

## Original Article

# Bone Marrow-Derived Cells Participate in Composition of the Satellite Cell Niche in Intact and Regenerating Mouse Skeletal Muscle

(satellite cells / bone marrow cells / satellite cell niche / transplantation / skeletal muscle regeneration)

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**Abstract.** The cellular components of the satellite cell niche participate in the regulation of skeletal muscle regeneration. Beside myogenic cells at different developmental stages, this niche is formed by cells of the immune system, the interstitial connective tissue and the vascular system. Unambiguous determination of the origin of these cell types could contribute to optimization of the cell-based therapy of skeletal muscle disorders. In our work, we intravenously transplanted mouse GFP<sup>+</sup> unseparated bone marrow cells into whole-body lethally irradiated immunocompetent mice four weeks before cardiotoxin-induced injury of the recipients' skeletal muscles. Seven and 28 days after the toxin injection, the injured regenerating and contralateral intact muscles were examined for identification of GFP<sup>+</sup> bone marrow-derived cells by direct fluorescence, protein immunohistochemistry and immunogold transmission electron microscopy. In both the intact and injured muscles, GFP positivity was determined in immune cells, mainly in macrophages, and in interstitial spindle-shaped cells. Moreover, in the injured muscles, rare GFP<sup>+</sup> endothelial cells of the blood vessels and newly formed myotubes and muscle fibres were present.

Our results confirmed the ability of bone marrow-derived cells to contribute to the cellular component of the satellite cell niche in the intact and regenerating skeletal muscle. These cells originated not only from haematopoietic stem cells, but obviously also from other stem or progenitor cells residing in the bone marrow, such as multipotent mesenchymal stromal cells and endothelial progenitors.

## Introduction

The term niche denotes a specialized local environment formed by cellular and acellular components where stem cells reside. It has a crucial role in maintaining the stem cells in undifferentiated and self-renewable state and in their production of differentiated progeny, i.e., in basic properties determining their stemness. The composition of the niche is dynamically changing during various processes such as development, regeneration, aging, tumorigenesis or other pathological conditions and can be influenced by exogenous factors. The adult stem cells of the skeletal muscle called satellite cells are located on the surface of the muscle fibre, between the plasmalemma and basal lamina (Mauro, 1961). The cellular components of the unique satellite cell niche comprise myogenic cells at various developmental stages, cells of the immune system, the interstitial tissue and the vascular system (Bentzinger et al., 2013; Yin et al., 2013; Mashinchian et al., 2018). The roles of these cell types that together participate in the regulation of adult skeletal muscle regeneration have been recently extensively studied; nevertheless, the origin of some of them remains to be elucidated.

The bone marrow represents a remarkable heterogeneous cell population composed by many cell types at different stages of maturity including haematopoietic stem cells and their progeny, multipotent mesenchymal stromal cells (MSCs; mesenchymal stem cells) and en-

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Received October 22, 2018. Accepted November 21, 2018.

This work was supported by grant project No. 15-09161S from the Grant Agency of the Czech Republic and grant project PROGRES Q40/06 and Q40/09 from the Ministry of Education, Youth and Sports of the Czech Republic.

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Abbreviations: MSCs – multipotent mesenchymal stromal cells, PBS – Dulbecco's phosphate-buffered saline.

endothelial progenitors. The haematopoietic stem cell-derived lymphoid progenitors differentiate into B and T lymphocytes, and myeloid progenitors give rise to granulocytes, monocytes, erythrocytes and megakaryocytes forming blood platelets. These cells circulate in the blood, but lymphocytes, granulocytes and monocytes are able to migrate into the connective tissue, where they play important roles in the immune system; for example, B lymphocytes can differentiate into antibody-producing plasma cells and monocytes can change into macrophages capable of phagocytosis, secretion of cytokines, and antigen processing and its presentation to other cells. Interestingly, mast cells and osteoclasts also originate from the haematopoietic stem cells. The already mentioned haematopoietic stem cell-derived immune cells are important cellular components of the skeletal muscle satellite cell niche. In resting conditions, mast cells and macrophages are the most abundant, but after muscle damage, circulating granulocytes, mainly neutrophils, are the most numerous at early stages of the reaction (Chazaud et al., 2003; Dumont and Frenette, 2010; Bentzinger et al., 2013). Contribution of haematopoietic stem cells to skeletal muscle regeneration has been described (Ferrari et al., 1998; Palermo et al., 2005; LaBarge et al., 2002; Brazelton et al., 2003; Doyonnas et al., 2004; Sacco et al., 2005; Abedi et al., 2005, 2007; de la Garza-Rodea et al., 2011). Even following transplantation of a single haematopoietic stem cell, the progeny of this cell was detected in muscle fibres after regeneration (Camargo et al., 2003; Corbel et al., 2003). Nevertheless, this phenomenon occurs only rarely and by a mechanism in which myeloid precursors could be incorporated by fusion during the formation of new muscle fibres (Camargo et al., 2003; Doyonnas et al., 2004; Sacco et al., 2005).

Multipotent mesenchymal stromal cells (MSCs) are multipotent adult stem cells originally isolated from the bone marrow (Friedenstein et al., 1966; Tavassoli and Friedenstein, 1983; Caplan, 1991). They can also be obtained from other tissues, such as the fat tissue or umbilical cord, and can differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* (Bianco and Gehron Robey, 2000; Ankrum et al., 2014). They have been extensively studied *in vitro*; however, their stem cell properties have to be confirmed in the living organisms *in vivo*. In addition to the classical trilineage potential required as a minimal criterion for their defining (Dominici et al., 2006), they may also differentiate to other mesodermal cell types *in vitro* such as fibroblasts, and after induction by 5-azacytidine to myoblasts (Wakitani et al., 1995).

Dezawa et al. (2005) introduced a method of induction of skeletal muscle lineage cells from adherent MSCs *in vitro*, which after their transplantation differentiated into muscle fibres in degenerated muscles or muscles of mdx-nude mice. Nevertheless, according to the recent knowledge, the ability of MSCs to directly differentiate into skeletal myoblasts under normal conditions in the adult organism has not been documented (Galli et al., 2014).

