

Inflammatory markers CD11b, CD16, CD66b, CD68, myeloperoxidase and neutrophil elastase in eccentric exercised human skeletal muscles

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Abstract The aim of the present study was to investigate leucocyte markers, CD11b, CD16, CD66b, CD68, myeloperoxidase and neutrophil elastase on skeletal muscle biopsies from biceps brachii after unaccustomed eccentric exercise followed by the second bout of exercise 3 weeks later. The subjects (10 subjects received COX-2 inhibitor (Celecoxib) and 13 subjects received placebo) were divided into three categories: mild, moderate and severe effect of eccentric exercise, according to the reduction and recovery of muscle force-generating capacity after performing 70 maximal eccentric actions with elbow flexors on an isokinetic dynamometer. The results showed that the CD66b antibody was applicable for localization of neutrophils in human skeletal muscle, whereas the other studied neutrophil markers recognized also other leucocytes than neutrophils. The number of CD66b positive cells

in skeletal muscle was very low and was not affected by the exercise. The macrophage marker CD68 showed reactivity also against satellite cells and fibroblast-like cells in skeletal muscle and therefore cannot be applied as a quantitative value for inflammatory cells. Skeletal muscle fibre injury, shown as dystrophin negative fibres, was observed approximately in half of the biopsies at 4 and 7 days after the first exercise bout in the categories moderate and severe effect of eccentric exercise. These subjects represent the most prominent loss in muscle force-generating capacity both at the category and the individual levels. Furthermore, deformed skeletal muscle fibres were observed in five subjects in these categories after the second bout of exercise. The present results suggest that neutrophils are not involved in skeletal muscle fibre injury and the reduction in muscle force-generating capacity after a single bout of eccentric exercise is a good indirect indicator of muscle damage in humans. Furthermore, prolonged regeneration process could be one of the reasons for impaired peripheral muscle function after high-force eccentric exercise.

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Abbreviations

BSA	Bovine serum albumin
CD68	Cluster of differentiation 68
CEL S01	Celecoxib subject 01
DAPI	4',6-Diamidino-2-phenylindole
<i>g</i>	Gravity
<i>M_r</i>	Molecular weight
O.C.T	Optimal cutting temperature
PBO S01	Placebo subject 01
ROI	Region of interest

Introduction

The aim of the present study was to test leucocyte markers, which have been applied for localization and counting the number of inflammatory cells, especially neutrophils and macrophages in human skeletal muscles after damaging exercise. The complexity of inflammation is currently acknowledged (see for review Scott et al. 2004). The affected tissue might be influenced by proinflammatory signalling molecules, even in the absence of inflammatory cell invasion. Antibodies against the cell surface molecules CD11b, CD16 and CD66b, the lysosomal enzymes CD68 and myeloperoxidase, as well as the serine protease neutrophil elastase were included in the present study. According to our knowledge, all of these antibodies have been used for counting the number of inflammatory cells in human skeletal muscles after damaging exercise. Investigators have applied CD11b (Malm et al. 2000, 2004), CD16 (Mikkelsen et al. 2009), CD66b (Paulsen et al. 2010a), myeloperoxidase (Beaton et al. 2002; MacNeil et al. 2011; Mahoney et al. 2008; Stupka et al. 2001) and neutrophil elastase (Beaton et al. 2002) to identify neutrophils and CD68 to identify macrophages (Beaton et al. 2002; Cramer et al. 2004, 2007; MacNeil et al. 2011; Mahoney et al. 2008; Mikkelsen et al. 2009; Paulsen et al. 2010a, b; Peterson et al. 2003; Przybyla et al. 2008; Stupka et al. 2001). On the other hand, CD11b (Hellsten et al. 1997) and CD16 (Paulsen et al. 2010b) have been used to monitor leucocytes in general, whereas Przybyla et al. (2008) have applied CD11b as a marker for a subpopulation of macrophages. While several specific markers for neutrophils have been reported, CD68 is widely applied as monocyte/macrophage specific marker, not only in exercised skeletal muscles but also in diagnostics and in other areas of research. In contrast to this generally accepted concept, it has been reported that anti-human CD68 antibodies show also some reactivity against other haematopoietic cells such as lymphocytes and also against non-haematopoietic cells, such as fibroblasts and endothelial cells (Gottfried et al. 2008). According to our knowledge it has not been investigated earlier on human skeletal muscles, whether these neutrophil and macrophage markers exclusively recognize the corresponding inflammatory cells.

Maximal eccentric exercise with the knee-extensors or elbow flexors on an isokinetic dynamometer has frequently been used to induce and to study skeletal muscle damage in humans. A typical response to such high-force, single joint eccentric exercise protocols is on average a 50 % reduction of the force-generating capacity immediately post-exercise, followed by gradual recovery over the next days or weeks (Clarkson and Hubal 2002). There are

clearly individual differences both in force-generating capacity immediately post-exercise and in the length of the force recovery period (Sayers and Clarkson 2001). Prolonged recovery of muscle force is thought to be related to distortion of the myofibrillar structure and disturbed calcium homeostasis and/or prolonged inflammatory response (Clarkson and Hubal 2002). Consequently, it has been suggested that reduction in muscle force-generating capacity may be a valuable indicator for monitoring muscle damage following exercise (Warren et al. 1999). Physiological adaptation takes place after a single bout of unaccustomed eccentric exercise by making muscles more resistant against structural changes from the second bout of eccentric exercise (Clarkson et al. 1987). In the present study, isokinetic eccentric actions with elbow flexors on an isokinetic dynamometer were applied to induce skeletal muscle damage (Paulsen et al. 2010a). In this model, the force-generating capacity reduced approximately 50 % immediately after the first bout of eccentric exercise in both COX-2 inhibitor (Celecoxib) and placebo groups. Furthermore, a COX-2 inhibitor reduced muscle soreness, but did not influence the recovery and the adaptation after eccentric exercise (Paulsen et al. 2010a). Exercise with elbow flexors is less frequently used protocol in studies investigating muscle damage from muscle biopsies. For the present study the exercise protocol using elbow flexors was chosen, because the muscle damage has been shown to be more pronounced compared to corresponding exercise with knee extensors (Chen et al. 2011).

The purpose of the present study was to investigate the leucocyte markers, CD11b, CD16, CD66b, CD68, myeloperoxidase and neutrophil elastase in skeletal muscle biopsies from biceps brachii muscles after unaccustomed eccentric exercise followed by the second bout of exercise 3 weeks later. All 23 subjects (10 subjects received COX-2 inhibitor [Celecoxib] and 13 subjects received placebo) were divided into three categories: mild, moderate and severe effect of eccentric exercise, according to the reduction and recovery of force-generating capacity. Studied leucocyte markers were first tested on leucocyte smears prepared from extracted leucocytes from human whole blood. Second, typical immunohistochemical staining patterns, such as cell localization or cell shape, for studied leucocytes markers in skeletal muscle sections were evaluated in the three categories representing different severity of skeletal muscle damage. Third, transmission electron microscopy sections were used to study the cell types corresponding to the characteristic immunohistochemical staining patterns for each category. Finally, the numbers of CD16, CD66b and CD68 positive cells were counted from immunohistochemical staining of muscle cross-sections.

Methods

Ethical approval

The present study was approved by the Regional Ethics Committee of Southern Norway and conformed to the standards set by the Declaration of Helsinki.

Subjects

This exercise experiment and the subjects have previously described by Paulsen et al. (2010a). Twenty-three healthy, physically active subjects (7 females; 16 males: 25 ± 3 years, 73 ± 9 kg) gave their written, informed consent to participate in the study. None of the subjects were engaged in any kind of specific resistance training with the elbow flexors. The subjects were asked to continue their regular diet, not to take any form of medication (except contraceptives) or prescription-free supplements (such as anti-oxidants) and avoid exercise during the experiment. Ten subjects were administered 400 mg celecoxib (Celebra, Pfizer, Oslo, Norway; 200 mg morning and evening) for 9 days starting 30–45 min before the first bout of eccentric exercise. 400 mg per day is the highest recommended dosage in the Norwegian Pharmaceutical Product Compendium. Correspondingly, thirteen subjects received two placebo tablets (lactose, Rikshospitalet University Hospital Pharmacia, Oslo, Norway) per day. The subjects were contacted every morning and evening to ascertain that the tablets were taken. The second bout of eccentric exercise was performed without drug administration 3 weeks after the first exercise bout. In addition, two subjects PBO (placebo) S13 and PBO S14 had microdialysis in both exercised and non-exercised arm 2 h after the first bout of exercise (Paulsen et al. 2010a).

Familiarization of the subjects to the testing for muscle force-generating capacity

Prior to the testing for the baseline of muscle force-generating capacity, all subjects were familiarized with exercising on the dynamometer 1–2 times on separate days during 1 week before the first bout of eccentric exercise. Before each familiarization session, subjects did arm-cranking (load 30–50 W; Lode B.V., Groningen, The Netherlands) for 3 min warm-up. After that each subject performed four submaximal concentric isokinetic actions (range of motion 175–40°, 180° equals full extension of elbow joint), two maximal voluntary isokinetic concentric actions (velocity of motion 60°/s) and two maximal voluntary isometric actions at 90° in elbow joint for 5 s with both arms on the dynamometer (described in detail below)

per each familiarization session. The subjects did not perform any eccentric contractions in the familiarisation sessions.

Baseline measurement of muscle force-generating capacity

Immediately before the first eccentric exercise bout, maximal voluntary isokinetic concentric (range of motion 175–40°; 180° equals full extension of elbow joint) elbow flexion from both arms were tested after warming up and after four submaximal concentric isokinetic actions on the dynamometer. The highest peak torque at velocity 60°/s of two consecutive attempts was chosen for baseline value. In addition to this, two maximal voluntary isometric actions at 90° in elbow joint for 5 s with both arms on the dynamometer were tested (data not shown). The subjects were verbally encouraged to perform maximally in each attempt.

Exercise protocol

Subjects performed two identical bouts of maximal eccentric exercise with one arm. There were 3 weeks between these two exercise bouts. Exercised arm was randomly chosen and the contralateral arm served as a control. The exercise protocol contained 70 maximal eccentric actions with elbow flexors on an isokinetic dynamometer REV 9000 Technogym (Technogym, Gambettola, Italy). The subjects were fastened with three belts (over the hip, over the shoulder of the exercising arm and diagonally over the chest from the shoulder of non-exercising arm to the opposite hip), on the chair of the dynamometer in sitting position. A cushion was placed between the upper arm and the chair to support and to keep the shoulder joint in slightly flexed position (30–35° from vertical axis) and to fix the elbow during the exercise. The subjects grasped the handle connected to the lever arm of the dynamometer. It was possible to rotate the handle so that the wrist was in a comfortable position. The range of motion was 40–175° (180° equals full extension of elbow joint) and the velocity of motion 30°/s. Seventy eccentric actions were divided into 14 sets of 5 repetitions. As the subjects only performed eccentric contractions with their elbow flexors, they rested their muscles during the flexion movement that lasted for 4.5 s. There was a 30 s rest period between sets. The subjects were instructed to supinate their forearm (elbow joint) and to resist the movement of the dynamometer arm with their maximal force throughout the full range of motion and during each repetition. They also received real time visual feedback about their performance from a computer screen and were verbally encouraged during the exercise bout to ensure maximal effort for each repetition.

Muscle force-generating capacity after exercise

Recovery of muscle function was assessed with repeated tests of maximal voluntary isokinetic concentric elbow flexion and maximal voluntary isometric actions (data not shown) as described above. Tests were performed immediately, 6 h, 1, 2, 3, 4 and 7 days after the both exercise bouts. Both exercised and contralateral arm were tested. The intra-individual coefficient of variation was $<5\%$ for this tests.

Serum creatine kinase analysis

Blood samples were collected before the first and the second exercise bouts, 1 (data shown only in Suppl. Table 3) and 8 h, 1, 2, 3, 4 and 7 days after the both exercise bouts. Blood was drawn from an antecubital vein into a 10 ml serum vacutainer tube. After coagulating for 30–45 min at room temperature ($\sim 20\text{ }^{\circ}\text{C}$), the blood was centrifuged at 2,700g for 10 min at $4\text{ }^{\circ}\text{C}$. Serum was then immediately pipetted into eppendorf tubes and stored at $-80\text{ }^{\circ}\text{C}$ until analysis. Creatine kinase (CK) was analysed with the Hitachi 917 Automated Biochemistry Analyzer (Roche[®], Basel, Switzerland) at Oslo University Hospital, Rikshospitalet (Oslo, Norway); analytic coefficient of variation being $<2.8\%$.

Scintigraphy

The method has been earlier described in detailed by Raastad et al. (2003). In brief, 50 ml blood was drawn, and leucocytes were isolated and labelled with $^{99\text{m}}\text{Tc}$ (99mTc), before being re-infused. Accumulation of $^{99\text{m}}\text{Tc}$ -leucocytes in the subjects' arms was detected using a gamma camera 6 and 20 h after the first and second bout of exercise. The radioactivity was quantified with custom-made software (from GE Healthcare, Oslo, Norway) from scintigrams of subjects' upper arm elbow flexors. The accumulation of radioactivity was calculated as a ratio between a region of interest (ROI) in the exercised arm and radioactivity in the same ROI in the control arm, corrected for background radiation. The radiolabelling and scintigraphy were carried out at the Department of Nuclear Medicine, Oslo University Hospital, Norway.

