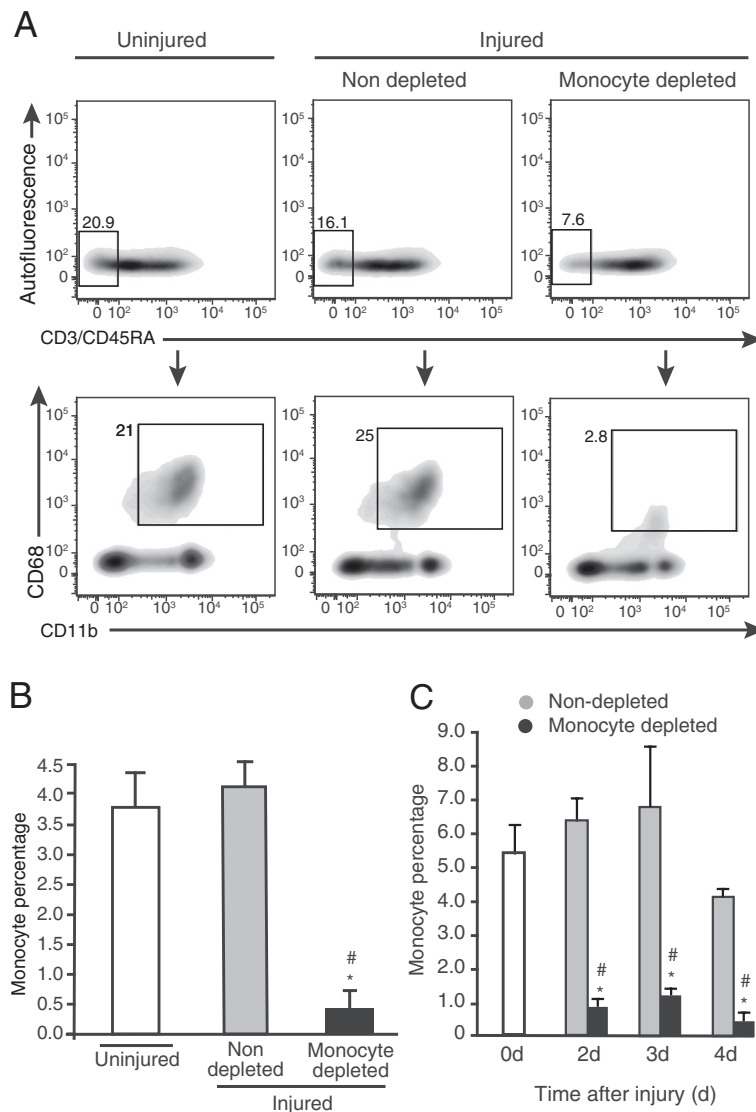
#### Statistical analysis

All values are expressed as means and standard error. The construct of these experiments allowed comparisons between groups to be performed by Student's t-test or one-way ANOVA followed by Tukey-Kramer post-hoc test, when appropriate (InStat GraphPad Software Inc., La Jolla, CA, USA). Significance was defined as  $p < 0.05$ .

## Results

### Blood monocyte depletion modifies macrophage accumulation in injured muscle

Before assessing the contribution of blood monocyte infiltration to the accumulation of macrophages in injured muscle, we validated that daily liposome-encapsulated clodronate injection led to profound blood monocyte depletion. Blood monocytes correspond to the population negative for lymphocyte markers CD3 and CD45RA, but positive for myeloid marker CD11b and the monocyte/macrophage marker CD68 (Figure 1A). We used values obtained at 4 d post-injury to demonstrate that when compared with uninjured animals that contain 20.9% of non-lymphoid  $\text{CD3}^-\text{CD45RA}^-$  circulating cells, muscle injury slightly decreased this frequency to 16.1%, while clodronate treatment lowered the frequency of  $\text{CD3}^-\text{CD45RA}^-$  cells to 7.6% in injured rats. When gated from  $\text{CD3}^-\text{CD45RA}^-$  cells, the frequency of  $\text{CD11b}^+\text{CD68}^+$  monocytes corresponded to 21% in uninjured animals. This percentage was not significantly affected by the induction of the injury, but was decreased to 2.8% by clodronate injection in injured rats. Clodronate treatment thus led to a 90% decrease of the percentage of  $\text{CD3}^-\text{CD45RA}^-\text{CD11b}^+\text{CD68}^+$  monocytes, relatively to the total PBMCs, in comparison with non-depleted animals injured