Endothelial progenitors represent a group of different cell types that contribute to endothelial cell formation *in vitro* and postnatal neovascularization *in vivo*, firstly isolated from the adult peripheral blood by Asahara et al. (1997). All the haematopoietic stem and progenitor cells, MSCs and vascular cells from the bone marrow have been proposed as sources of the endothelial progenitors (Chao and Hirschi, 2010).

The goal of our work was to unambiguously determine the cell types of bone marrow origin contributing to the cellular component of the satellite cell niche in regenerating and intact skeletal muscles. Therefore, we intravenously transplanted mouse GFP<sup>+</sup> freshly isolated unseparated bone marrow cells into whole-body lethally irradiated immunocompetent mice, and after four weeks during which the recipients had recovered, experimental injury was induced by intramuscular injection of cardiotoxin in the recipients' skeletal muscles. In this skeletal muscle regeneration model, cardiotoxin causes necrosis of the muscle fibre sarcoplasm, but does not affect their basal laminae, the satellite cells, blood vessels and muscle nerves. Seven and 28 days after the cardiotoxin injection, the injured and contralateral intact recipients' muscles were examined for the presence of GFP<sup>+</sup> cells by direct fluorescence, protein immunohistochemistry and immunogold transmission electron microscopy. So far, the ultrastructure of the transplanted GFP<sup>+</sup> bone marrow-derived cells settled in the mouse intact and regenerating skeletal muscle revealed in transmission electron microscope has not been described in the literature. The results of our study could contribute to optimization of the cell-based therapy of skeletal muscle disorders.

## Material and Methods

### Animals

C57Bl6-Tg(CAG-EGFP)C14-Y01-FM131-Osb mice were purchased from Riken Laboratories (Saitama, Japan) and C57Bl/6 mice from the Animal House of Masaryk University (Brno, Czech Republic). The mice used for the experiments were housed in groups of two in a temperature- and humidity-controlled colony room that was maintained on a 12-h light/dark cycle. Food and water were available *ad libitum* throughout the experiment. For this study, in total 36 mice were used, out of which six were donors and 30 were recipients or mice of a control group. The investigation was approved by the Ethical Committee supervising procedures in experimental animals at the Faculty of Medicine in Hradec Králové, Charles University, Czech Republic.

### Skeletal muscle injury

Muscle injury was induced in C57Bl/6 recipient mice by injecting 75 µl cardiotoxin (*Naja mossambica mossambica*; 0.06 µg/µl diluted in 0.9% NaCl solution; Sigma-Aldrich, St. Louis, MO) into the right tibialis anterior muscle. Before the cardiotoxin injection, the recipient mice were anesthetized by intraperitoneal ad-

ministration of ketamine (100 mg/kg; Narkamon 5%, Spofa, Prague, Czech Republic) and xylazine (10 mg/kg; Rometar 2%, Spofa). A short section of the skin above the right tibialis anterior muscle facilitated accurate injection of the cardiotoxin solution along the longitudinal axis of the muscle, deeply from the ankle to the knee of the mouse leg, using a 27-gauge needle and a 1-ml syringe. After the cardiotoxin injection, the cut skin was sutured using Premilene (B/Braun, Tuttlingen, Germany). All surgical procedures were performed under aseptic conditions.

### *Bone marrow cell transplantation*

Bone marrow cells were harvested from 8- to 10-week-old C57Bl6-Tg(CAG-EGFP)C14-Y01-FM131-Osb donor mice. All cells with a nucleus including all bone marrow cells of these mice express the GFP protein. Single-cell suspension was prepared from the marrow contents of the femurs of mice in Dulbecco's phosphate-buffered saline (PBS, Sigma-Aldrich) containing 2% foetal calf serum.

Recipient animals, 8- to 10-week-old C57Bl/6 mice, were exposed to 9 Gy of  $\gamma$  radiation from a  $^{60}\text{Co}$  source (Chisotron, Chirana, Prague, Czech Republic). Subsequently, three hours after whole-body irradiation, suspensions of  $5 \times 10^6$  unseparated freshly isolated GFP<sup>+</sup> bone marrow cells/mouse were transplanted intravenously via the tail vein to the recipients.

The described method of bone marrow cell transplantation was used in our previous work (Čížková et al., 2011), where its efficacy was confirmed by analysis of the donor-specific gene DNA content in the bone marrow of transplanted mice using the qPCR method.

Two types of control experiments were conducted. In the first type, unseparated bone marrow cells freshly isolated from donor mice were transplanted intravenously via the tail vein ( $5 \times 10^6$  bone marrow cells/mouse) to recipient mice (C57Bl/6) 3 h after their whole-body lethal irradiation, but the muscle injury was omitted. In the other type of control experiments, the cardiotoxin-induced injury of the recipients' tibialis anterior muscles was the only intervention, since whole-body irradiation and bone marrow cell transplantation were not performed.

### *Histology and GFP fluorescence*

The recipient mice (N = 18) and the mice of control experiments (N = 6) were sacrificed by CO<sub>2</sub> inhalation and subsequent exsanguination and then perfused with 4% paraformaldehyde in PBS. Their right injured and left intact tibialis anterior muscles were excised and immersed in 4% paraformaldehyde in PBS either for 48 h at room temperature, dehydrated and embedded in paraffin, or for 24 h at 4 °C, soaked in 10% sucrose in PBS for 4 h and then in 30% sucrose overnight at 4 °C, embedded in OCT (Tissue-Tek, Sakura Finetek, Radnor, PA) and snap-frozen in liquid nitrogen-chilled isopentane (Sigma-Aldrich). Serial 10- $\mu\text{m}$ -thick cryosections were cut using a cryostat (Leica Biosystems, Nussloch,

Germany) and every tenth slide was counterstained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and coverslipped with DAKO (Santa Clara, CA) Fluorescence Mounting Medium for GFP fluorescence observation. Six- $\mu\text{m}$ -thick serial sections were cut from the paraffin blocks using a microtome and every tenth slide was stained with haematoxylin-eosin for histological examination.