Statistics

Variability of the muscle force-generating capacity and creatine kinase results were expressed as means \pm standard deviations and scintigraphy results as mean \pm standard error of the means. A non-parametric equivalent of variance analysis for related samples, Friedman test, was used to search for statistically significant differences over

time for the muscle force-generating capacity and creatine kinase results in the three different categories. The statistical significances of different time points compare to baseline in each category was determined by non-parametric Wilcoxon Signed Rank test for two related samples, when Friedman's test showed $p < 0.05$. Non-parametric Kruskal–Wallis H tests for independent samples were performed to test the statistically significant differences between different categories. When Kruskal–Wallis H test showed $p < 0.05$, non-parametric Mann–Whitney U for two independent samples was used to compare differences between different categories for muscle force-generating capacity, creatine kinase and scintigraphy. Differences were considered statistically significant at $p < 0.05$.

Time points of muscle biopsies

Muscle biopsies were obtained from both exercised and contralateral biceps brachii muscle. Biopsies were sampled from each subject at two of the four time points; 1 h ($n = 9$), 2 ($n = 15$), 4 ($n = 10$), or 7 ($n = 10$) days after the first exercise bout and at one of the two time points; 1 h ($n = 9$) or 2 ($n = 11$) days the second bout of exercise (3 weeks between the two exercise bouts). Muscle sampling time points were randomly selected for each subject.

Muscle biopsies

A 5 or 6 mm biopsy needle was used to obtain tissue samples from m. biceps brachii. Biopsies for the first time point from both exercised and contralateral arm were obtained from the mid part of m. biceps brachii. The needle insertion for the second time point was placed 1–2 cm medial to the first insertion and correspondingly the third biopsy was placed 1–2 cm laterally. Subjects were in supine position during the muscle biopsy procedure. Local anaesthesia (Xylocain[®] 10 mg/ml, adrenaline 5 $\mu\text{g/ml}$, AstraZeneca, Södertälje, Sweden) was injected to the area of incision (skin and fascia; not into the muscle tissue). Incision of scalpel was made through the skin and subcutaneous fat all the way through fascia. If there was not enough muscle sample from the first insertion of biopsy needle, the situation was evaluated together with the subject whether it would be possible to take the second one or possible the third one. Biopsy needle was not inserted more than three times into the incision of scalpel. Each of the successive biopsies was collected at different depth or directions from the previous biopsy to avoid collecting muscle tissue from the same place as previous biopsy. Muscle biopsies were always obtained first from exercised arm. The same biopsy needle was used for both exercised and contralateral arm. Tissue was carefully removed from biopsy needle with sterile syringe needle to avoid any

contamination. The average of wet weights of muscle biopsies was 146 ± 46 mg.

After removing the tissue from biopsy needle with sterile syringe needle, muscle tissue was rinsed in ice-cold saline (0.9 % NaCl, B. Braun Melsungen AG, Melsungen, Germany) while visible fat and connective tissue were removed under stereomicroscopy Opton Zeiss at 60–100 \times magnification. Compact and large piece of muscle tissue was selected for histology sample. Selected muscle piece was subsequently frozen in isopentane cooled on dry ice and stored at -80 °C until analysis. For transmission electron microscopy small compact pieces of muscle tissue were immersed in fixative (4 % paraformaldehyde and 0.1 % glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4) and stored at $+4$ °C until embedding.

Preparation of leucocyte smears from extraction of leucocytes from whole blood

Leucocytes were extracted from whole blood to test the antibodies (Table 1) against different types of circulating leucocytes. Blood was drawn from an antecubital vein into a 10 ml EDTA-vacutainer tube from three healthy subjects. The tube was gently turned up and down few times. Whole blood was gently mixed with 6 % dextran ($M_r \sim 500,000$, Fluka Biochemika, Buchs, Switzerland) in saline (blood:dextran 4:1 v/v) and incubated at the room temperature for 60 min. The overlying leucocyte rich plasma was transferred to an eppendorf tube. Small amount of red blood cells were removed by a short spinning and by transferring the overlying leucocyte rich plasma to a fresh tube. After centrifugation at 1,000g for 5 min, plasma was discarded and the pellet of leucocytes was mixed gently with 5–10 μ l of remaining plasma. A small drop (1–2 μ l) of leucocyte–plasma suspension was placed on the microscope glass slides and dispersed using other slide. The leucocyte smears were immediately air-dried and fixed

10 min in methanol at -20 °C and stored for further analysis at -20 °C.

Immunostaining of leucocyte smears

Leucocyte smears prepared of extracted leucocytes from whole blood were stained with six different antibodies, CD11b, CD16, CD66b, CD68, myeloperoxidase and neutrophil elastase, in order to test the antibodies against different types of circulating leucocytes. Detailed information of these antibodies such as clone, supplier, dilution and expression and/or function according to supplier's information is listed in Table 1.

In the single primary antibody staining procedure for methanol fixed leucocytes smears, a circle was drawn around the smear with a PAP pen (ImmEdge Hydrophobic Barrier Pen, Vector Laboratories, Burlingame, California, USA) and the smear was rehydrated in a drop of PBS for 5 min at room temperature. The smears were kept in horizontal position in a humidified chamber during all incubations and washes. Unspecific binding sites were blocked with 1 % BSA (bovine albumin serum) in PBS for 30 min in room temperature. Smears were incubated overnight with primary antibodies diluted (dilutions are listed in Table 1) in 1 % BSA in PBS at $+4$ °C. After overnight incubation the slides were washed for 2×5 min in PBS and incubated for 45 min with secondary antibodies diluted 1:200 in 1 % BSA in PBS. Secondary antibody used together with CD66b was Alexa Fluor[®] 488 F(ab')₂ fragment of goat anti-mouse IgG (Invitrogen, Eugene, Oregon, USA), with CD11b, CD16, CD68 and neutrophil elastase was Alexa Fluor[®] 594 F(ab')₂ fragment of goat anti-mouse IgG (Invitrogen) and with myeloperoxidase was Alexa Fluor[®] 594 F(ab')₂ fragment of goat anti-rabbit IgG (Invitrogen). The fluorochrome-stained leucocyte smears were washed for 2×5 min in PBS and mounted on Pro-Long[®] Gold Antifade reagent with DAPI (Invitrogen).

Table 1 Antibodies

Antigen	Clone/Nr	Supplier	Dilution	Expression/localization
CD11b	2LPM19c	DC	1:40	Granulocytes, macrophages, subset of monocytes and lymphocytes
CD16	DJ130c	DC	1:300	Natural killer cells, macrophages, subset of monocytes, T cells and neutrophils
CD66b	CLB-B13.9	Sanquin	1:500	Granulocytes
CD68	EBM11	DC	1:300	Macrophages
Myelo-peroxidase	A 0398	DC	1:2,000	Granules of neutrophil granulocytes, lysosomes of monocytes
Neutrophil elastase	NP57	DC	1:100	Neutrophils, reacts weakly with a minor population of monocytes
Dystrophin	ab15277	Abcam	1:2,000	Inner surface of muscle fibers
Laminin	Z 0097	DC	1:1,000	Basement membranes of myofibers, capillaries and vessels

Clone, supplier, dilution and expression and/or localization, according to supplier's information of the antibodies used in the present study
Supplier: Abcam (Cambridge, UK), DC (DakoCytomation, Glostrup, Denmark), Sanquin Reagents (Amsterdam, The Netherlands)

Leucocyte smears without primary antibody were used as negative controls.

For double primary antibody stainings, leucocyte smears were stained with CD66b antibody combined together with CD11b, CD16, CD68, myeloperoxidase or neutrophil elastase antibody. Three different protocols were used depending on the antibody combination. The first double-staining protocol monoclonal CD66b antibody was stained together with monoclonal CD11b, CD16 or CD68 antibodies. Leucocyte smears were first stained with CD11b, CD16 or CD68 antibodies according to single staining protocol as described above. Next CD66b antibody was labelled according to manufactures protocol with Zenon[®] mouse IgG₁ Alexa Flour 488 labelling reagent (Zenon[®] Tricolor Mouse IgG₁ Labelling Kit, Invitrogen). In brief, 1 µg CD66b antibody was mixed with 5 µl Zenon[®] mouse IgG₁ Alexa Flour 488 labelling reagent and incubated for 5 min at room temperature. After this 5 µl Zenon[®] blocking reagent (Zenon[®] Tricolor Mouse IgG₁ Labelling Kit, Invitrogen) was added to the mixture and incubated for 5 min at the room temperature. The mixture was further diluted by adding 400 µl 1 % BSA in PBS. The CD11b, CD16 and CD68 stained leucocyte smears were incubated with this mixture for 1 h at room temperature. The fluorochrome-stained leucocyte smears were washed 2 × 5 min in PBS and mounted on ProLong[®] Gold Antifade reagent with DAPI (Invitrogen). The second double-staining protocol monoclonal CD66b antibody was stained together with monoclonal neutrophil elastase antibody. CD66b antibody was labelled with Zenon[®] mouse IgG₁ Alexa Flour 488 labelling reagent, whereas neutrophil elastase was labelled with Zenon[®] mouse IgG₁ Alexa Flour 594 labelling reagent as described above. After labelling both antibodies separately, they were mixed together and further diluted by adding 400 µl 1 % BSA in PBS. Blocked leucocyte smears were incubated with this mixture for 1 h at room temperature. The fluorochrome-stained leucocyte smears were washed 2 × 5 min in PBS and mounted on ProLong[®] Gold Antifade reagent with DAPI (Invitrogen). The third double-staining protocol monoclonal CD66b antibody was stained together with polyclonal myeloperoxidase antibody. Leucocyte smears were first stained with polyclonal myeloperoxidase antibody according to single staining protocol as described above. After fluorochrome-detection of myeloperoxidase, the leucocyte smears were stained with monoclonal CD66b antibody according to the single staining protocol, except that diluted CD66b antibody was incubated for 2 h at room temperature.

Images of the stained leucocyte smears were captured using Olympus DP72 camera (Olympus Imaging Europa GmbH, Hamburg, Germany) mounted on Olympus BX61 microscope (Olympus Life Science Europa GmbH, Hamburg, Germany) and Cell F 3.3 software (Olympus Soft Imaging Solutions GmbH, Hamburg, Germany).

Immunohistochemical stainings of skeletal muscle sections

Histology samples of muscle biopsies, which were frozen in isopentane cooled on dry ice, were mounted with Tissue-Tek[®] O.C.T. Compound (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) on a specimen holder in a cryostat (Leica CM3050, Nussloch GmbH, Germany). Muscle fibres were oriented in upright position to the direction towards the specimen holder. 7 µm thick serial transverse sections were cut with a cryostat microtome at −22 °C. Sections were pick up on Superfrost Plus microscope glass slides (Menzel-Gläser, Braunschweig, Germany), air-dried and stored at −80 °C until further analysis. Whenever possible, each subjects' muscle sections from all time points were mounted on the same microscope slide. At least subjects' sections from both exercised and contralateral muscle from the same time point were placed on the same microscope slide. Two or three sections were cut from each muscle histology sample.

Selected cross-sections containing areas that typically were observed to contain leucocyte positively stained cells, were stained with CD66b antibody combined together with CD11b, CD16, CD68, myeloperoxidase or neutrophil elastase antibody to evaluate the suitability of these antibodies for detecting inflammatory cells in skeletal muscle. The same three double staining protocols described above for staining leucocyte smears were also applied to these muscle cross-sections.

Serial muscle cross-sections were immunohistochemically stained with three different leucocyte antibodies, CD16, CD66b and CD68, in order to count the number of different leucocytes after eccentric exercise. Furthermore, the same sections were also stained with dystrophin or laminin antibody for visualizing borders of muscle fibres. Detailed information of primary antibodies is listed in Table 1. In the double-staining procedure for combining leucocyte antibody staining together with staining the borders of muscle fibres, a circle was drawn around muscle sections with a PAP pen (Sigma-Aldrich, St. Louis, Missouri). Sections were fixed in 4 % paraformaldehyde solution in a staining jar for 10 min at room temperature and rinsed 3 × 10 min in TRIS buffer containing 0.5 % Tween 20 (Sigma-Aldrich). The microscope slides were moved into a humidified chamber and unspecific binding sites were blocked with a drop of 10 % calf or horse normal serum in TBS-Tween on the section for 30–60 min in room temperature. Sections were incubated overnight with leucocyte antibodies diluted (dilutions are listed in Table 1) in 10 % calf or horse normal serum in TBS-Tween at 4 °C. After overnight incubation the slides were washed for 3 × 10 min in TBS-Tween in a staining jar.

Slides were moved back to the humidified chamber and sections were incubated for 45 min with secondary antibodies diluted 1:200 in 10 % calf or horse normal serum in TBS-Tween at room temperature. Alexa Fluor[®] 594 F(ab')₂ fragment of goat anti-mouse IgG (Invitrogen) was used as a secondary antibody. The fluorochrome-stained sections were washed for 3 × 10 min in TBS-Tween. For the staining the borders of muscle fibres, the same procedure was used as staining leucocytes on muscle sections except that the leucocyte stained sections were incubated in a drop of dystrophin or laminin antibody diluted (dilutions are listed in Table 1) in 10 % calf or horse normal serum in TBS-Tween the humidified chamber for 2 h in the room temperature and the secondary antibody used together with dystrophin or laminin was Alexa Fluor[®] 488 F(ab')₂ fragment of goat anti-rabbit IgG (Invitrogen). After the last washing in TBS-Tween the sections were mounted on ProLong[®] Gold Antifade reagent with DAPI (Invitrogen). Muscle sections stained without primary antibody were used as negative controls.