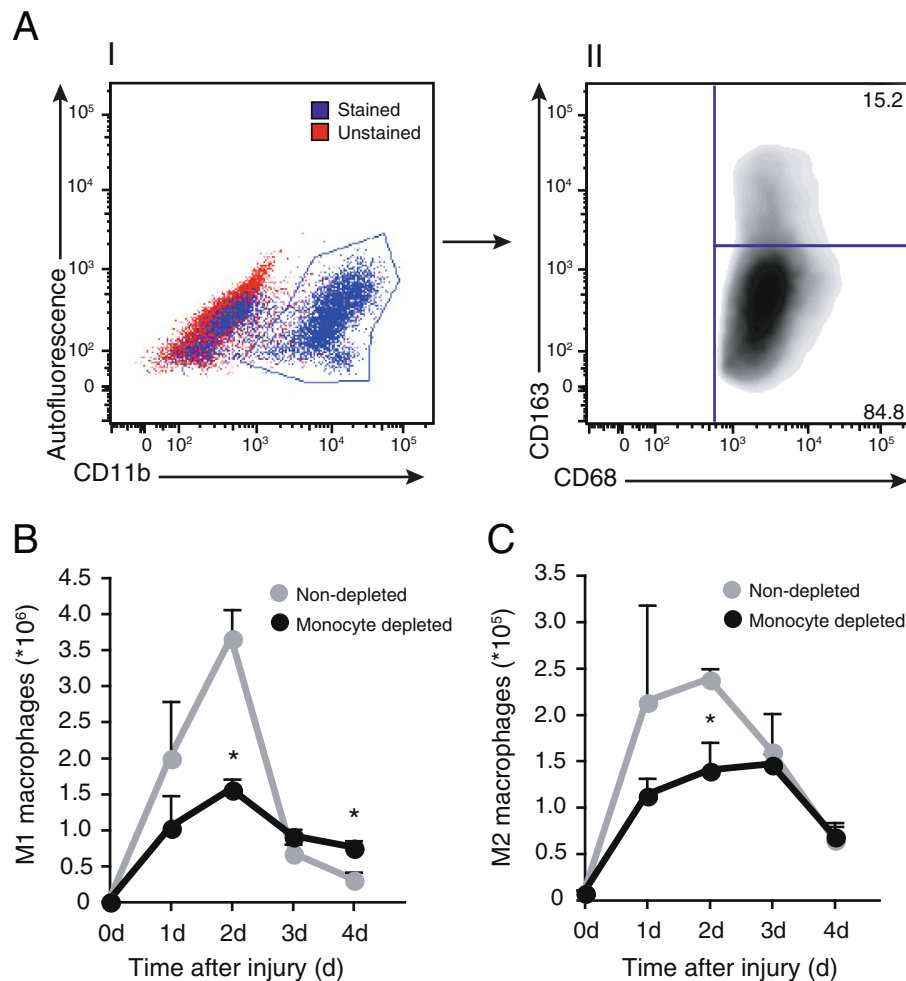


**Figure 1 Liposome-encapsulated clodronate eliminates more than 80% of blood monocytes.** (A) Rats received daily injections of liposome-encapsulated clodronate starting 24 h prior to intra-muscular injection of bupivacaine until euthanasia at 4 d post-injury. Clodronate treatment induces a decrease of the CD3<sup>+</sup>CD45RA<sup>-</sup> non-lymphoid cell frequency (A, upper lane), and of the frequency of CD11b<sup>+</sup>CD68<sup>+</sup> monocytes found in the CD3<sup>+</sup>CD45RA<sup>-</sup> population (A, lower lane). Gates were set using either omission of primary antibodies (omission of CD3/CD45RA or CD11b) and using an isotype control for CD68. n = 3. (B) When expressed relatively to total PBMCs, the frequency of CD3<sup>+</sup>CD45RA<sup>-</sup>CD11b<sup>+</sup>CD68<sup>+</sup> monocytes was decreased by 90% by the clodronate treatment in injured animals, in comparison to uninjured (\*) and injured non-depleted (#) animals. Student's t-test, p < 0.05. n = 3. (C) Using the same gating strategy, we show that clodronate treatment significantly decreased the frequency of CD3<sup>+</sup>CD45RA<sup>-</sup>CD11b<sup>+</sup>CD68<sup>+</sup> monocytes at 2 d and 3 d by 86% and 82%, respectively in injured animals, in comparison to uninjured (\*) and injured non-depleted (#) animals. Student's t-test, p < 0.05. n = 3.

with bupivacaine (Figure 1B). The degree of monocyte depletion was verified for each experience at all time points and similar depletion levels were observed: 86% at 2 d and 82% at 3 d (Figure 1C). Thus, the clodronate depletion procedure was effective throughout the experimental protocol.