### *Immunohistochemistry and immunofluorescence*

Immunohistochemical detection of the GFP protein was performed by the indirect three-step LSAB method in paraffin-embedded sections. After deparaffinization and rehydration of sections, HistoStation (Milestone, Sorisole, Italy) was applied for antigen retrieval. Endogenous peroxidase was blocked in 5% H<sub>2</sub>O<sub>2</sub> (3  $\times$  10 min) and sections were then incubated in 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were incubated with primary rabbit anti-GFP, clone D5.1 (Cell Signaling Technology, Danvers, MA; 1 : 400) antibody overnight at 4 °C and after washing in PBS, they were exposed to anti-rabbit secondary biotinylated antibody (Jackson ImmunoResearch Laboratories, USA) for 45 min at room temperature. After rinsing, sections were incubated with streptavidin conjugated to horseradish peroxidase (DAKO) for 45 min and the reaction was then developed with 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Sections were dehydrated, counterstained with haematoxylin and mounted in DPX (Sigma-Aldrich).

Immunofluorescent detection of desmin was performed by an indirect two-step method in cryosections. Five per cent normal donkey serum (Jackson ImmunoResearch Laboratories, USA) was used for blocking, and cryosections were incubated with primary rabbit anti-desmin, clone Y66 (Abcam, Cambridge, UK) antibody for 1 h at room temperature and then with anti-rabbit Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, USA) for 45 min at room temperature. Cryosections were counterstained with DAPI (Sigma-Aldrich). To avoid false immunopositivity, serial sections were processed according to the same protocol, but primary antibodies were omitted.

Tissue sections were examined in an Olympus BX51 microscope (Tokyo, Japan) equipped with epifluorescence and a DP71 camera.

### *Immunogold transmission electron microscopy*

For immunogold transmission electron microscopy, the recipient mice (N = 6) were sacrificed by CO<sub>2</sub> inhalation and subsequent exsanguination and then perfused with 3% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M SB (Sorensen buffer, pH 7.2–7.4, Sigma-Aldrich). Their right injured and left intact tibialis anterior muscles were excised, cut to small blocks and immersed in the same fixative solution for 4 h at room temperature. After rinsing in 0.1 M SB, the tissue blocks were dehydrated in graded alcohols (30%, 50%, 70%, 90%, 96%) at 4 °C and embedded in LR White resin

(Sigma-Aldrich; polymerized for 1 day at 50 °C). Semithin sections were stained with haematoxylin and eosin. Ultrathin sections were cut with Ultratome Nova (LKB, Broma, Sweden), collected onto formvar carbon-coated nickel grids and processed for immunogold detection.

Immunohistochemical detection of the GFP protein was performed by an indirect two-step method in ultrathin sections. Ten per cent normal goat serum (Jackson ImmunoResearch Laboratories, USA) was used for blocking, and ultrathin sections were then incubated with primary rabbit anti-GFP, clone D5.1 (Cell Signaling Technology, USA; 1 : 40) or rabbit anti-desmin, clone Y66 (Abcam, 1:25) antibody for 3.5 h at room temperature and after thorough washing, with anti-rabbit goat EM grade 18 nm colloidal gold-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, USA, 1 : 20) for 2 h at room temperature.

Ultrathin sections counterstained with uranyl acetate and lead citrate were examined either under a Tesla BS500 transmission electron microscope (at 90 kV; Tesla, Czech Republic) equipped with a Megaview G2 digital camera and iTEM software (Olympus, Tokyo, Japan) or a JEOL JEM-1400Plus transmission electron microscope (at 120 kV; JEOL, Tokyo, Japan) equipped with an integrated 8Mpix CCD camera and software (JEOL).

## Results

### *Bone marrow-derived cells in the regenerating skeletal muscle 7 days after injury*

Seven days after induction of injury in skeletal muscles of whole-body irradiated and BMC-transplanted mice, newly formed myoblasts, myotubes and immature muscle fibres with centrally located nuclei were present in the regenerating area together with numerous cells of the inflammatory infiltrate. GFP positivity was observed in frequent immune cells, mainly in macrophages and neutrophils (Fig. 1 A, B, D arrowheads), which was confirmed by GFP protein immunohistochemical detections in deparaffinised (Fig. 1 E, F arrowheads) as well as ultrathin TEM sections (Fig. 2 A, A1). Many cells located in the connective tissue of the endomysium revealed GFP fluorescence (Fig. 1 A, B, D “empty” arrows). Although they had a spindle-like shape, they did not express muscle marker desmin, and thus they did not belong to the myogenic lineage and could not represent myoblasts (Fig. 1 D “empty” arrows). These endomysial GFP<sup>+</sup> cells were identified in deparaffinised (Fig. 1 E, F “empty” arrows) and importantly, also in ultrathin sections. Their elongated body with one nucleus was arranged parallel to the uninjured muscle fibres and contained very few organelles (Fig. 2 B, B1). Detection of desmin in the cryosections (Fig. 1 C, D) and in ultrathin TEM sections (Fig. 2 C, C1) revealed the presence of spindle-shaped myoblasts in the regenerating area. Importantly, GFP positivity and desmin positivity had never overlapped in spindle-shaped cells; therefore, in

our experimental setting, myoblasts were not bone marrow derived. Desmin was also expressed by myotubes and maturing muscle fibres, but only rarely; its signal had been co-localized with a weak GFP signal. GFP<sup>+</sup> structures resembling muscle fibres were noticed in the cryosections using small magnifications (Fig. 1 C arrow); nevertheless, using larger magnifications, it became apparent that they were degenerated muscle fibres packed with GFP<sup>+</sup> macrophages phagocytosing the necrotic sarcoplasm. Surprisingly, the tendon of the injured muscles contained numerous GFP<sup>+</sup> fibroblasts called tendinocytes. GFP positivity was infrequently observed in the wall of the blood vessels, mainly in endothelial cells (Fig. 1 E, F asterisks), but rarely also in some other cell types located in the deeper layers.

### *Bone marrow-derived cells in the regenerated skeletal muscle 28 days after injury*

Twenty-eight days after the toxin injection, injured skeletal muscles of whole-body irradiated and BMC-transplanted mice were fully regenerated. The damaged area could be estimated according to the smaller diameters of the regenerated muscle fibres; however, their nuclei were located at the periphery, similarly as in the uninjured muscle fibres. The number of GFP<sup>+</sup> immune cells substantially decreased in comparison with their number in the regenerating muscle examined 7 days after the injury (Fig. 3 A, B arrowheads, Fig. 4 C, C1). The endomysial spindle-shaped cells represented the cell population that expressed GFP the most frequently (Fig. 3 A, B, C, D, F “empty” arrows). Both in cryosections and in deparaffinised sections, we succeeded in finding GFP<sup>+</sup> muscle fibres whose frequency of occurrence following BMC transplantation was described to be very low (Fig. 3 A, E arrows). GFP positivity was observed in several tendinocytes in the tendon (Fig. 4 B, B1) and rarely in endothelial cells of the blood vessels (Fig. 4 A, A1), similarly to regenerating muscles evaluated 7 days after the toxin injection.