Counting the number of leucocytes

Number of CD16 and CD68 positive cells was counted from images taken from CD16 and dystrophin, and CD68 and laminin stained skeletal muscle sections. Images were captured using Axiovision Imaging Software 4.3 and AxioCam camera (Zeiss, Oberkochen, Germany) mounted on Axioskop-2 light microscope (Zeiss) at 200× magnification. Four images were captured from the muscle section, which showed the best morphology and staining. Two or three sections were stained per each muscle biopsy. The exposure time was adjusted for each image to obtain the highest contrast. At first the captured image of the borders of skeletal muscle fibres visualized by dystrophin or laminin staining was uploaded to TEMA image analysis system (Devitech Holding ApS, Vodskov, Denmark) to count the total number of skeletal muscle fibres. The captured image contained typically 50–100 muscle fibres. Next the corresponding image of CD16 or CD68 stained section was uploaded to TEMA image analysis system and the number of CD16 or CD68 positive cells were counted using capillary counting tool. Areas of captured images, which contained freeze damage or were folded due to the cutting procedure, were not included to the analysis. The number of CD66b positive cells as well as the total number of skeletal muscle fibres from the area included for counting the positive stained cells was counted directly from microscopy. Data was calculated as percentage of CD16, CD66b or CD68 positively stained cells per 100 skeletal muscle fibres.

Embedding, sectioning and contrast staining of samples for transmission electron microscopy

Fixed (4 % paraformaldehyde and 0.1 % glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4) samples were embedded in epoxy resin. First, skeletal muscle splices were post fixed with 2 % OsO₄ in 0.1 M cacodylate buffer for 2 h at 4 °C and washed in 0.1 M cacodylate buffer 3 × 5 min at room temperature. Samples were placed into eight samples baskets and were dehydrated in a series of ethanol, in 2 % uranyl in absolute ethanol and in dehydrated acetone followed by infiltration in mixtures of epoxy resin Agar 100 (Agar Scientific, Cambridge, UK) and acetone in an automatic tissue processor (Leica EM TP). Next the sample baskets were incubated in epoxy resin mixture containing accelerator DMP-30 (Agar Scientific) for 6 h at room temperature. After this the samples were removed into plastic embedding moulds filled with epoxy resin mixture containing accelerator DMP-30 and muscle fibres were oriented in horizontal position. Finally, the plastic embedding moulds were place overnight in the oven at 60 °C. Semithin sections were cut with glass knife on fully motorized rotary microtome (Leica RM 2155, Leica Microsystems AG) and stained with Toluidine Blue solution. Ultrathin sections (80 nm) were cut with a diamond knife (ultra 45°, Diatome Ltd, Biel, Switzerland) on ultramicrotome (Leica Ultracut R, Leica Microsystems AG, Wetzlar, Germany) and mounted on Formvar-coated copper slot grids. Ultrathin sections were contrasted in 4 % uranyl acetate in 40 % ethanol for 12 min followed by 5 min in lead citrate. The sections were analyzed in a transmission electron microscope (Tecnai 12, Philips Electronics N.V., Eindhoven, Netherlands). A few samples were embedded at low temperature as described earlier by Lauritzen et al. (2009).

Results

Division of the subjects into three categories based on muscle force-generating capacity after the exercise

Subjects were divided into three categories depending on the muscle force loss immediately after the first bout of eccentric exercise and how fast muscle force was recovered during the following 7 days. Muscle force-generating capacity was measured at the highest peak torque at velocity 60°/s of maximal voluntary isokinetic concentric elbow flexion. Six subjects (three celecoxib and three placebo subjects) were included in the first category, which represents the mild effect of eccentric exercise. Immediately after the first bout of eccentric exercise, muscle force-generating capacity for these subjects was on average 64 %

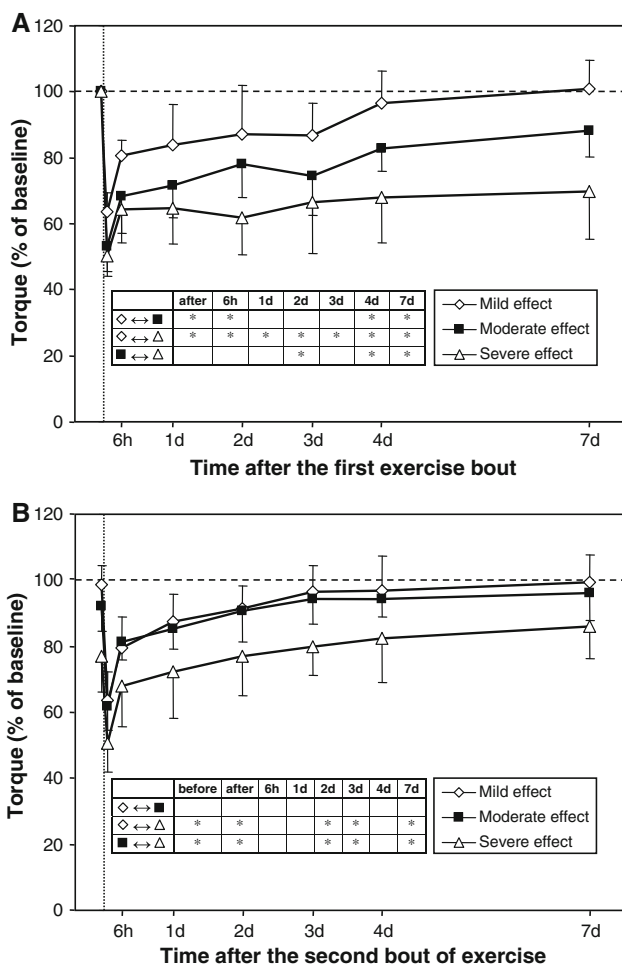


Fig. 1 Post-exercise muscle force-generating capacity of the exercised arm. **a** After the first exercise bout and, **b** after the second exercise bout, which was performed 3 weeks after the first bout. Subjects were divided into three categories: mild ($n = 6$), moderate ($n = 10$) and severe ($n = 7$) effect of eccentric exercise, based on the loss and the recovery of muscle force. Vertical dashed line indicates the time of eccentric exercise bout and horizontal dashed line is the baseline for muscle force. Error bars are standard deviation

of pre-exercise force (Fig. 1a). Muscle force recovered to the pre-exercise level 7 days after the first bout of eccentric exercise. On the second category, which represents moderate effect of eccentric exercise, post-exercise muscle force-generating capacity for 10 subjects (two celecoxib and eight placebo subjects) was on average 53 % of pre-exercise force (Fig. 1a). Furthermore, muscle force was still below baseline at the end of the observation period. Finally, seven subjects (five celecoxib and two placebo subjects) were included to the third category, which represents severe effect of eccentric exercise. Immediately after the eccentric exercise muscle force-generating capacity was on average 50 % of pre-exercise force. Seven days after the first bout of eccentric exercise muscle force was on average 70 % of pre-exercise force (Fig. 1a).

Before the second bout of eccentric exercise (3 weeks after the first bout), muscle force-generating capacity was on average 99, 92 and 77 % of pre-exercise force in mild, moderate and severe categories, respectively (Fig. 1b). Immediately after the second bout of eccentric exercise, muscle force decreased in a same way as after the first bout. On average the muscle force was 64, 62 and 51 % of pre-exercise force in mild, moderate and severe categories, respectively (Fig. 1b). At the end of the observation period muscle force was slightly below the baseline in mild and moderate categories, 99 and 96 % of pre-exercise force, respectively. Whereas in the category severe effect of eccentric exercise, muscle force was still 86 % of pre-exercise force (Fig. 1b).

Friedman test showed statistically significant differences in all of the three categories for exercised arm. Further statistical evaluation by Wilcoxon Signed Rank test showed statistically significant differences in all time points compared to muscle force generating capacity before the first exercise bout in the category severe effects of eccentric exercise. Similarly in the category moderate effects of exercise statistically significant differences were observed in all time points except 7 days after the second exercise bout, whereas in the category of mild effects of eccentric exercise statistically significant differences were observed immediately and 6 h after both exercise bouts and also 1 and 2 days after the second exercise bout. Non-parametric Kruskal–Wallis H test for independent samples showed statistically significant differences in all time points after the first exercise bout, whereas the second bout of exercise showed statistically significant differences before the second bout of exercise and immediately, 2, 3 and 7 days after the second exercise bout. Further comparison with the category severe effects of eccentric exercise between mild and moderate categories by Mann–Whitney U test showed statistically significant differences after the first exercise bout in all time points and 2, 4 and 7 days, respectively, whereas the second exercise bout showed statistically significant differences before the second bout of exercise and immediately, 2, 3 and 7 days after the second exercise bout. Comparison between categories mild and moderate effects of eccentric exercise showed differences only immediately, 6 h, 4 and 7 days after the first exercise bout.

The force-generating capacity for the non-exercised arm ranged between 90 and 110 % of the baseline in 287 of the total 315 force measurements performed after the first and the second bout of eccentric exercise. The rest of post-exercise force measurements, which were outside of this range, were observed in most cases in categories mild and severe effects of eccentric exercise. In some subjects muscle forces below 90 % of the pre-exercise force were observed to take place on the following day (or days) after

the muscle biopsy was obtained. Friedman test did not show any statistically significant differences in any of the three categories. Kruskal–Wallis *H* test showed statically significant differences at 4 days after the second exercise bout between the three categories. Mann–Whitney *U* test showed that these significant differences were between mild and severe categories and between moderate and severe categories. Individual results for each subject are presented as percentage of pre-exercised torque for both exercised and non-exercised arm in the Suppl. Tables 1 and 2.

Serum creatine kinase

The average values of serum creatine kinase before the first and the second bout of eccentric exercise were similar in all three categories (Fig. 2). The average values before the

first exercise bout were 207, 228 and 140 U/l and before the second bout 251, 181 and 181 U/l in categories mild, moderate and severe effects of eccentric exercise, respectively. After the first exercise bout the average values of serum creatine kinase changed the most in categories moderate and severe effects of eccentric exercise ($p < 0.05$ Friedman test, $p < 0.05$ all time point compared to creatine kinase before the first exercise bout by Wilcoxon Signed Rank test). The highest average creatine kinase values, 2,929 and 10,266 U/l, in these categories were observed 4 days after the first exercise bout, whereas in the mild effects of eccentric exercise category, the highest average value, 538 U/l, was observed 7 days after the exercise (Fig. 2a). There was no statistically significant differences in the category mild effects of exercise ($p > 0.05$ Friedman). Kruskal–Wallis *H* test showed statistically significant differences at 3, 4 and 7 days after the first exercise bout between the three categories. Mann–Whitney *U* test showed significant differences between categories mild and moderate effects of eccentric exercise at 3 and 4 days, between mild and severe categories at 3, 4 and 7 days and between moderate and severe categories at 7 days the first exercise bout. After the second bout of exercise the average serum creatine kinase values ranged from 171 to 333 U/l (Fig. 2b). Friedman test showed statistically significant differences at only in category moderate effects of eccentric exercise ($p < 0.05$ at 8 h, 3 and 4 days compared to creatine kinase before the second exercise bout by Wilcoxon Signed Rank test). There were no statistically significant differences between different categories. Individual serum creatine kinase results are presented in the Suppl. Table 3.

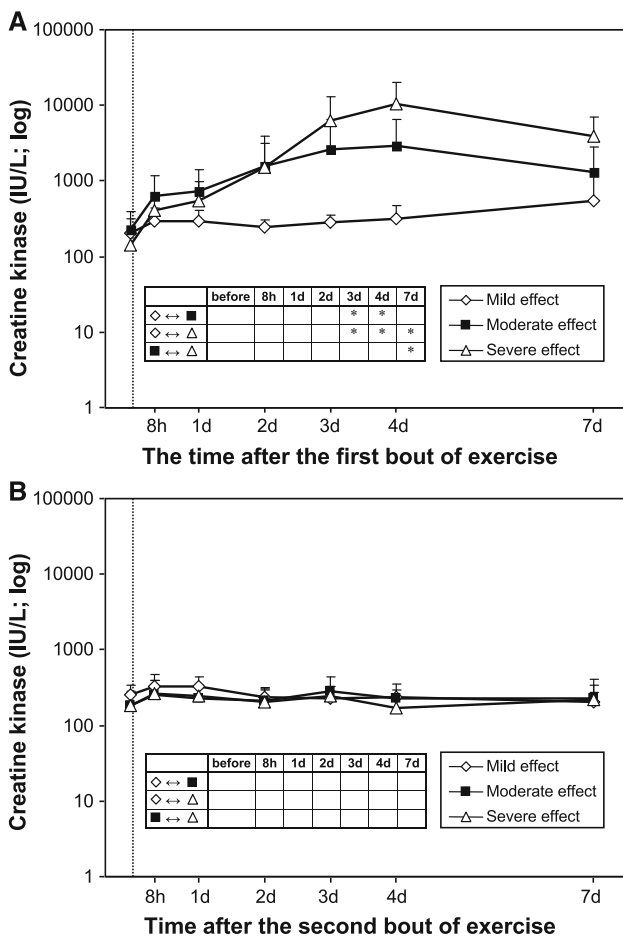


Fig. 2 Serum creatine kinase. **a** After the first exercise bout and, **b** after the second exercise bout, which was performed 3 weeks after the first bout. Subjects were divided into three categories: mild ($n = 6$), moderate ($n = 10$) and severe ($n = 7$) effect of eccentric exercise, based on the loss and the recovery of muscle force. Vertical dashed line indicates the time of eccentric exercise. Y-axis is logarithmic. Error bars are standard deviation