We next characterized the effect of blood monocyte depletion on the accumulation of M1 and M2 macrophage subsets into TA muscle following muscle injury. The

gating strategy consisted in prior selection of myeloid cells (CD11b<sup>+</sup>) from total cells (Figure 2A I), followed by selection of total CD68<sup>+</sup> cells (not shown), and discrimination of the M1 and M2 populations based on the expression of CD163 by CD11b<sup>+</sup>CD68<sup>+</sup> monocytes/macrophages (Figure 2A II). Prior to injury (0 d), only a few CD11b<sup>+</sup>CD68<sup>+</sup>CD163<sup>-</sup> (M1) and CD11b<sup>+</sup>CD68<sup>+</sup>CD163<sup>+</sup> (M2) macrophages were present in the TA muscle (Figure 2B and C). In injured animals non-depleted of monocytes,



**Figure 2 Numbers of M1 and M2 macrophages in TA muscle are modified by blood monocyte depletion.** Rats were treated once a day with intravenous injection of liposome-encapsulated clodronate starting 24 h prior to injury until euthanasia at 1, 2, 3 and 4 d post-injury, for analysis of M1 and M2 macrophage accumulation into injured muscle. **(A)** Gating strategy (at 3 d post-injury) in single cell suspensions from skeletal muscles was based on omission of primary antibody for CD11b, and on isotype control stainings for CD68 and CD163 to identify M1 (CD163<sup>-</sup>) and M2 (CD163<sup>+</sup>) subsets. Liposome-encapsulated clodronate induced a significant decrease in accumulation of M1 **(B)** and M2 **(C)** macrophages at 2 d post-injury and a significant increase in M1 macrophage number at 4 d post-injury. \*Significantly different from non-depleted groups, Student's t-test,  $p < 0.05$ ,  $n =$  at least 3, for all the time points and conditions evaluated.

a classical accumulation of M1 macrophages occurred following injury, which peaked at 2 d with  $3.67 \times 10^6$  M1 macrophages per muscle and then dramatically decreased by 81% at 3 d before returning to baseline at 4 d post-injury (Figure 2B). A similar pattern was observed with M2 macrophages, with a peak at 2 d ( $2.38 \times 10^5$  M2 macrophages per muscle). However M2 macrophage accumulation resolved more progressively than the M1 population, with a 33% decrease between 2 and 3 d, and above baseline accumulation remaining at 4 d (Figure 2C). When monocytes were depleted, we observed a 57% decrease in accumulation of M1 macrophages at 2 d post-injury, when compared to non-depleted injured animals (Figure 2B). Monocyte depletion had no impact on M1 macrophage number at 3 d, but it led to a 2.4-fold increase

of M1 macrophage accumulation at 4 d, when compared with non-depleted injured rats (Figure 2B). As for the M2 macrophages, monocyte depletion induced a significant 41% decrease of their accumulation that was only detectable at 2 d after injury (Figure 2C). Taken together, these results show that a 90% blood monocyte depletion only leads to a partial (about 50%) decrease of macrophage accumulation at 2 d, and causes an increase in the number of the M1 subset at 4 d post-injury, suggesting an alternative mechanism of accumulation besides monocyte infiltration.