### *Bone marrow-derived cells in the contralateral skeletal muscle*

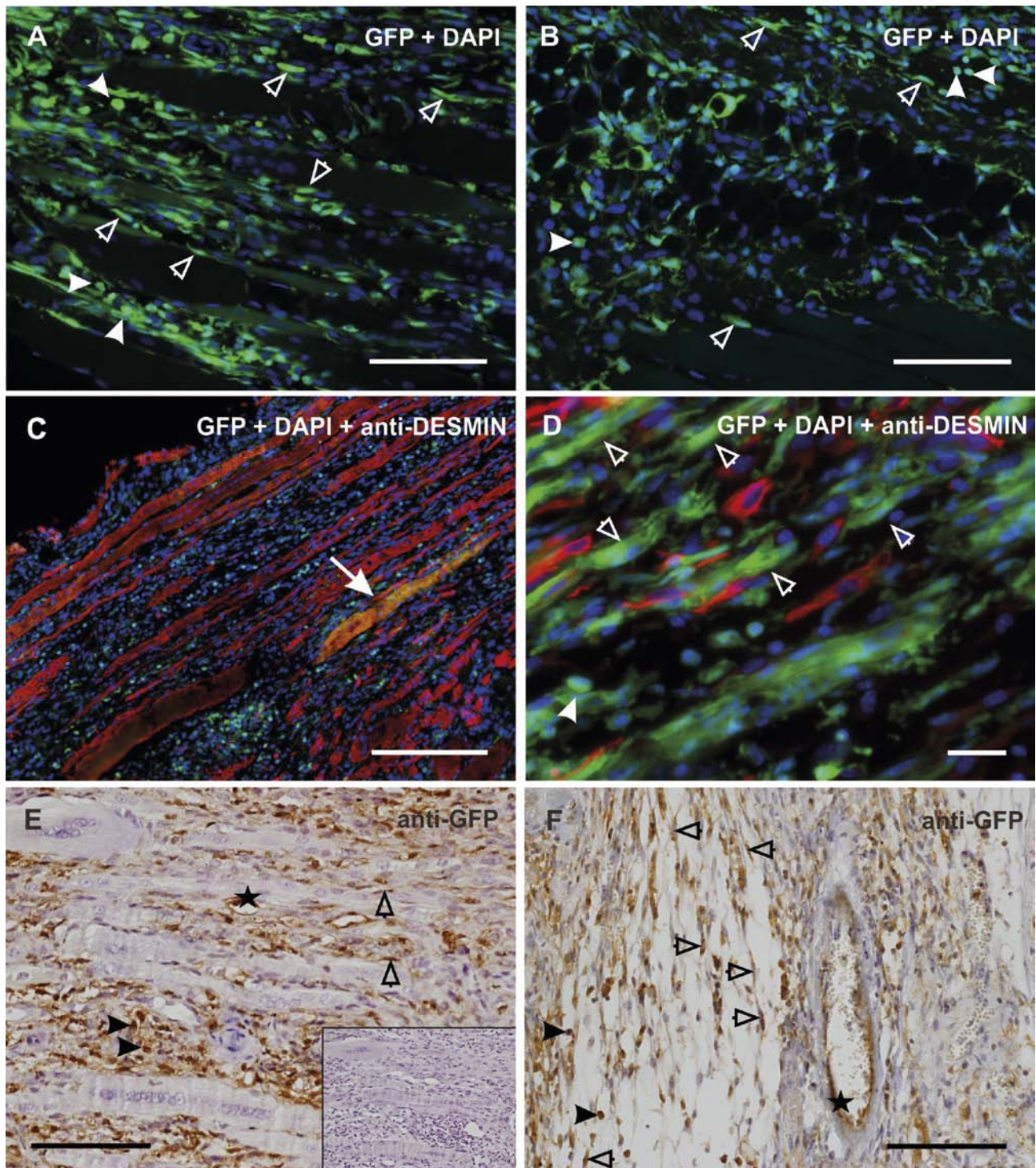
In contralateral skeletal muscles of whole-body irradiated and BMC-transplanted mice, GFP positivity was observed in infrequent immune cells, namely in macrophages and mast cells, and in occasional endomysial spindle-shaped cells in both time periods, 7 as well as 28 days after the toxin injury. In samples obtained from our experiments, we did not detect GFP in muscle fibres or in cells of the blood vessels.

### *Bone marrow-derived cells in the skeletal muscle in control experiments*

In the first type of control experiments, the recipient mice were whole-body irradiated and BMC-transplanted, but the muscle injury was not induced. The recipients'

uninjured skeletal muscles were excised at the same time as the injured skeletal muscles, e.g. 5 and 8 weeks after the whole-body irradiation and BMC transplanta-

tion; however, GFP positivity was only detected in infrequent endomysial spindle-shaped cells and in sparse immune cells including macrophages and mast cells in