Scintigraphic monitoring of leucocyte accumulation

The accumulation of radiolabelled leucocytes was observed to be the highest in the category severe effect of eccentric exercise 20 h after the first exercise bout (on average 2.6 times higher than contralateral arm, $n = 6$, Fig. 3). The average values for other measured time points, 6 h after the first exercise bout ($n = 6$) and 6 and 20 h after the second bout ($n = 4$), varied from 1.7 to 2.0 times higher compared to contralateral arm. The highest ratio (on average 1.8 higher than contralateral arm, $n = 9$, Fig. 3) in the category moderate effects of eccentric exercise was observed as well 20 h after the first exercise bout. Whereas the average values for the rest of the measured time points in the category moderate effects of eccentric exercise ($n = 9$ in the first bout and $n = 5$ in the second bout of exercise) and all time points in category mild effects of eccentric exercise ($n = 6$ in the first bout and $n = 3$ in the second bout of exercise) varied from 1.3 to 1.4 times higher compared to contralateral arm. Intensive local

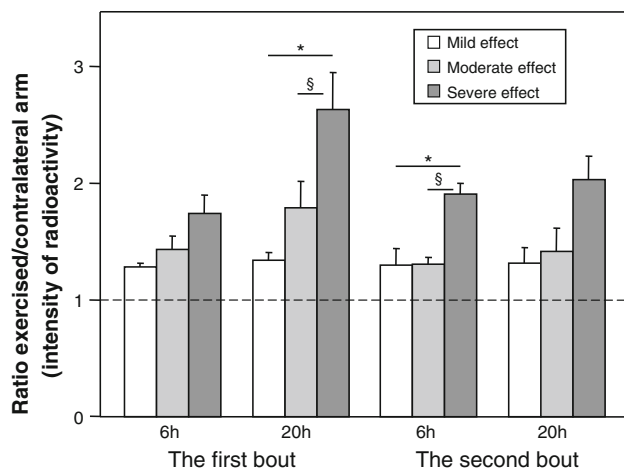


Fig. 3 Scintigraphic monitoring of leucocyte accumulation. Values are expressed as a ratio of radioactive intensity between the exercised and contralateral arm, corrected for background radiation at 6 and 20 h after the first exercise bout and after the second exercise bout, which was performed 3 weeks later. Subjects were divided into three categories: mild, moderate and severe effect of eccentric exercise, based on the loss and the recovery of muscle force. *Horizontal dashed line* is the baseline for muscle force. *Error bars* are standard error of the mean

accumulation of radioactivity (1,000 % higher compared same area in the contralateral arm) was observed the mid part of *m. biceps brachii* in subject PBO6, CEL11 and CEL26 at 20 h after the first exercise bout. Kruskal–Wallis *H* test showed statistically significant differences between the three categories at 20 h after the first exercise bout and at 6 h after the second exercise bout. Mann–Whitney *U* test showed that the statistically significant differences were between mild and severe and between moderate and severe categories at both time points. There were no statistically significant differences between mild and moderate categories. Individual results are presented in the Suppl. Table 4. Values are expressed as a ratio of radioactive intensity between the exercised and contralateral arm, corrected for background radiation.

CD11b, CD16, CD66b, CD68, myeloperoxidase and neutrophil elastase antibodies against circulating leucocytes

Immunohistochemical stainings of leucocytes extracted from whole blood showed that the CD66b antibody recognized only neutrophils (Fig. 4; Table 2). No cross-reaction was observed with other leucocytes. The CD11b antibody stained all types of leucocytes (Fig. 4; Table 2), although the staining intensity in lymphocytes was weak or there were no staining at all in some lymphocytes. Moderate staining intensity for the CD16 antibody was observed in neutrophils (Fig. 4; Table 2), whereas in monocytes and lymphocytes the staining intensity was

weak or there was no staining at all in some monocytes and lymphocytes. The neutrophil elastase antibody stained neutrophils very intensively (Fig. 4; Table 2). Furthermore, monocytes and some, but not all bi-lobed nucleated cells showed moderate staining. The myeloperoxidase antibody showed intensive or moderate staining of neutrophils, monocytes and all bi-lobed nucleated cells (Fig. 4; Table 2), whereas staining in lymphocytes was weak or there were no staining in some lymphocytes. The CD68 antibody stained monocytes and a portion of cells with bi-lobed nuclei (Fig. 4; Table 2).

Distinguishing different leucocytes from each other was based on the shape of their nuclei. Neutrophils had segmented nuclei containing typically 3–5 lobes, which were connected by fine nuclear filaments. Nuclei of monocytes were horseshoe shaped, whereas nuclei of lymphocytes were round and filling a large portion of the cytoplasm. Both basophils and eosinophils had bi-lobed nuclei. Distinguishing basophils and eosinophils based on the shape of their nuclei was very difficult; however, it seemed that there were two different looking bi-lobed nuclei; one with condensed and sharp-edged nuclei and the other with faint and amorphous-edged nuclei.

Double immunostaining with CD66b antibody and the antibodies CD11b, CD16, CD68, myeloperoxidase or neutrophil elastase of circulating leucocytes

Zenon[®] mouse IgG₁ Alexa Flour 488 labelled monoclonal CD66b antibody was stained together with CD11b, CD16 or CD68 antibody. In these double stainings, Zenon[®] labelled CD66b antibody stained only neutrophils, no cross reactivity was observed with any other leucocytes (Suppl. Fig. 1, 2 and 3). Correspondingly, the CD11b, CD16 and CD68 antibodies showed the same staining pattern against different leucocytes as in single antibody staining protocols described above (compare Fig. 4 to Suppl. Fig. 1, 2, and 3). Next, the CD66b antibody and the neutrophil elastase antibody were labelled with Zenon[®] mouse IgG₁ Alexa Flour 488 and Zenon[®] mouse IgG₁ Alexa Flour 594 complex, respectively. Both Zenon[®] labelled antibodies showed positive staining of neutrophils, whereas the Zenon[®] labelled neutrophil elastase antibody stained also monocytes and a portion of cells with bi-lobed nuclei (compare Fig. 4 to Suppl. Fig. 4). Finally, double immunostaining with the monoclonal CD66b antibody and the polyclonal myeloperoxidase antibody showed positive staining with both antibodies of neutrophils (Suppl. Fig. 5). In addition to that positive staining, myeloperoxidase antibody staining was observed on monocytes, a portion of the lymphocytes and all the cells with bi-lobed nuclei (compare Fig. 4 to Suppl. Fig. 5). In summary, all studied

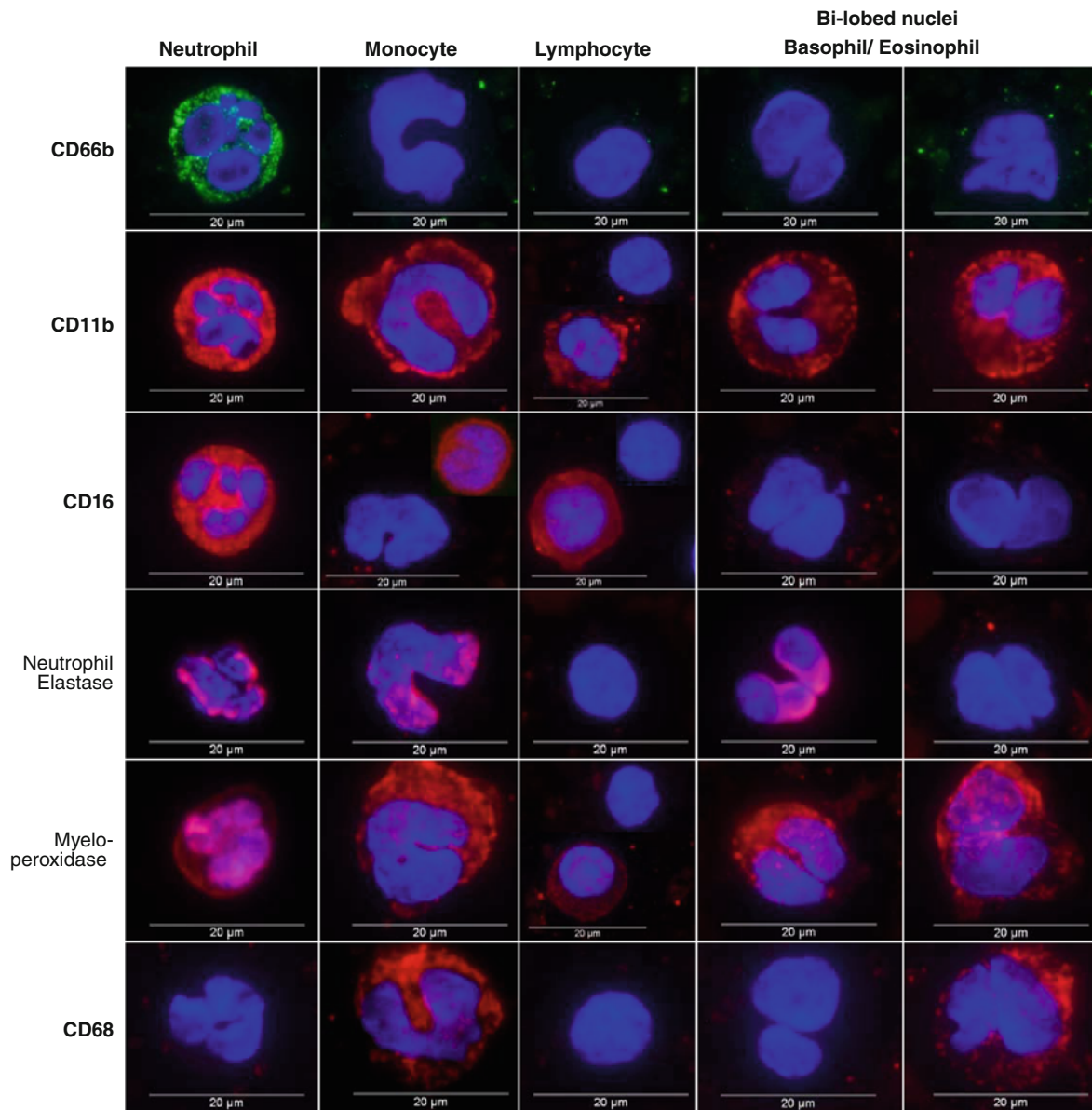


Fig. 4 Immunostaining of CD11b, CD16, CD66b, CD68, myeloperoxidase and neutrophil elastase antibodies on circulating leucocytes extracted from whole blood. Secondary antibody for CD66b Alexa Fluor® 488 anti-mouse, for CD11b, CD16, CD68 and neutrophil elastase Alexa Fluor® 594 anti-mouse and for myeloperoxidase Alexa Fluor® 594 anti-rabbit. *Scale bar* 20 µm

Table 2 Immunostaining intensity of leucocyte smears

Staining intensity of studied antibodies of leucocytes extracted from whole blood and prepared as smear slides
 +++ intensive,
 ++ moderate, + weak, - no staining

	Neutrophils	Monocytes	Lymphocytes	Cells with bi-lobed nuclei basophil/eosinophil
CD66b	+++	-	-	-
CD11b	++	++	±	++
CD16	++	±	±	-
Elastase	+++	++	-	++
MPO	+++	++	±	++
CD68	-	+	-	+

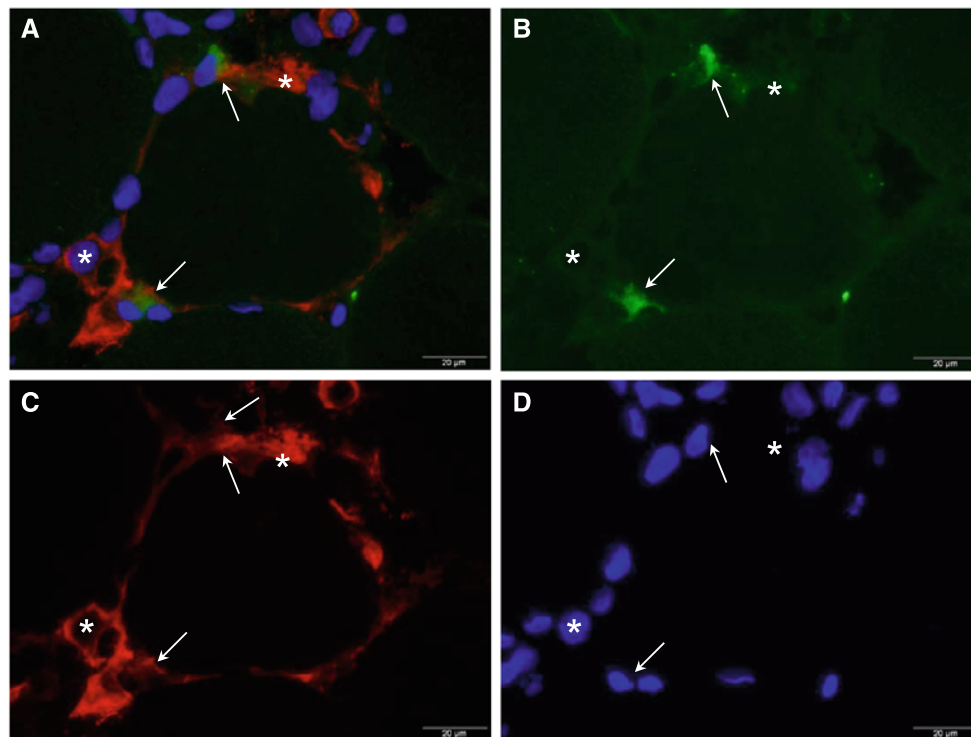


Fig. 5 CD66b and CD16 staining in endomysial cells in the around swollen skeletal fibre. Both CD66b and CD16 antibodies showed positive staining on cells (*arrows*) located in endomysium around slightly swollen skeletal muscle fibre. CD16 antibody showed also staining in the areas (*asterisks*), which were negative for CD66b

antibody staining. **a** Merged image of **b** Zenon[®] mouse IgG1 Alexa Flour 488 labelled CD66b antibody, **c** CD16 antibody detected by Alexa Flour 594 goat anti-mouse secondary antibody and **d** nuclear counterstain DAPI. *Scale bar* 20 μ m

antibodies gave similar staining patterns for the different leucocytes both with single and with double-staining protocols.