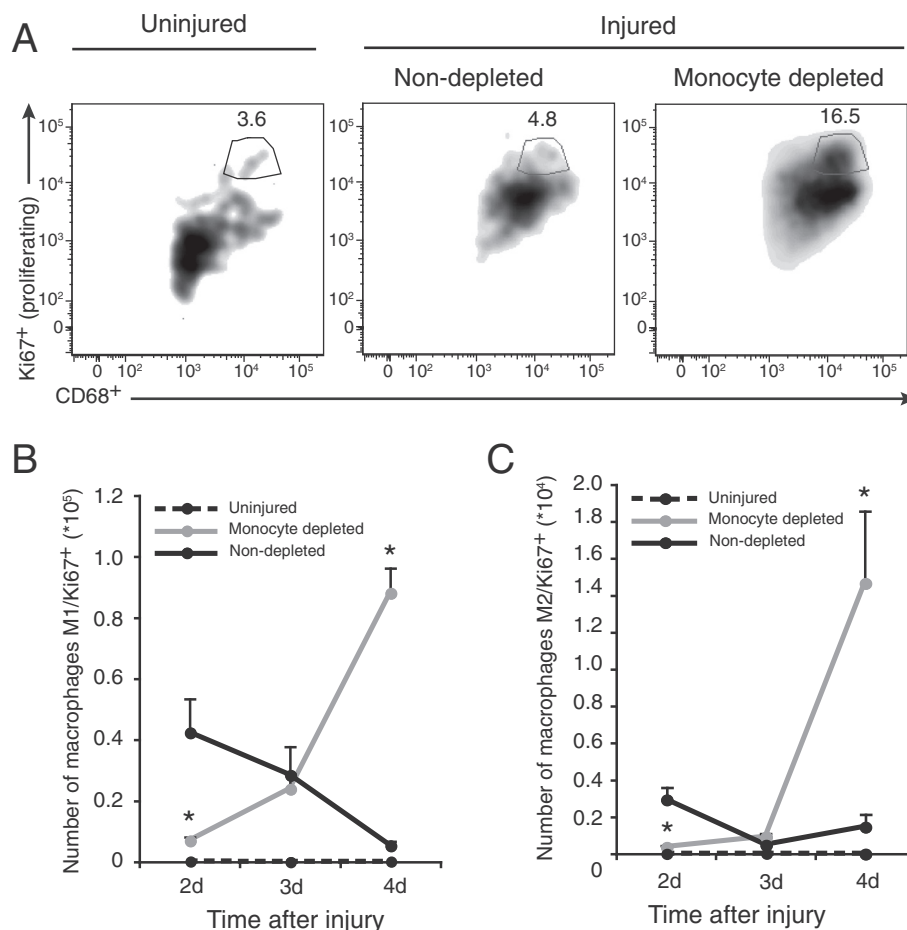
#### Monocyte depletion is associated with proliferation of macrophages in injured muscles

Given that profound monocyte depletion led to modest decrease of macrophage numbers in injured muscles at

2 d and even to an increase at 4 d, we aimed at determining if local proliferation could occur in macrophages found within skeletal muscle. Using the gating strategy previously described (prior gating on CD11b<sup>+</sup> cells, from viable cells), we then measured the proportion of M1 and M2 macrophages positive for Ki-67, which detects proliferative cells, irrespectively of their position within the cell cycle. In muscles of rats that were uninjured or that were injured but not submitted to monocyte depletion, 3.6% and 4.8% of all CD11b<sup>+</sup>CD68<sup>+</sup> cells were positive for Ki-67 marker, respectively. The percentage of CD11b<sup>+</sup>CD68<sup>+</sup>Ki-67<sup>+</sup> proliferative macrophages was increased by 3.4-fold when injured rats were treated with clodronate (Figure 3A). In uninjured rat muscles, the absolute numbers of Ki-67<sup>+</sup>M1 and Ki-67<sup>+</sup>M2 macrophages were barely detectable at all time points tested (Figure 3B and C). At 2 d, numbers of proliferating M1

and M2 macrophages were respectively 6- and 7.5-fold higher in non-depleted animals in comparison to depleted animals (Figure 3B and C), but these absolute numbers represented only a small fraction (approximately 1%) of the total M1 and M2 macrophages found in tissue at this time point under the same conditions (Figure 2B and C). Inversely, at 4 d, monocyte depletion led to significant 16- and 10-fold increases in the numbers of proliferating M1 and M2 macrophages (Figure 3B and C), respectively, which correspond to 10% of total M1 and 20% of M2 macrophage populations (Figure 2B and C). There is clearly an increased local proliferation of macrophages following skeletal muscle injury when monocyte infiltration is blocked.