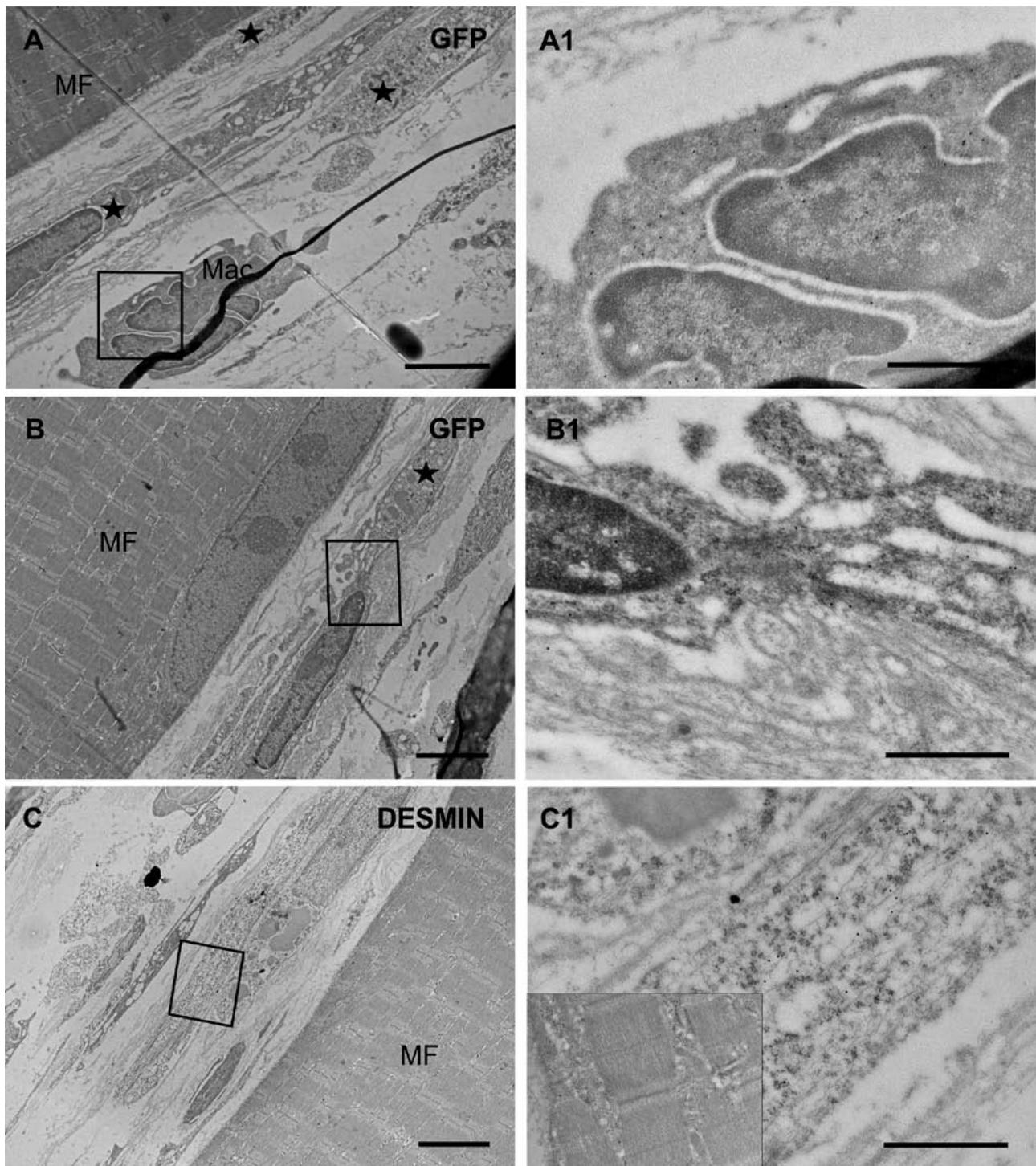


**Fig. 1.** Identification of donor-derived GFP<sup>+</sup> cells in the injured muscles of recipients by GFP fluorescence (green; A, B, C, D) and GFP immunohistochemistry (brown; E, F). In the regenerating muscle 7 days after injury, GFP positivity was observed in numerous immune cells, mainly in macrophages (A, B, D, E, F arrowheads), in some spindle-shaped cells located in the endomysium (A, B, D, E, F “empty” arrows) and rarely in endothelial cells of the blood vessels (E, F asterisks). The arrow in panel C points at a degenerated desmin<sup>+</sup> (red) muscle fibre filled with GFP<sup>+</sup> macrophages phagocytosing its necrotic sarcoplasm. The insert in panel E shows a negative control to GFP immunohistochemical detection when the primary antibody was omitted. A, B, C, D are counterstained with DAPI, E, F with haematoxylin.

Bars A, B, E, F 200 μm; C 400 μm; D 50 μm.

both time periods. We did not find the GFP signal in any muscle fibres or in the cells of the blood vessels in our samples.

In the other control experiments, the recipient mice were not whole-body irradiated nor BMC transplanted, but their skeletal muscles were injured. We examined