Double immunostaining with the CD66b antibody and the antibodies CD11b, CD16, CD68, myeloperoxidase or neutrophil elastase in skeletal muscle

Three different areas, which typically were observed to contain immunostained cells, were selected for the evaluation of double immunostainings of skeletal muscle sections. At first, areas, which showed some effects of the eccentric exercise such as accumulation of cells in endomysium, second, skeletal muscle fibres, which were infiltrated by cells, and, finally, vessels. The same double staining protocols as were used for staining leucocyte smears were applied for skeletal muscle sections.

First, double staining with the Zenon[®] labelled monoclonal CD66b antibody and the monoclonal CD16 antibody showed occasionally stained cells with both antibodies in the endomysium of skeletal muscle fibres with signs of damaging exercise, such as swelling and accumulation of cells in the endomysium (Fig. 5). In addition, CD16

antibody staining was observed to occupy also areas, which were completely negative for CD66b antibody staining (Fig. 5). This was also observed when the CD66b antibody was combined with CD11b, myeloperoxidase and neutrophil elastase antibodies (data not shown).

Second, double staining with the monoclonal CD66b and the polyclonal myeloperoxidase antibodies showed stained cells with both antibodies inside skeletal muscle fibres (Fig. 6). In addition, the myeloperoxidase antibody staining seemed to occupy much larger area inside damaged skeletal muscle fibre compared to the CD66b antibody staining (Fig. 6). A similar staining pattern was observed when the CD66b antibody was combined with the CD11b, CD16 (data not shown) or neutrophil elastase antibodies (Suppl. Fig. 6). The Zenon[®] labelled monoclonal CD66b antibody and the monoclonal CD68 did not show any cross reactivity of the same cells (Suppl. Fig. 7).

Third, double staining with the Zenon[®] labelled monoclonal CD66b and the neutrophil elastase antibodies showed staining with both antibodies in cells located in the lumen of vessels (Suppl. Fig. 8), attached to the inner wall of vessels (data not shown) and areas of the vessel walls (data not shown). Similar staining patterns were also observed when combining the CD66b antibody with the

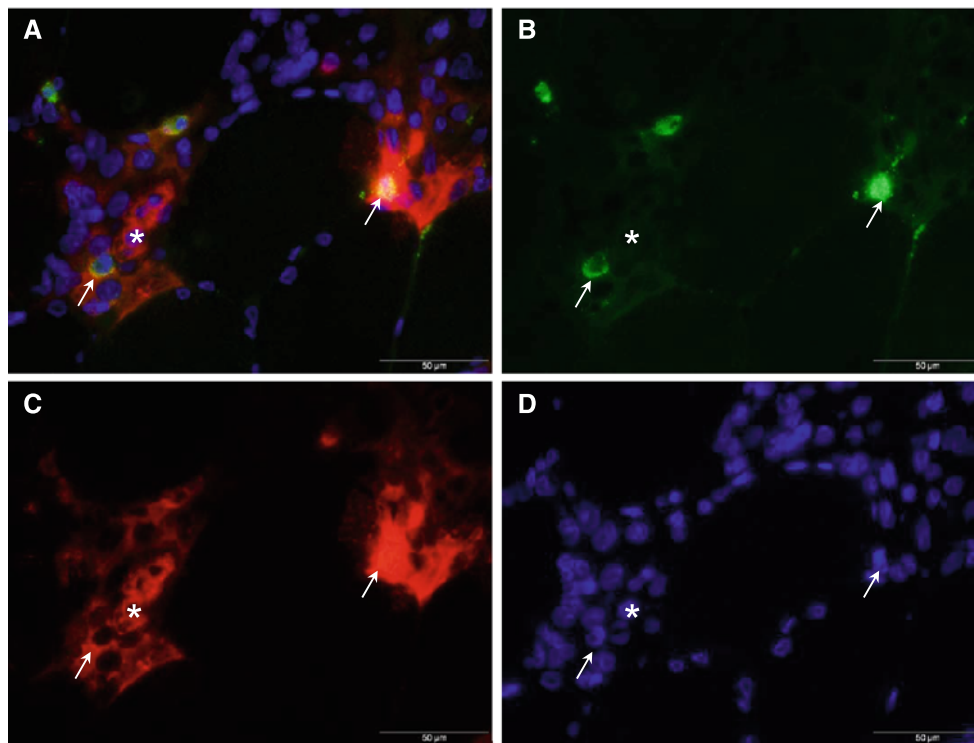


Fig. 6 CD66b and myeloperoxidase staining in damaged skeletal muscle fibre. Both CD66b and Myeloperoxidase antibodies showed positive staining on cells (*arrows*) located inside skeletal muscle fibres, which were infiltrated by many cells. Myeloperoxidase antibody staining occupied much larger area inside damaged skeletal

muscle fibre compared to CD66b antibody staining (*asterisk*). **a** Merged image of **b** monoclonal CD66b antibody detected by Alexa Flour 488 anti-mouse secondary antibody, **c** polyclonal MPO antibody detected by Alexa Flour 594 goat anti-rabbit secondary antibody and **b** nuclear counterstain DAPI. Scale bar 50 µm

CD11b, CD16 or the myeloperoxidase antibodies (data not shown). In addition, the CD11b, CD16, myeloperoxidase and neutrophil elastase antibodies showed more stained cells in the cellular rich wall of vessels (data not shown) than the CD66b antibody. Furthermore, double staining with CD66b and CD68 did not show any cross reactivity in the cellular rich wall of vessels or its surrounding (Suppl. Fig. 9). In conclusion, the CD66b antibody stained some of the same cells as the CD11b, CD16, myeloperoxidase and neutrophil elastase antibodies, while no cross reactivity was detected between CD66b and CD68 antibodies. In addition, the CD11b, CD16, myeloperoxidase and neutrophil elastase antibodies seemed to stain more cells than the CD66b antibody.

The number of CD66b positive cells and their localization in skeletal muscles after eccentric exercise

There were CD66b positive cells in 34 of the 122 biopsies included for the counting (Suppl. Table 5). Thirty-two muscle samples contained one to five CD66b positive cells per 100 muscle fibres. The highest numbers of CD66b positive cells were observed in one subject (PBO S18) at 2 days after the first bout of exercise both in exercised

(30 CD66b positive cells per 100 fibres) and in non-exercised (20 CD66b positive cells per 100 fibres) samples. The CD66b positive cells seemed to be randomly distributed between exercised (18 of 62 exercised samples) and non-exercised samples (16 of 60 non-exercised samples) as well as between different time points. However, only one of 34 samples containing CD66b positive cells belonged to the category mild effect of eccentric exercise.

In 23 (marked with # in Suppl. Table 5) of the 34 biopsies containing CD66b positive cells, the stained cells were located inside capillaries or vessels, attached to the wall of the vessels or in blood clots containing leucocytes. In the remaining 11 (marked with § in Suppl. Table 5) of the 34 biopsies containing CD66b positive cells, single CD66b positive cells were observed in the endomysium of affected muscle fibres or in the sarcoplasm of damaged fibres.

The number of CD16 antibody positive cells in skeletal muscles after eccentric exercise

Eighteen (12 biopsies after the first and 6 biopsies after the second bout of exercise) of the 62 exercised biopsies showed high number of CD16 stained cells (more than 50

CD16 positive cells per 100 fibres, Suppl. Table 6). In the rest of the exercised samples, the number of CD16 positive cells varied from no stained cells to 37 stained cells per 100 muscle fibres. Whereas in the 61 non-exercised biopsies, five samples (all after the first bout of exercise) showed high numbers of stained cells (more than 50 CD16 positive cells per 100 fibres). In the rest of non-exercised samples, the number CD16 positive cells varied from no stained cells to 31 stained cells per 100 muscle fibres.

The most of the 23 exercised and non-exercised samples, which contained high number of CD16 positive stained cells, contained one or more dystrophin negative fibres (biopsies marked on the black background on the Suppl. Table 6). Furthermore, 22 of these samples were from subjects, which belonged to the categories moderate and severe effect of eccentric exercise and only one sample belonged to the category mild effect of exercise. All in all, the number of CD16 positive cells per 100 fibres was higher than the number of CD66b positive cells.

The number of CD68 positive cells in skeletal muscles after eccentric exercise

Twenty-nine (19 biopsies after the first and 10 biopsies after the second bout of exercise) of the 61 exercised biopsies showed high number of CD68 stained cells (more than 50 CD68 positive cells per 100 fibres, Suppl. Table 7). In the rest of the exercised samples, the number of CD68 positive cells varied from 7 to 49 stained cells per 100 muscle fibres. On the other hand, in the 62 non-exercised biopsies, 7 samples (5 biopsies after the first and 2 biopsies after the second bout of exercise) showed high numbers of stained cells (more than 50 CD68 positive cells per 100 fibres). In the rest of non-exercised samples the number CD68 positive cells varied from 5 to 49 stained cells per 100 muscle fibres.

Half of the exercised and non-exercised samples, which contained high number of CD68 positive stained cells, contained one or more dystrophin negative fibres (biopsied marked on the black background on Suppl. Table 7). Furthermore, only one of these samples belongs to category mild effect of exercise. Overall the number of CD68 positive cells per 100 fibres seemed to be two to three times higher than the corresponding number of CD16 positive cells.

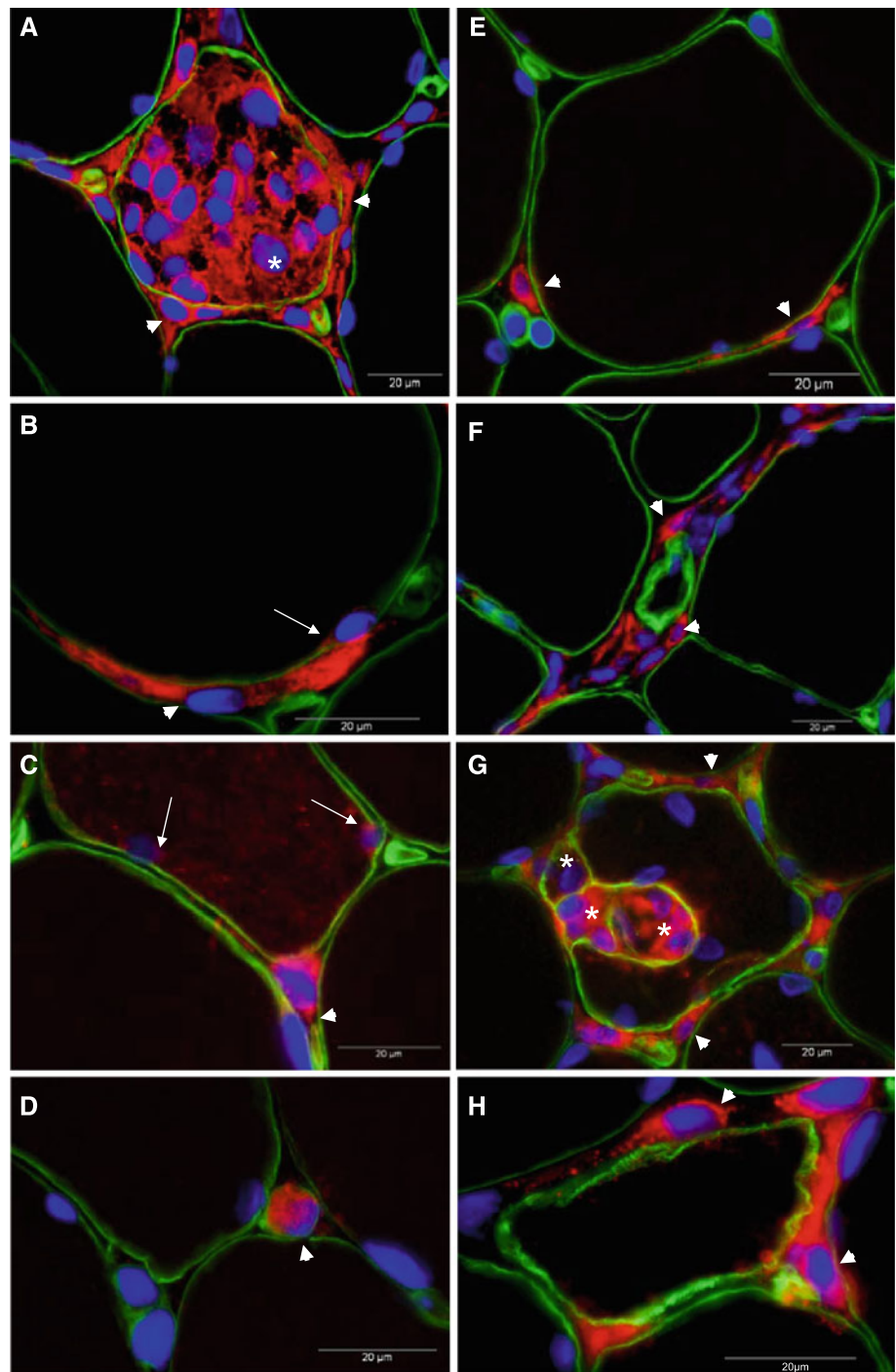
CD68 immunostaining in the sarcoplasm and endomysium of skeletal muscle fibres

Three different types of sarcoplasmic CD68 immunostaining were observed in skeletal muscle fibres in the present study. First, intensive staining was observed in cells in the sarcoplasm of skeletal muscle fibres (Fig. 7a,

asterisk) with intact laminin staining in the basement membrane around muscle fibre, but lacked dystrophin staining. Dystrophin negative fibres were observed in 9 of the 61 exercised biopsies (biopsies containing at least one dystrophin negative fibre were marked with on black background on Suppl. Table 7). These biopsies belonged to the categories moderate and severe effect of eccentric exercise at 2, 4 and 7 days after the first exercise bout. Large areas of fibres, which were entirely occupied by CD68 stained cells in their sarcoplasm, were observed in 2 of these 9 biopsies. The rest of these biopsies showed varying number of single dystrophin negative fibres scattered throughout the skeletal muscle section. These dystrophin negative and laminin positive fibres were typically infiltrated by variable number of CD68 stained cells, ranging from a few stained cells in the sarcoplasm to the CD68 stained cells entirely occupying the sarcoplasm. Dystrophin negative fibres were also observed in four of 62 non-exercised biopsies after the first bout of exercise. The second typical sarcoplasmic CD68 immunostaining pattern was observed in single cells aligned next to laminin staining (Fig. 7b, arrow). These CD68 stained cells often showed long extensions next to the laminin staining and were occasionally observed both in non-exercised and exercised biopsies. Third, in some exercised biopsies CD68 staining was scattered throughout the sarcoplasm in small intensive dots typically accumulating around myonuclei located next to sarcolemma (Fig. 7c, arrow).