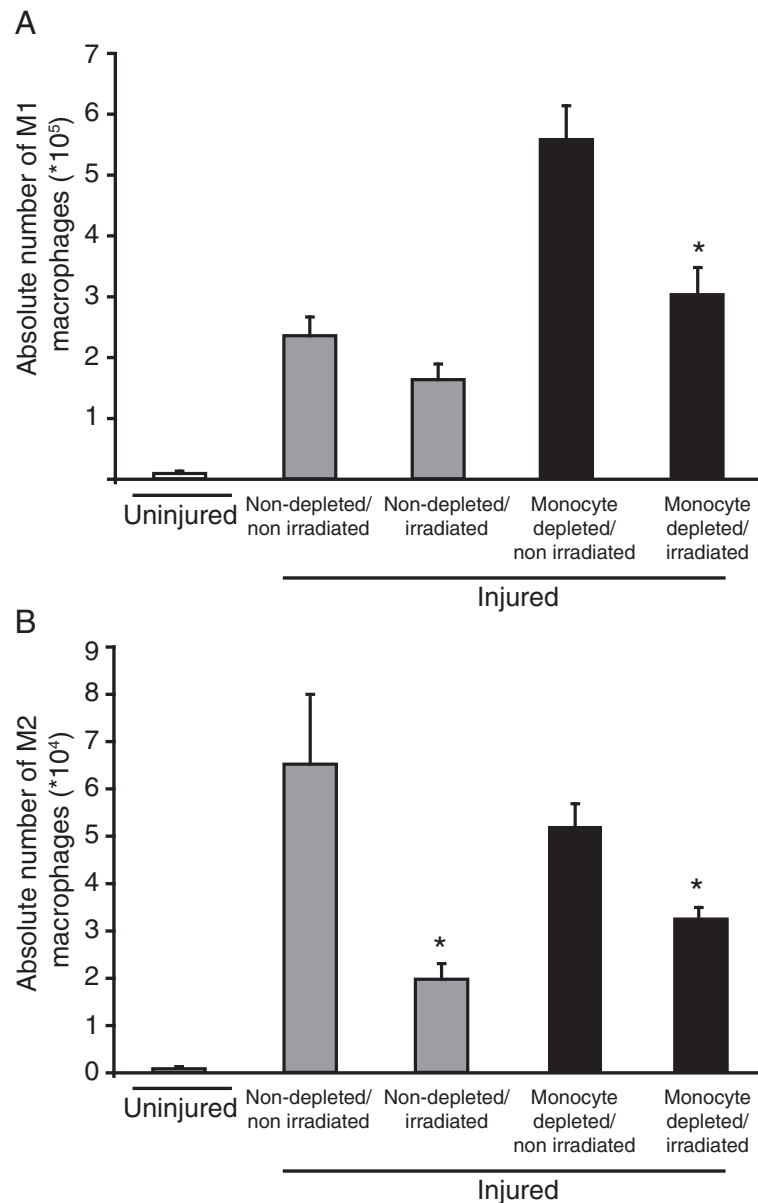
To rule out the contribution of resident cells to M1 and M2 macrophage accumulation following injury, we used single leg irradiation before muscle injury. Irradiation



**Figure 3 Monocyte depletion induced significant increase in the number of proliferative macrophages in injured TA muscles.** Rats were treated once a day with intravenous injection of liposome-encapsulated clodronate starting 24 h prior to injury until euthanasia at 2, 3 and 4 d post-injury for analysis of the number of proliferating Ki-67<sup>+</sup>M1 and Ki-67<sup>+</sup>M2 macrophages into injured muscle. **(A)** Total CD11b<sup>+</sup>CD68<sup>+</sup> proliferating macrophages also positive for Ki-67<sup>+</sup> (not shown) were identified. Gates were set using isotype controls for Ki-67 and CD68. **(B)** Ki-67<sup>+</sup>M1 and **(C)** Ki-67<sup>+</sup>M2 macrophage subsets were quantified at 2, 3 and 4 d post-injury. Liposome-encapsulated clodronate induced a significant increase of Ki-67<sup>+</sup>M1 and Ki-67<sup>+</sup>M2 macrophage numbers at 4 d post-injury. \*Significantly different from non-depleted groups, Student's t-test, p < 0.05. n = at least 4, for all the time points and conditions evaluated.

did not affect the number of M1 macrophages in non-depleted animals 4 d after injury, supporting that infiltration is the main source of accumulation and that the population is contracting normally in those conditions (Figure 4A). However, irradiation reduced almost completely the depletion-induced increase of M1 macrophage number when compared to the non-depleted groups suggesting the implication of a local precursor (Figure 4A). Relatively to the uninjured group, M2 macrophages accu-

mulated in spite of irradiation in non-depleted animals but to a lower extent than in non-irradiated/non-depleted animals, suggesting that their accumulation relies on both proliferation of local progenitor and an infiltrating precursor (Figure 4B). With irradiation, monocyte depletion did not induce a greater decrease in M2 macrophage accumulation, suggesting that M2 progenitors were unaffected when monocyte infiltration is artificially blocked (Figure 4B). Taken together, these results suggest that