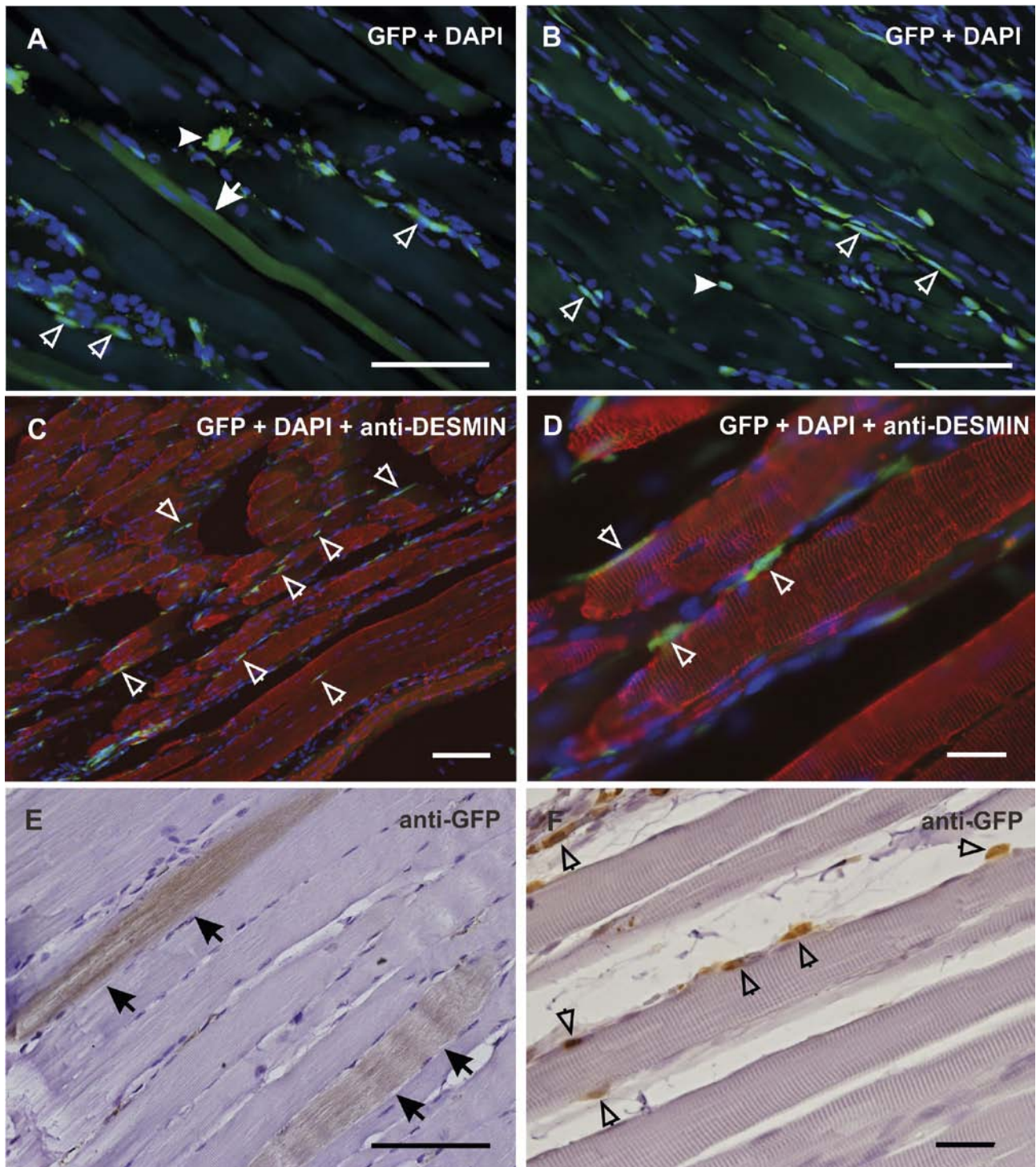


*Fig. 2.* Identification of donor-derived GFP<sup>+</sup> cells in the injured muscles of recipients by immunogold transmission electron microscopy (A, B) 7 days after the toxin injection. Areas of the rectangles in panels A, B, C are enlarged in panels A1, B1, C1, where 18 nm gold particles conjugated to the secondary antibody are visible. A shows a GFP<sup>+</sup> macrophage (Mac; rectangular area). Elongated cells in the vicinity of a muscle fibre (MF) labelled with asterisks also express GFP (enlarged images not shown). Similar GFP<sup>+</sup> elongated cells located next to a muscle fibre in the endomysium are captured in panel B (rectangular area and asterisk). Panel C shows immunogold detection of desmin. An elongated cell in the neighbourhood of a muscle fibre expresses this protein. In the insert, desmin immunopositivity in a muscle fibre is visible. Bars A, B, C 3  $\mu$ m; A1, B1, C1 1  $\mu$ m.

the injured skeletal muscles both 7 and 28 days after the toxin injection and did not detect any GFP positivity.

The results of semi-quantitative evaluation of the amount of GFP<sup>+</sup> cells in the injured, contralateral and control recipients' skeletal muscles examined 5 (7D, 7

days after the toxin injection in the injured recipients' muscles) and 8 (28D, 28 days after the toxin injection in the injured recipients' muscles) weeks after the whole-body irradiation and BMC transplantation are summarized in Table 1.



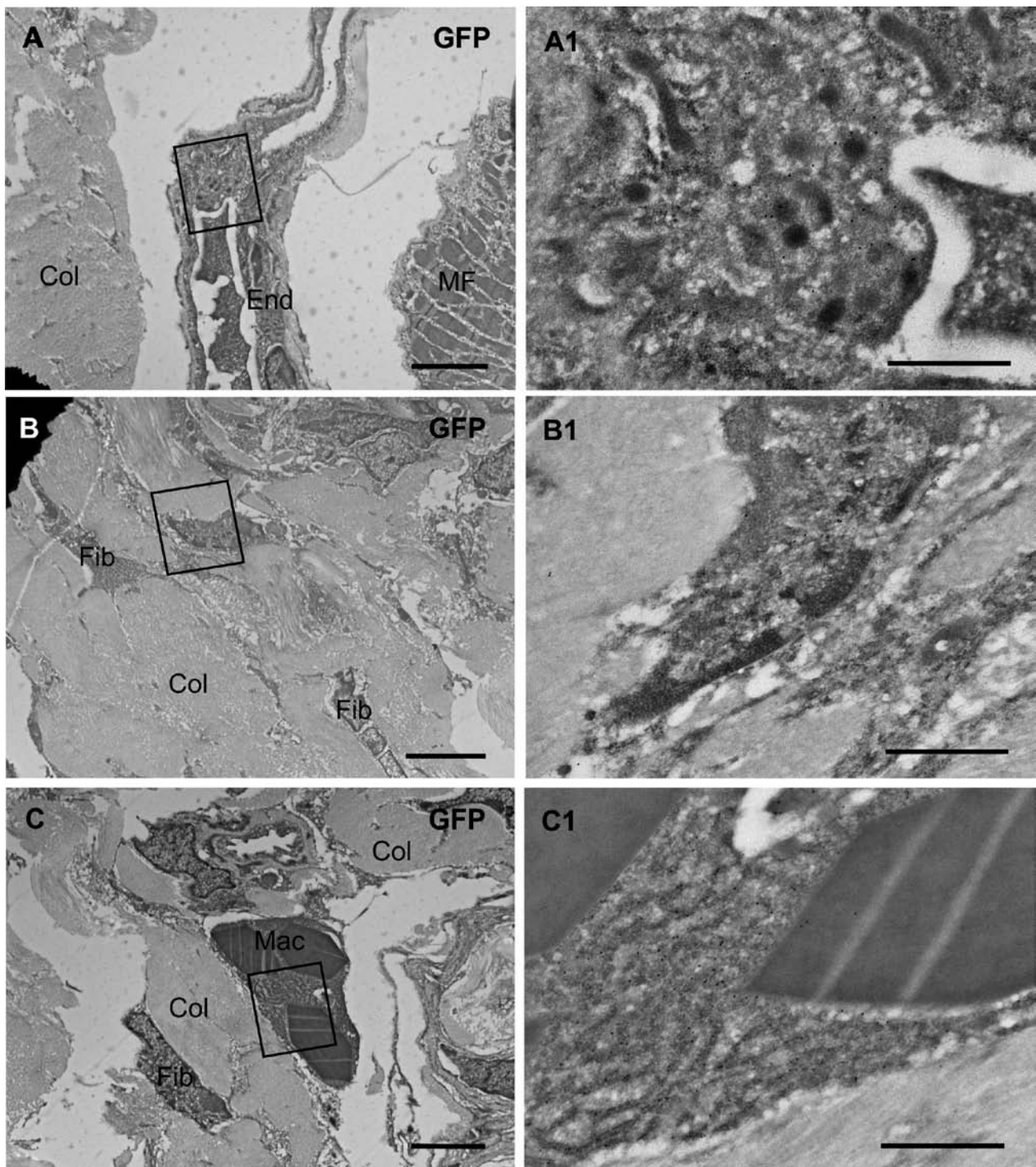
*Fig. 3.* Identification of donor-derived GFP<sup>+</sup> cells in the injured muscles of recipients by GFP fluorescence (green; A, B, C, D) and GFP immunohistochemistry (brown; E, F). In the regenerated muscle 28 days after injury, GFP<sup>+</sup> immune cells (A, B arrowheads) and spindle-shaped cells in the endomysium (A, B, C, D “empty” arrows) became less numerous, whereas infrequent GFP<sup>+</sup> endothelial cells (Fig. 4) and desmin<sup>+</sup> GFP<sup>+</sup> muscle fibres (A, E arrows) were still detected. A, B, C, D are counterstained with DAPI, E, F with haematoxylin.