Endomysial CD68 positive cells seemed to be typically located close to capillaries in both non-exercised and exercised biopsies (Fig. 7e, arrowheads) similarly after both exercise bouts. Furthermore, in some cases these CD68 stained cells seemed to have long extensions (Fig. 7b, arrowhead) and even form a chain with other stained cells around fibres, especially in necrotic fibres (Fig. 7a, arrowheads). Occasionally, CD68 positive cells were observed inside capillaries (Fig. 7d, arrowheads) and vessels (data not shown) both in non-exercised and exercised biopsies. A dense accumulation of CD68 stained cells were observed in the surroundings of vessels (Fig. 7f, arrowhead). It is not known whether this accumulation is due to exercise since vessels appeared quite seldom in studied biopsies. Furthermore, CD68 positive cells were located in peripheral nerves (Suppl. Fig. 10). In addition, CD68 positive cells were observed in skeletal muscle fibres, which showed some degree of deformation in their structure. Several small fibres or split fibres were observed in biopsies for 5 subjects (these biopsies are marked on dark grey background on Suppl. Table 7) after the second bout of exercise. Split fibres showed variable pattern such as in Fig. 7g, where fibre is divided by basement membrane into four parts. CD68 stained cells were located both in the endomysium and inside the divided areas. Finally,

Fig. 7 CD68 and laminin staining on eccentric exercised skeletal muscle. CD68 staining around DAPI stained nuclei together with laminin stained basement membranes around muscle fibers and capillaries. **a** Necrotic fiber infiltrated by CD68 stained cells (*asterisk*). In the endomysium CD68 stained cells seemed form a chain around necrotic fibre (*arrow heads*). **b** CD68 stained cells with long extensions in sarcoplasm (*arrow*) and in the endomysium (*arrow head*). **c** CD68 staining is scattered throughout the sarcoplasm in small intensive dots typically accumulating around myonuclei (*arrow*) located next to sarcolemma. CD68 stained cells (*arrow heads*) next to **b**, **c**, **e** and inside **d** capillary and in the surrounding of a vessel (**f**). **g** Fibre is divided by basement membrane into four parts (*asterisk*). CD68 stained cells are located both in the endomysium (*arrowheads*) and inside the divided areas (*asterisk*). **h** CD68 positive cells (*arrowheads*) around fibre, which is one-fifth of normal size of skeletal muscle fibre. Muscle sections from exercised samples **a–c** Subject PBO S06 7 days, **d** Subject PBO S04 2 days, **e** Subject PBO S13 2 days and **f** Subject PBO S18 2 days after the first bout exercise. **g** PBO S29 1 h and PBO S6 2 days after the second bout of exercise. Scale bar 20 μm

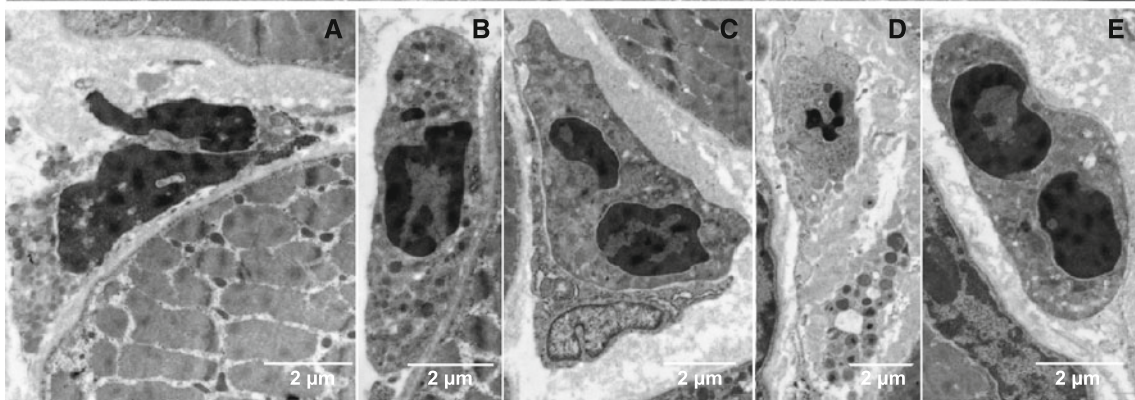
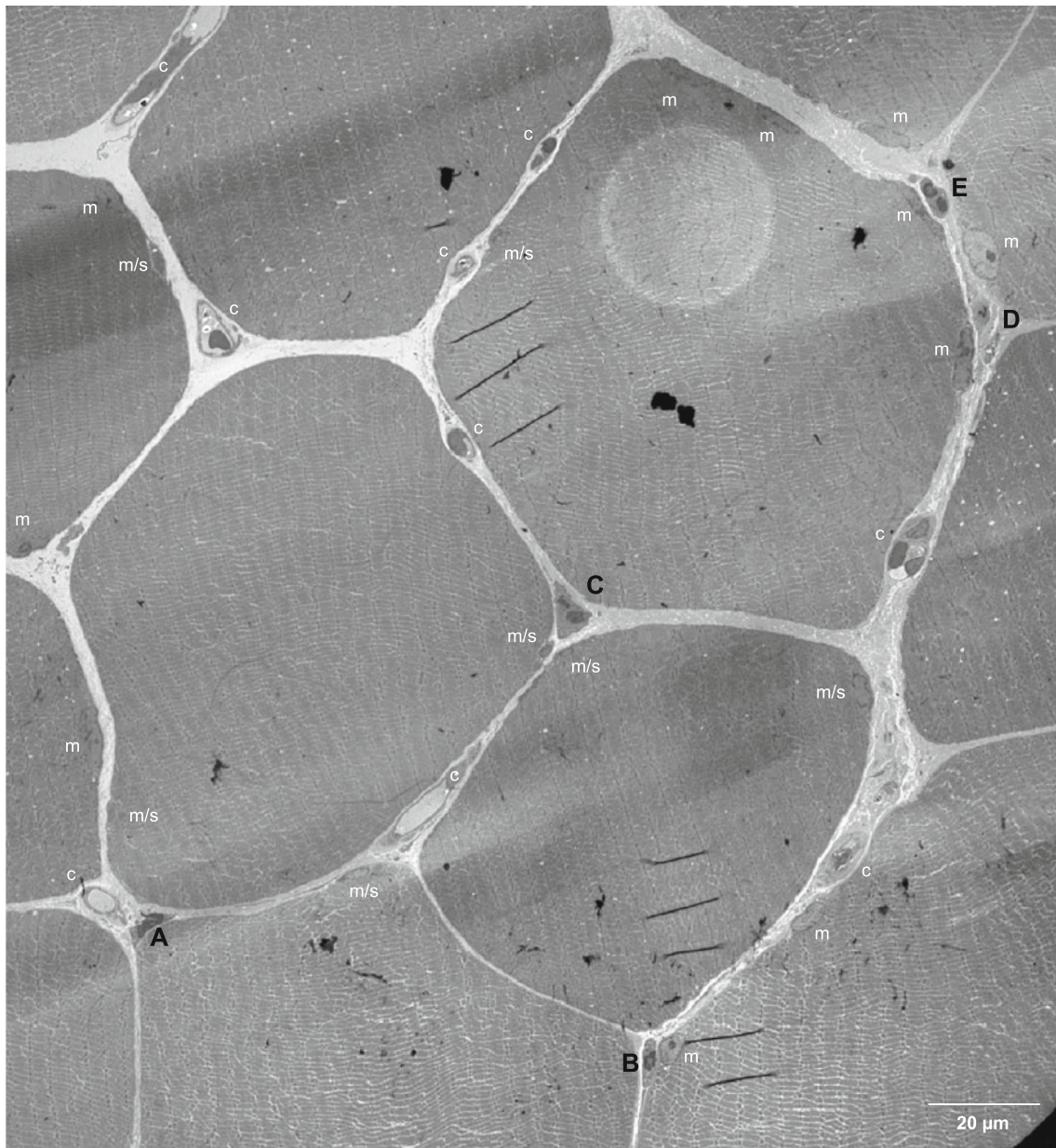


intensively stained CD68 cells were observed in the endomysium around small fibres typically one-third to one-fifth of the normal size of skeletal muscle fibres (Fig. 7h).

Identification of cell types by transmission electron microscopy

Single neutrophils were observed in the electron microscopy samples in same locations as the CD66b positive cells

in immunohistochemical stained samples. CD66b positive cells were observed in the endomysium of affected muscle fibres or in the sarcoplasm of damaged fibres in 11 (marked with § on Suppl. Table 5) of 34 biopsies containing CD66b positive cells. One of these biopsies was the exercised biopsy from the subject PBO S18 at 2 days after the first exercise bout. A few endomysial neutrophils were also observed in this subject's transmission electron microscope sections (Fig. 8). In addition, signs of apoptosis were



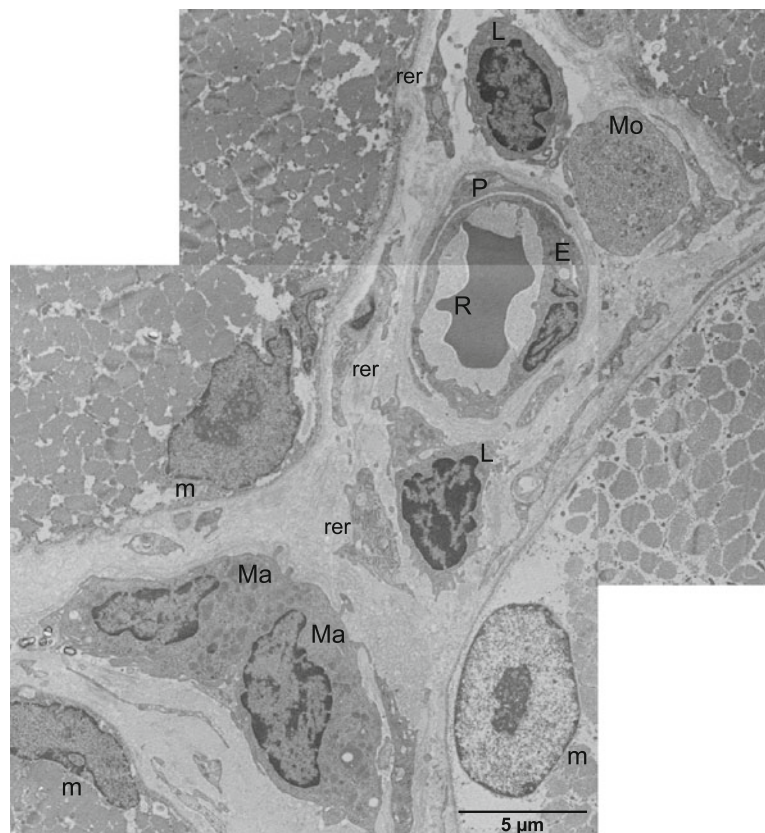
◀ **Fig. 8** Neutrophils in endomysium. Electron micrographs of low temperature embedded exercised skeletal muscle biopsy from subject PBO S18 at 2 days after the first bout of exercise (moderate category). *Large figure above: Black uppercases indicate the corresponding magnification of cell in the small figures below. White lowercases represent capillary (c), myonuclei (m) and satellite cell (s).* Small figures below: (A) and (D) signs of apoptosis such as *small round structures* possible from neutrophil. (B), (C), and (E) neutrophils. (C) unknown cell connected to the neutrophil

observed in a limited number of cells possibly neutrophils. In the remaining 23 (marked with # on Suppl. Table 5) of 34 biopsies containing CD66b positive cells, the stained cells were located in blood clots containing also leucocytes, attached to the wall of the vessels, or located inside capillaries or larger vessels (Suppl. Fig. 8). Blood clots, containing cluster of red blood cells and occasionally single leucocytes, were observed quite often in semi-thin toluidine blue stained sections both in exercised and in non-exercised samples (Suppl. Fig. 11A). Neutrophils, monocytes (Suppl. Fig. 11B) and platelets (Suppl. Fig. 11C) were from time to time observed inside capillaries and vessels in transmission electron microscopic sections as well. Furthermore, a dense accumulation of CD68 stained cells were observed in the surroundings of vessels (Fig. 7f, arrowhead). By electron microscopy these cells appeared to be lymphocytes and macrophages (Suppl. Fig. 12). However, since vessels were observed quite

seldom in the studied biopsies, it was difficult to get an overview of these accumulated cells and whether exercise might have had an effect on the number of cells.