**Figure 4 Irradiation modulates M1 and M2 macrophage accumulation in TA muscle.** Hind limb was submitted to 20 Gy 24 h before injury to inhibit proliferation of resident cells. Rats were treated, or not, with daily intravenous injection of liposome-encapsulated clodronate starting 24 h prior to injury until euthanasia at 4 d post-injury, for analysis of the numbers of M1 and M2 macrophages into injured muscle. **(A)** Irradiation significantly decreased M1 macrophage accumulation when monocytes were depleted. **(B)** Irradiation impaired the accumulation of M2 macrophages following depletion or not. \*Significantly different from the non-irradiated condition homologous, Student's t-test,  $p < 0.05$ .  $n =$  at least 3, for all the time points and conditions evaluated.

following muscle injury, M1 macrophage accumulation is mostly dependent on monocyte infiltration but that a local progenitor can contribute when monocyte infiltration is impaired. Conversely, M2 macrophage accumulation relies on both infiltrating and local precursors.

#### The M2 Marker CD163 is not expressed by circulating monocytes

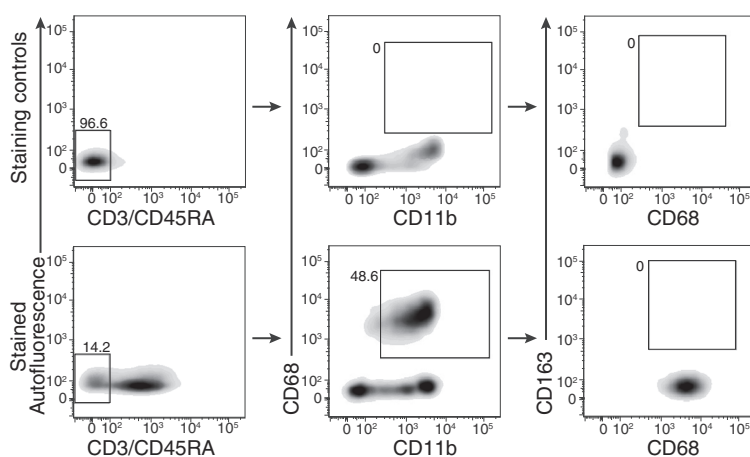
Since an infiltrating precursor seems to participate to M2 macrophage accumulation, we verified if a monocyte subset expressing CD163 marker was present in circulation. We used the previously defined strategy where CD3<sup>-</sup>/CD45RA<sup>-</sup> stained PBMCs were analyzed based on expression of CD11b, CD68 and CD163. Gates were based on the omission of primary antibody for CD3/CD45RA and CD11b while isotype controls were used for CD68 and CD163 (Figure 5). We found that blood monocyte subset CD3<sup>-</sup>CD45RA<sup>-</sup>CD11b<sup>+</sup>CD68<sup>+</sup> does not express CD163 (Figure 5). Our results are in accordance with previous literature showing that CD68 is largely used as human and rat monocyte marker, but CD163 expressed by human blood monocytes appears restricted to M2 macrophages in rat [25-27]. Taken together, our results support that in rat, circulating CD11b<sup>+</sup>CD68<sup>+</sup> cells do not express the M2 CD163 marker.

#### Discussion

Macrophages adopt various phenotypes based on their environment and participate to many physiological processes [4,8,28,29]. Substantial efforts have been deployed to characterize these various phenotypes with the hope that a better understanding of macrophage subsets could help delineate detrimental from the beneficial effects. It is now known that both M1 and M2 macrophages

positively influence myogenesis, but can also contribute to pathological conditions [30-33]. Although mechanisms of macrophage replenishment were described in other tissues [34-36], the intrinsic mechanisms supporting their accumulation in the specific context of muscle injury are still a matter of debate and how these mechanisms are modulated under various anti-inflammatory strategies remains unknown. The main findings of this paper are that following muscle injury 1) both M1 and M2 macrophages can proliferate locally and 2) the major source of M1 macrophages are circulating precursors while M2 accumulation relies about equally on local (radiation sensitive) and infiltrating precursors. 3) We show for the first time that proliferation of macrophages is increased under condition of monocyte depletion.