Bars A, B, E 200 μm; C 400 μm; D, F 50 μm.

## Discussion

In this study, GFP positivity indicating the bone marrow origin was identified during skeletal muscle regen-

eration in both time periods, 7 and 28 days after the muscle injury, in all cell types that represent important constituents of the satellite cell niche, namely in cells of the immune system, cells of the endomysial connective



*Fig. 4.* Identification of donor-derived GFP<sup>+</sup> cells in the injured muscles of recipients by immunogold transmission electron microscopy (A, B, C) 28 days after the toxin injection. Areas of the rectangles in panels A, B, C are enlarged in panels A1, B1, C1, where 18 nm gold particles conjugated to the secondary antibody are visible. Panel A shows a GFP<sup>+</sup> endothelial cell of a blood vessel (rectangular area; End) located between a muscle fibre (MF) and bundles of collagen fibres (Col), part of the tendon. The tendon of the muscle contains fibroblasts (B; Fib). One of the fibroblasts (rectangular area) was proved to express GFP (B1). Panel C shows a GFP<sup>+</sup> macrophage (Mac; rectangular area) in the region of the tendon.

Bars A, B, C 3  $\mu$ m; A1, B1, C1 1  $\mu$ m.



*Table 1. Summarized results of semi-quantitative evaluation of the amount of GFP<sup>+</sup> cells in the injured, contralateral and control recipients' skeletal muscles examined 5 (7D, 7 days after the toxin injection in the injured recipients' muscles) and 8 (28D, 28 days after the toxin injection in the injured recipients' muscles) weeks after whole-body irradiation and BMC transplantation*

		GFP <sup>+</sup> cells			
		Immune cells	Endomysial spindle-shaped cells	Myotubes, muscle fibres	Endothelial cells of the blood vessels
Injured muscles	7D	+++	+++	+	+
	28D	++	++	+	+
Contralateral muscles	7D	+	+	-	-
	28D	+	+	-	-
Control muscles	7D	(+)	+	-	-
	28D	(+)	+	-	-

tissue, blood vessel-associated cells and cells of the myogenic lineage. In contralateral and control uninjured muscles, GFP positivity was determined in less numerous immune cells and endomysial cells.

GFP<sup>+</sup> immune cells, mainly macrophages, were abundant in the regenerating muscle 7 days after the injury and were still detected in the regenerated muscle 28 days after the cardiotoxin injection. In the contralateral and control uninjured skeletal muscles, GFP<sup>+</sup> macrophages and sparse mast cells were sporadically observed. The cells of the immune system derived from haematopoietic stem cells are indispensable for the initial step of regeneration of the injured skeletal muscle (Lescaudron et al., 1999). Resident leukocytes activated by the injury secrete e.g. TNF- $\alpha$  and interleukin-6, which together with other factors promote activation and proliferation of the satellite cells and induce attraction of circulating granulocytes, mainly neutrophils. Neutrophils release pro-inflammatory signals necessary for recruitment of monocytes, which become very numerous and differentiate into macrophages. Initially, pro-inflammatory M1 macrophages phagocytose necrotic sarcoplasm of the degenerated muscle fibres and promote proliferation of myoblasts arisen from the activated dividing satellite cells. Subsequently, anti-inflammatory M2 macrophages regulate myogenic differentiation and stimulate myotube formation (Bentzinger et al., 2013; Yin et al., 2013; Mashinchian et al., 2018).

Thus, all types of the above-mentioned immune cells precisely participate in the regulation of skeletal muscle regeneration, and any changes in their appropriate proportions can lead to regeneration failure (Arnold et al., 2007; Segawa et al., 2008; Liu et al., 2017). This was also documented in our previous study in which the recipients were whole-body irradiated 4 h after the muscle injury to eradicate dividing satellite cells and suppress host haematopoiesis, and subsequently transplanted with donor bone marrow cells. Seven days after the cardiotoxin injection, no donor bone marrow-derived immune cells were found in the degeneration area, which led to persistence and even calcification of the necrotic sarcoplasm of the injured muscle fibres. Two and four

weeks following muscle damage, when in the course of proper regeneration new muscle fibres were already present, we found many donor-derived large multinucleated cells resembling foreign body giant cells formed by fusion of activated macrophages in the close vicinity of the remaining necrotic muscle fibres (Čížková et al., 2011).