CD68 positive cells in the endomysium were typically located near capillaries (Fig. 7c, e, arrowheads). Electron microscopy showed that larger cells near capillaries most likely were macrophages (Fig. 9). These cells contained various sizes of vesicles and vacuoles and many pseudopodia on the cell surface. The smaller cells with their cytoplasm almost entirely occupied by the nucleus were monitored as lymphocytes (Fig. 9). Furthermore, cells containing larger amount of rough endoplasmic reticulum appeared in the surrounding of these cells. CD68 stained also single cells with long extensions aligned next to laminin staining both in the sarcoplasm of skeletal muscle fibre (Fig. 7b, arrow) and in the endomysium (Fig. 7b, arrowhead). Cells in the sarcoplasm aligned next to basement membrane were possibly satellite cells (Fig. 10a, b). As shown in the electron micrographs, these cells were separated from the sarcoplasm of skeletal muscle fibre by a plasma membrane, but were located inside the basement membrane of skeletal muscle fibre (Fig. 10b). Cells with long extensions in the endomysium contained substantial amounts of rough endoplasmic reticulum (Fig. 10c, d) indicating the cell's ability to synthesize proteins like collagen.

Fig. 9 Monocytes/macrophages and leucocytes nearby a capillary. Electron micrograph of low temperature embedded exercised skeletal muscle biopsy of subject PBO S13 at 2 days after the second bout of exercise (severe category). Larger cells nearby capillary are possibly monocytes (Mo), macrophages (Ma) and the smaller cells, which cytoplasm is almost entirely occupied by nucleus, are probably lymphocytes (L). Segments of cells containing rough endoplasmic reticulum (rer) are located in the surrounding of these cells. Endothelia cell (E), myonuclei (m), pericyte (P) and red blood cell (R)



Thirteen biopsies, all after the first bout of exercise, were observed to contain dystrophin negative fibres infiltrated by variable numbers of CD68 positive cells (biopsies containing at least one dystrophin negative fibre were marked on black background on the Suppl. Table 5–7). In electron microscopy sections these fibres showed disorganized sarcolemmal structure and the plasma membrane was not visible. The cells in the sarcoplasm and in the endomysium of these damaged fibres were most likely either monocytes/macrophages or lymphocytes (Fig. 11). These cells showed many different shapes and sizes occasionally occupying the whole sarcoplasm. Different sizes of digestive vacuoles were observed in these invading cells.

After the second bout of exercise five biopsies (marked on grey background on the Suppl. Table 5–7) showed some degree of deformation in their structure such as split fibres and small-sized fibres. Many CD68 positive cells were observed in these deformed fibres (Fig. 7g, h). According to electron microscopy several cell types were observed in the surrounding of such fibres. As shown in the electron micrographs (Fig. 12), a small part of larger fibre was separated by basement membrane. There were several capillaries and endomysial cells with long extensions containing rough endoplasmic reticulum connected to this fibre. Furthermore cells, which showed similarities to monocytes/macrophages, were connected to this fibre.

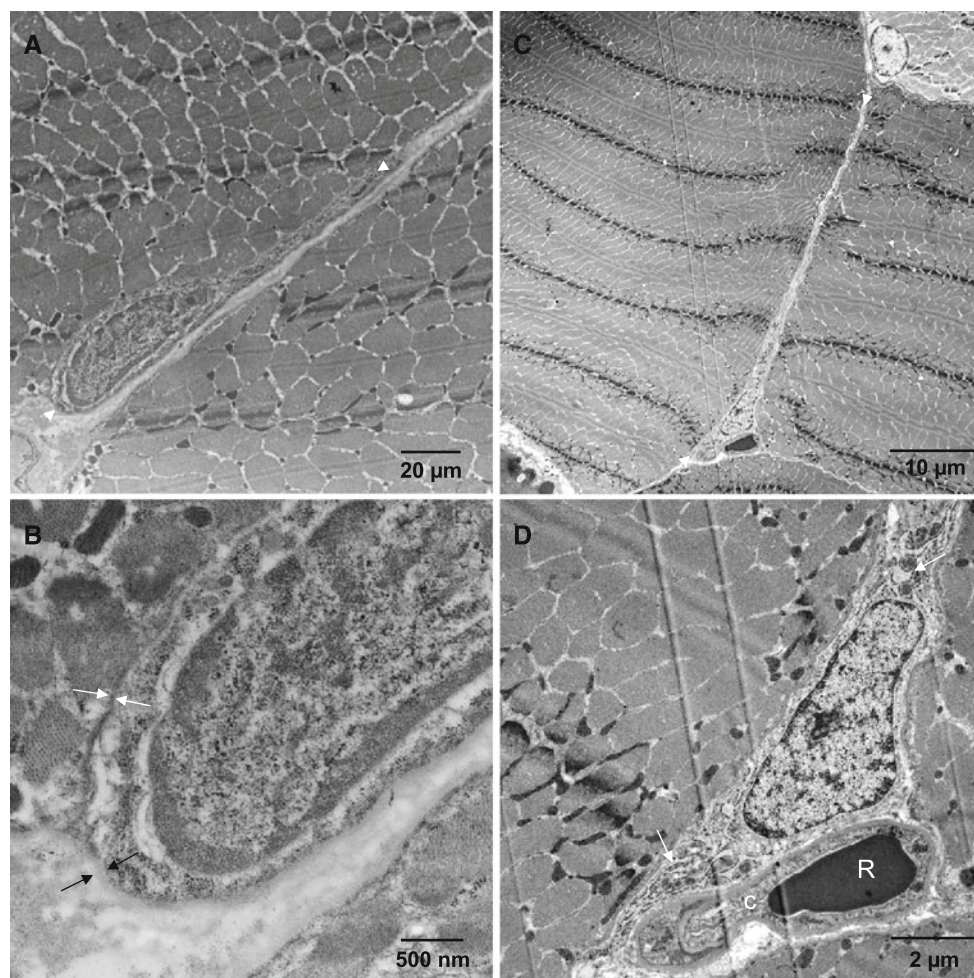


Fig. 10 Satellite cell and cell containing rough endoplasmic reticulum. **a** Electron micrograph of low temperature embedded non-exercised skeletal muscle biopsy of subject PBO S18 2 at days after the first bout of exercise (moderate category). The cell on the sarcoplasm aligned next to basement membrane is possibly a satellite cell. *White arrow heads* point out the length of the cell. **b** Higher magnification of part of **a**. The cell is separated from sarcoplasm of skeletal muscle fibre by plasma membrane (*white*

arrows) and is located inside the basement membrane of skeletal muscle fibre (*black arrows*). **c** Electron micrograph of epoxy embedded exercised skeletal muscle biopsy of subject PBO S14 at 7 days after the first bout of exercise (moderate category). Cell with long extension in the endomysium. *White arrow heads* point out the length of the cell. **d** Higher magnification of part of **c**. Rough endoplasmic reticulum (*white arrows*) indicates cell's ability to produce collagen. Red blood cell (*R*) is located inside capillary (*c*)

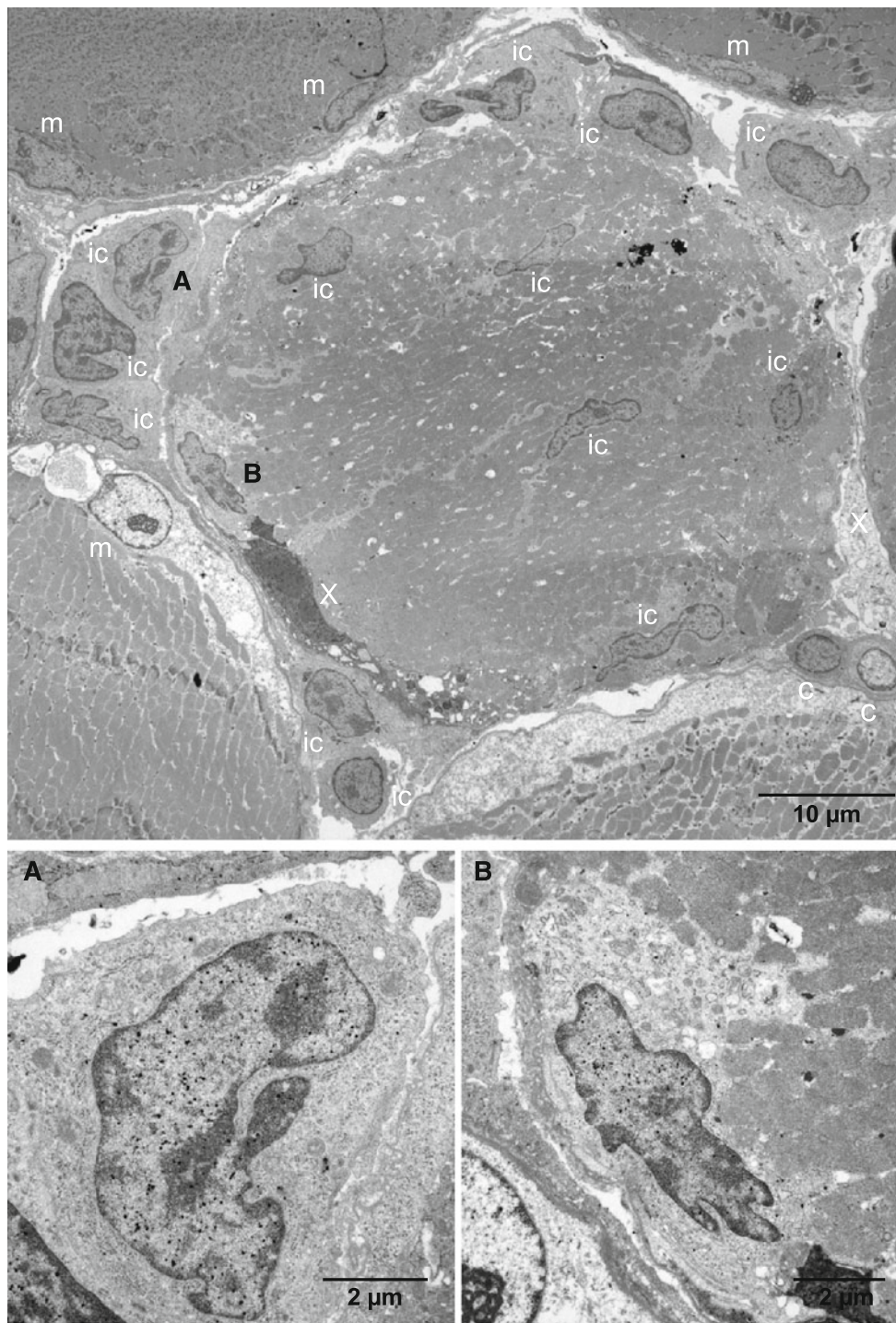


Fig. 11 Damaged skeletal muscle fibre. Electron micrograph of epoxy embedded exercised skeletal muscle biopsy of subject PBO S27 at 4 days after the first bout of exercise (moderate category). Skeletal muscle fibre infiltrated by inflammatory cells (*ic*), possibly

macrophages and lymphocytes (*above*). There are also many inflammatory cells in the endomysium. Myonuclei (*m*), capillary (*c*) and unknown cell (*x*). Higher magnification of inflammatory cells in the endomysium (*A*) and in the sarcoplasm (*B*)

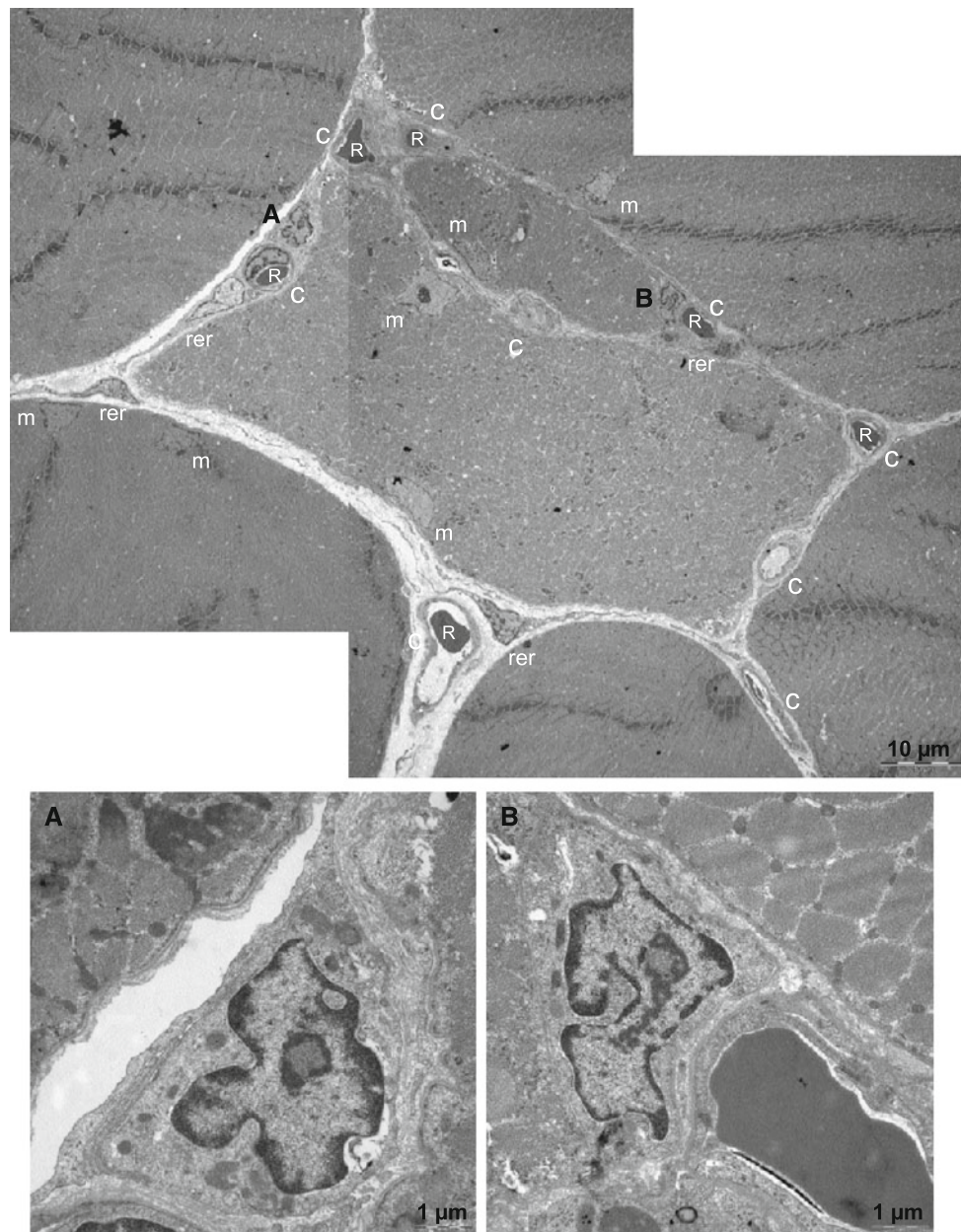


Fig. 12 Spilt fibre. Electron micrograph of epoxy embedded exercised skeletal muscle biopsy of subject PBO S02 at 2 days after the second bout of exercise (moderate category). A smaller part of skeletal muscle fibre is separated by basement membrane from the