We observed a low but quantifiable proliferation of M1 and M2 macrophage subsets following muscle injury at 2 d post-injury in non-depleted animals. This is in accordance with the reported proliferative ability of a number of macrophage subtypes including macrophages located in brain [35,37] or peritoneum [38], bone marrow-derived macrophages in culture [39], and the macrophage cell line RAW-264.7 [40]. Moreover, Hashimoto et al. have recently demonstrated that some tissue-resident macrophages repopulate locally via cellular proliferation with minimal contribution from monocytes, but these findings were observed under steady-state situation in alveolar macrophages, which are known to possess unique features such as a half-life 3 to 4 times longer than other macrophage subsets [34]. Ajami et al. also found no evidence of microglia progenitor recruitment from the circulation, but these results were observed in denervation and CNS neurodegenerative disease models [35]. Macrophage proliferation was also observed during chronic inflammation



**Figure 5 CD68<sup>+</sup> blood monocytes do not express CD163.** Blood was isolated from uninjured rat. Total white blood cells were collected and submitted to antibody staining for flow cytometric analyses to assess CD163 expression on CD3<sup>-</sup>CD45RA<sup>-</sup>CD11b<sup>+</sup>CD68<sup>+</sup> cells. Gates were set using omission of primary antibody for CD3/CD45RA and for CD11b; and using isotype control staining for CD68 and CD163. Results shown are representative of 4 individual observations.



[41] and Khmelewski et al. (2004) revealed that both subsets of macrophages accumulated around collateral vessels in spite of monocyte depletion during femoral artery occlusion-induced arteriogenesis [42]. However, in these studies the authors failed to discriminate the proliferative capacity of M1 and M2 macrophages [41,42]. Jenkins et al. (2011) clearly showed that tissue macrophages with M2-like phenotype and M1 macrophages can undergo proliferation, but this was done in a model of  $T_H$ -2-driven inflammation where the presence of IL-4 was essential [15]. Thus, macrophage proliferation has been demonstrated in various tissues and experimental models quite distinct from the one presented here. This is the first evidence that macrophage proliferation occurs specifically in skeletal muscle tissue and that proliferation contributes to macrophage accumulation following muscle injury. Our results are therefore unique and represent, to the best of our knowledge, the first demonstration that proliferation of M1 and M2 macrophages normally occurs following sterile muscle injury.

#### **M1 macrophage accumulation**

Up to now, most studies pointed toward the infiltration of “inflammatory monocytes” to explain the accumulation of tissue macrophages after non-infectious tissue injury like skeletal muscle trauma. However, recent evidence shows that macrophages can undergo local proliferation in  $T_H$ 2-mediated inflammation [15]. This prompted us to verify the effect of sustained monocyte depletion, in an attempt to delineate if monocyte infiltration is the sole contributor to macrophage accumulation in muscle injury. In accordance with other studies [3,43,44], monocyte depletion induced a significant decrease in the absolute number of M1 macrophages present at 2 d post-injury. We surprisingly observed a higher number of M1 macrophages observed in the monocyte-depleted animals at 4 d post-injury, when compared to non-depleted injured animals. It was very unlikely that this phenomenon could be explained by an increased recruitment at 4 d, since current literature suggests that signals for monocyte recruitment are not produced at this time point [2]. Importantly, numerous reports showed delayed peaks of macrophage accumulation following acute muscle injury when using diverse anti-inflammatory strategies; our results are the first to suggest that the mechanisms leading to this delayed accumulation differ from that of normal accumulation and relies on local proliferation. One could argue that the preconditioning with clodronate has enriched the residual monocyte population for the highly proliferative monocytes. To verify it, we have assessed the effect of clodronate treatment on the percentage of proliferative monocytes into PBMCs population. We observed that clodronate treatment tended to enrich  $CD11b^+CD68^+Ki-67^+$  proliferative blood monocyte population by about 2.5-fold

at all time points tested in comparison to untreated animals (data not shown). This increase represents a small absolute percentage of proliferative monocytes into PBMCs population (0.01% in non-treated vs. 0.025% in treated animals), and given the short timeframe of this study, those could have contributed partially the large increase of  $CD11b^+CD68^+Ki-67^+$  proliferative macrophages observed into muscle.