Interestingly, in this study the GFP signal was recognised in many desmin<sup>-</sup> spindle-shaped cells located in the endomysium of the injured muscles examined both 7 and 28 days after cardiotoxin injection. In the contralateral and control uninjured muscles, these cells were also noticed, but in lower quantity as estimated. In the interstitial tissue of endomysium, different cell types occur; however, their origin and functions in the muscle regeneration have not been fully elucidated (Bentzinger et al., 2013; Yin et al., 2013; Mashinchian et al., 2018). Fibroblasts proliferate after muscle injury very quickly and are indispensable for remodelling of the satellite cell niche during regeneration by secreting constituents of the extracellular matrix and basement membranes. Moreover, they support expansion of the satellite cells, as documented by a study in which partial ablation of fibroblasts during regeneration led to reduced numbers of satellite cells and their premature differentiation, and to a smaller size of formed muscle fibres (Murphy et al., 2011). Fibroblasts can arise from the resident mesenchymal stem cells; nevertheless, e.g. in the tumour stroma, also from the donor bone marrow (Dikerze et al., 2004; Barcellos-de-Souza et al., 2013; Kurashige et al., 2018).

Fibroadipogenic progenitors are mesenchymal cells that express surface markers CD34, Sca-1, and PDGFR $\alpha$  and are capable of differentiation into fibroblasts, adipocytes, but not into muscle cells. After muscle injury, they are activated, proliferate, and together with fibroblasts synthesize extracellular matrix proteins during regeneration. Furthermore, they facilitate myogenic differentiation, e.g. by secreting interleukin-6 (Joe et al., 2010; Heredia et al., 2013; Fiore et al., 2016). To date, their origin has not been unambiguously determined. Mitchell et al. (2010) described resident stem cells in the

muscle interstitium which express stress mediator PW1, surface markers CD34, Sca-1, but not satellite cell markers Pax7 and PDGFR $\alpha$ , and have myogenic potential when cultured *in vitro*, unlike fibroadipogenic progenitors (Mitchell et al., 2010; Pannérec et al., 2013; Yin et al., 2013). These PW1<sup>+</sup> interstitial cells promote proliferation and differentiation of the satellite cells (Formicola et al., 2014; Mashinchian et al., 2018). Pax3 lineage tracing experiments indicate that PW1<sup>+</sup> interstitial cells do not arise from embryonic Pax3<sup>+</sup> myogenic progenitor cells; therefore, they are derived from a distinct lineage than the satellite cells (Mitchell et al., 2010; Yin et al., 2013).

Telocytes represent another interstitial cell type of undetermined origin occurring in the skeletal muscle. They are characterized according to their appearance in the transmission electron microscope as cells with a small body and very long thin moniliform prolongations, dichotomously branched, forming a 3D network (Popescu et al. 2011; Suciú et al., 2012; Yin et al., 2013). Unlike fibroblasts, they express c-kit and then also PDGFR $\beta$  and VEGF, but not Pax7. Their estimated functions are transmission of intercellular signalling by shedding or absorbing microvesicles enriched for proteins and RNAs and facilitation of vasculogenesis by secretion of VEGF (Popescu et al. 2011; Suciú et al., 2012; Yin et al., 2013). Dreyfus et al. (2004) identified donor bone marrow-derived cells expressing CD34 or Sca1 in the interstitium of the intact muscle. Although immunophenotyping of these cells is insufficient according to the recent knowledge, in accordance with our results this study shows the presence of bone marrow-derived cells in muscle interstitium. In conclusion, the interstitial cells of the endomysium are found to be a very intriguing cellular component of the satellite cell niche and it will be important to study their exact functions and origins. Which cell type the GFP<sup>+</sup> desmin<sup>-</sup> cells observed in the endomysium of both intact and regenerating muscles in our experiments represent remains to be clarified and has become the goal of our upcoming study.

Infrequently, GFP positivity was noticed in the endothelial cells of the blood vessels of injured muscles both 7 and 28 days after the toxin injection. These findings give evidence of the presence of endothelial progenitors in the donor bone marrow. Blood vessel-associated cells are an important component of the satellite cell niche. In both intact and regenerating muscle, capillaries occur in close vicinity to the satellite cells (Christov et al., 2007). After muscle injury, their number initially increases, despite that they were not directly damaged (Ochoa et al., 2007). The endothelial cells secrete a variety of mitogenic or anti-apoptotic factors, such as VEGF, that influence muscle cells (Abou-Khalil et al., 2010; Bentzinger et al., 2013) and vice versa, differentiating myogenic cells also produce VEGF and thus have a proangiogenic effect. Therefore, myogenesis and angiogenesis are closely co-regulated (Bentzinger et al., 2013; Lacroche et al., 2017).

We rarely detected GFP together with desmin in the myotubes of injured muscles 7 days and in the muscle fibres 28 days after the cardiotoxin injection. The contribution of haematopoietic stem cells to skeletal muscle regeneration has been described (Ferrari et al., 1998; Palermo et al., 2001; LaBarge et al., 2002; Brazelton et al., 2003; Camargo et al., 2003; Corbel et al., 2003; Doyonnas et al., 2004; Sacco et al., 2005; Abedi et al., 2005, 2007; de la Garza-Rodea et al., 2011) and our results confirmed occurrence of this rare phenomenon. Importantly, we did not find any GFP<sup>+</sup> desmin<sup>+</sup> myoblasts and the GFP signal appeared only in myotubes and muscle fibres, which supports the findings that haematopoietic stem cells are incapable of generating myogenic progenitors and that myeloid cells are incorporated by fusion during the formation of new myotubes (Camargo et al., 2003; Corbel et al., 2003). Myogenic cells at various developmental stages are important constituents of the satellite cell niche during reparative myogenesis. They secrete growth factors, provide regulatory signals and are involved in intercellular interactions. Notch and Wnt7a signalling pathways belong to the most important and thus recently studied (Brack et al., 2008; Bentzinger et al., 2013; Yin et al., 2013).

To conclude, our results confirmed the ability of bone marrow-derived cells to contribute to the cellular component of the satellite cell niche in the intact and regenerating skeletal muscle. These cells originated not only from the haematopoietic stem cells, but obviously also from other stem/progenitor cells residing in the bone marrow, such as MSCs and endothelial progenitors.

### Acknowledgements

The authors thank Mrs. Magda Voborníková, Ms. Petra Hajzlerová, Mrs. Simona Vrchotová, Mrs. Jaroslava Prokešová, Mrs. Milada Hetešová and Mrs. Jana Hošková for their skilful technical assistance.

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