Our data strongly suggest that a precursor located within skeletal muscles prior to injury can contribute to M1 macrophage accumulation when monocyte infiltration is blocked. Irradiation, which prevents replication of resident macrophages, did not have any effect on the absolute number of M1 macrophages in non-depleted animals following injury, suggesting that infiltration of blood-derived monocytes was the main mechanism in those conditions. Inversely, irradiation short-circuited the increase of M1 macrophage number that was induced at 4 d with continuous depletion of monocytes. We conclude that when monocyte infiltration is artificially decreased or delayed, an alternative mechanism based on proliferation of a local M1 precursor is triggered to ensure the accumulation of that specific subset.

#### **M2 macrophage accumulation**

Based on the present results, we conclude that M2 macrophage accumulation following injury relies on mixed mechanisms involving infiltration of blood monocytes as well as proliferation of local and radiation-sensitive precursors. Different mechanisms have been proposed to explain M2 macrophage accumulation; these include a specific circulating M2 precursor ( $CX_3CR1^{hi}/Ly6C^{lo}$  monocytes) [16], a switch of phenotype from M1 toward M2 [2], and lastly, the capacity of M2 macrophages to undergo local proliferation [15]. Data obtained in models of muscle injury led us to originally hypothesize that depletion of blood monocyte would eliminate M1 macrophages and thus indirectly decrease the number of M2 macrophages [2]. As predicted,  $CD163^+$  macrophage accumulation was significantly reduced at 2 d post-injury in monocyte-depleted animals, but this difference was lost at later time points. In an attempt to explain this phenomenon, we showed that irradiation had a significant impact on the absolute number of M2 macrophages in injured rats treated or not with clodronate. These results thus suggest that M2 macrophage accumulation is ensured, at least partially, by a local progenitor such as resident cells expressing M2 phenotype or resulting from the conversion of previously infiltrated M1 macrophages into M2 [7,16]. Conversely, M2 macrophage accumulation was still significant when muscle was irradiated, which argues against the hypothesis that local radiation-sensitive precursors are the only source for M2 macrophages. Overall, the data obtained by

combining monocyte depletion and irradiation suggest that different pools of infiltrating and local M2 precursors could differently contribute to their accumulation. In order to identify a circulating progenitor for M2 macrophages, we assessed if a circulating monocyte subset expressed the M2 marker CD163 but were unable to detect such cells. Thus, the possibility of a CD68<sup>+</sup>CD163<sup>-</sup> precursor or of an unknown circulating precursor for M2 macrophages remains open. Moreover, non-circulating progenitor cannot be excluded since it has been shown in brain that microglia are maintained throughout life independently of any blood input [45].

## Conclusions

In summary, the present study shows for the first time that macrophages have the capacity to proliferate following sterile muscle injury and that the number of proliferating macrophages is increased within muscles when monocyte infiltration is blocked. We conclude that under normal physiological conditions, the main source of M1 macrophages is circulating monocytes while M2 macrophage accumulation relies on both local and infiltrating precursors. As a whole, our study highlights overlapping mechanisms involved in macrophage accumulation after sterile skeletal muscle injury and suggests that these mechanisms are modulated when monocyte infiltration is impaired like is the case of anti-inflammatory conditions.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contribution

ED, CHC, DM conceived and designed the research. ED and PB performed experiments. ED analyzed the data. ED, CHC DM PB, NvR interpreted results of experiments. ED and PB drafted the manuscript. ED, CHC, DM, PB, NvR edited and revised the manuscript. NvR provided liposome-encapsulated clodronate. All authors approved the final version of manuscript.

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## Author details

<sup>1</sup>Centre de Recherche du CHUL (CHUQ), 2705 Boulevard Laurier, RC-9800 Québec, Québec, Canada. <sup>2</sup>Département de Réadaptation, Faculté de Médecine, Université Laval, Québec, Québec, Canada. <sup>3</sup>Department of Molecular Cell Biology, Faculty of Medicine, Vrije Universiteit, Amsterdam, The Netherlands. <sup>4</sup>Département de Médecine, Faculté de Médecine, Université Laval, Québec, Québec, Canada. <sup>5</sup>Centre de Recherche de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec, Québec, Canada. <sup>6</sup>Unité de Physiothérapie, Département des Sciences de la Santé, Université du Québec à Chicoutimi, Chicoutimi, Québec, Canada.

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