main fibre (*above*). Myonuclei (*m*), capillary (*c*) and cell or segments of cells containing rough endoplasmic reticulum (*rer*). Higher magnification of unidentified cells in the endomysium (*A*) and in the sarcoplasm (*B*)

Discussion

Neutrophils in skeletal muscle after eccentric exercise

The results from the present study indicate that the monoclonal CD66b antibody is more suitable for detecting neutrophils than other previously used neutrophil markers CD11b, CD16, myeloperoxidase and neutrophil elastase. Thus, immunostained leucocyte smears showed that CD66b stained only neutrophils, whereas CD11b, CD16,

myeloperoxidase and neutrophil elastase stained also other leucocytes than neutrophils (Fig. 4; Table 2). Furthermore, double immunohistochemical stainings with the CD66b antibody combined with CD11b, CD16, myeloperoxidase and neutrophil elastase of skeletal muscle sections showed that each combination recognized some of the same cells, but CD11b, CD16, myeloperoxidase and neutrophil elastase stained more cells than CD66b (Figs. 5, 6, Suppl. Figs. 6 and 8). The double-staining procedures applied to skeletal muscle sections were first tested on leucocyte

smears. These results showed similar staining pattern on different leucocyte types as in corresponding single-staining procedures on leucocyte smears (compare Fig. 4 to Suppl. Figs. 1–5), indicating that each visualization method was applicable to different primary antibodies.

Review articles on muscle damage after high-force eccentric exercise often conclude that unaccustomed eccentric exercise causes skeletal muscle fibre injury and inflammatory cell reaction is initiated by infiltration of neutrophils (e.g.: Clarkson and Hubal 2002; Evans and Cannon 1991; MacIntyre et al. 1995; Peake et al. 2005; Smith 1991). Increased number of neutrophils has been reported after high-force eccentric exercise in human vastus lateralis muscle biopsies using myeloperoxidase (Beaton et al. 2002; MacNeil et al. 2011; Mahoney et al. 2008; Stupka et al. 2001) and neutrophil elastase (Beaton et al. 2002) to identify neutrophils, whereas no increase was observed when CD11b (Malm et al. 2000, 2004) or CD16 (Mikkelsen et al. 2009) were applied as a neutrophil markers. Other authors have applied CD11b (Hellsten et al. 1997) and CD16 (Paulsen et al. 2010b) as indicator of total number of leucocytes, whereas Przybyla et al. (2006) applied CD11b as a marker for a subpopulation of macrophages. However, no attempt was made in any of these studies to confirm, which type of cells these antibodies actually recognized in the tissue.

Of the five neutrophil markers included in the present study, CD66b and CD16 were chosen for counting the number of positive cells in eccentric exercised biceps muscles (Suppl. Tables 5 and 6). Overall the number of CD16 positive cells was higher than the number of CD66b positive cells. This is in line with the finding that the CD16 antibody also stained some monocytes and lymphocytes in addition to neutrophils in leucocyte smears (Fig. 4). Only 34 of 122 biopsies included for counting contained CD66b positive cells (Suppl. Table 5). A closer examination showed that in two-thirds of these biopsies the CD66b stained cells were located inside capillaries or vessels, attached to the wall of the vessels or were detected in blood clots. In the remaining biopsies, single CD66b positive cells were observed in the endomysium of affected muscle fibres or in the sarcoplasm of damaged fibres. Furthermore, single neutrophils were observed by transmission electron microscopy samples in the same locations as CD66b positive cells were observed in immunohistochemical stained samples. There was no consistent pattern regarding how CD66b positive cells were distributed between exercised and non-exercised samples or between different time points. These results showed that neutrophils were not involved in exercise induced skeletal muscle fibre injury.

Interestingly, accumulation of radiolabelled leucocytes (considered to contain primarily neutrophils) detected by scintigraphy increased 6 and 20 h after the both exercise

bouts in all the categories, most prominently in the category severe effect of exercise. Leucocyte scintigraphy has been shown to positively predict and localize colonic acute inflammation with a high degree of confidence (Middleton et al. 1995). Paulsen et al. (2010a) suggested that radiolabelled leucocytes in skeletal muscle after eccentric exercise were probably either infiltrating the interstitial space of the muscle tissue or merely adhering to the luminal side of the local micro-vessels.

In the present study, muscle biopsies were obtained also from the non-exercised biceps muscles at the same frequent as from the exercised biceps muscle. Four non-exercised biopsies differed from normal skeletal muscle structure (Suppl. Tables 5–7, subjects CEL S05, PBO S08, S14 and S18). These biopsies showed signs indicative of tissue trauma such as dystrophin negative fibres, increased CD68 staining and CD66b stained cells located in the endomysium or inside fibres. The trauma was probably caused by the previous biopsy in three of these four biopsies and in the fourth one by microdialysis performed 2 days earlier.

Our results suggest that the CD66b antibody is more suitable for detecting neutrophils in skeletal muscle sections than CD11b, CD16, myeloperoxidase and neutrophil elastase. The number of CD66b positive cells was in general very low in both exercised and non-exercised biopsies and neutrophils are not involved in exercise induced skeletal muscle fibre injury. Unusual high numbers of CD66b stained cells located in the endomysium or inside fibres may indicate trauma from the previous biopsy.

Monocytes/macrophages in skeletal muscle after eccentric exercise

According to our observations the CD68 antibody recognized more cell types than monocytes/macrophages in skeletal muscle biopsies after eccentric exercise. The subjects' average CD68 positive cell counts are widely used for indication of monocyte/macrophage infiltration in skeletal muscle biopsies after single bout of eccentric exercise (Beaton et al. 2002; Crameri et al. 2004, 2007; MacNeil et al. 2011; Mahoney et al. 2008; Mikkelsen et al. 2009; Paulsen et al. 2010a, b; Przybyla et al. 2006; Peterson et al. 2003; Stupka et al. 2001). In the present study, subjects were divided into three categories based on muscle force loss immediately after the first exercise bout and how fast muscle force was recovered. In the category mild effect of eccentric exercise the number of CD68 positive cells in exercised biopsies was at the same level as in general in non-exercised biopsies of all three categories. The highest individual CD68 positive cell counts were related to skeletal muscle fibre injury, which was shown as dystrophin negative fibres, and were observed in exercised biopsies at 4 and 7 days after the first exercise bout in the

categories moderate and severe effect of eccentric exercise. In these biopsies, monocytes/macrophages were probably the most prominent CD68 positive cell type. On the other hand, in the exercised biopsies not containing dystrophin negative fibres delineation of the monocytes/macrophages proportion of CD68 positive cell in these two categories is not straightforward. The results from the present study showed that satellite cells and some endomysial cells containing rough endoplasmic reticulum, probably representing myofibroblasts or fibroblasts, were recognized by CD68 antibody. Both of these cell types were most likely involved in the skeletal muscle adaptation to increased mechanical loading. However, it is not known whether the number of such cells significantly affects the total number of macrophages measured as a number of CD68 positive cells. The number of CD68 positive cells cannot be applied as a quantitative value for inflammatory cells. There were also other biopsies, which contained dystrophin negative fibres, but in these biopsies the muscle damage was probably not induced by exercise (non-exercised biopsies CEL S05, PBO S8, S14 and S18, and exercised biopsy PBO S18; Suppl. Table 7). Our results support previous observations that muscle force-generating capability after a single bout of eccentric exercise is a good indirect indicator of muscle damage in humans (Sayers and Clarkson 2001) and that one of the reasons for the impaired peripheral muscle function could be skeletal muscle fibre injury (Hubal et al. 2007).

The second bout of eccentric exercise is known to result in markedly less symptoms of muscle damage than the initial bout, known as repeated bout effect (see for review McHugh 2003). In support, in the present study blood creatine kinase remained low and no dystrophin negative fibres were observed in any of the three categories after the second bout of exercise. The recovery of muscle force-generating capacity seemed to be faster after the second bout of exercise compared with the first bout, which is also in line with previous studies (see for review McHugh 2003). However, muscle force was clearly below the pre-exercised level among the subjects in the category severe effect of eccentric exercise and in some of the subjects in the category moderate effect of eccentric exercise right before the second exercise bout. This muscle force loss was probably related to regeneration following muscle damage and adaptation from the first exercise bout. Furthermore, deformed skeletal muscle fibres were observed in the exercised biopsies from five subjects at 1 h and 2 days after the second exercise bout. Intensive CD68 staining was observed in these deformed skeletal muscle fibres (Figs. 7g, h, and 12), which were most likely regenerating from the first bout of exercise rather than being affected by the second exercise bout. Finally, the muscle force-generating capacity for four subjects was clearly below the

pre-exercised level still 1 week after the second bout of eccentric exercise. Such a long lasting recovery phase after high force eccentric exercise for individual subjects has been reported previously (Sayers and Clarkson 2001; Hubal et al. 2007). In the future studies more attention should be paid for improving the muscle force recovery of these subjects after the experiment itself is over.

In the present study, CD68 positive cells in the sarcoplasm of damaged skeletal muscle fibres (Figs. 7a, 11), inside capillaries (Fig. 7d, Suppl. Fig. 11B) and vessels were most likely monocytes/macrophages, whereas endomysial CD68 positive cells could represent several cell types (for example Figs. 7b, c, 9, 10c, d). Correspondingly, it has been reported earlier that CD68 is expressed also in other cell types than macrophages in synovial tissue (Kunisch et al. 2004), in various primary cells and carcinoma cell lines (Gottfried et al. 2008). Sarcoplasmic CD68 immunostaining was observed in single cells aligned next to the laminin (Fig. 7b). These cells were most likely satellite cells (Figs. 10a, b). It has been reported previously that mesenchymal stem cells may express CD68 (La Rocca et al. 2009). Endomysial cells with long extensions were observed to be CD68 positive. The present transmission electron microscopy results showed that cells with similar appearance contained prominent rough endoplasmic reticulum indicating cells with high capacity for protein synthesis such as in fibroblasts/myofibroblasts (compare Figs. 7b, 10c, d). Interestingly, Gottfried et al. (2008) demonstrated that cultured fibroblasts and endothelial cells isolated from human normal skin and umbilical vein, respectively, expressed CD68. Furthermore, endomysial CD68 positive cells without pronounced extensions (Figs. C, E) were often observed close to capillaries. Based on the localization and the cell shape these cells probably were monocytes/macrophages and occasionally lymphocytes according to the observations by transmission electron microscopy (Fig. 9). Immunostainings of leucocyte smears showed no CD68 positive lymphocytes, whereas CD16 stained in addition to neutrophils also some lymphocytes and monocytes (Fig. 4). Furthermore, double staining with CD68 and CD16 showed overlapping staining pattern at least in damaged skeletal muscle fibres (Suppl. Fig. 13). Ten percent of circulating monocytes in healthy individuals have been shown to be CD16 positive, whereas in patients with active rheumatoid arthritis the CD16 positive portion is increased (Kawanaka et al. 2002). CD16 positive macrophages have been found most densely in the synovial lining layer from rheumatoid arthritis patients (Bröker et al. 1990). CD16 positive monocytes are considered to be able to produce proinflammatory cytokines more efficiently, migrate rapidly to the site of inflammation and readily differentiate into tissue macrophages than CD16 negative monocytes (Ziegler-Heitbrock 2007). In the present study,

the number of CD68 positive cells per 100 fibres seemed to be two to three times higher than the corresponding number of CD16 positive cells. Since the number of neutrophils was very low in the present biopsies, the CD16 positive cell counts could represent an important subpopulation of macrophages in skeletal muscle degeneration and regeneration. Further research is needed to study the occurrence of CD16 positive cells in skeletal muscle, and in particular which portion of the tissue macrophages that is overlapping with CD68. Finally, intensive CD68 staining was observed in skeletal muscle fibres, which were smaller than normal (Fig. 7h) and were divided into several parts by basement membrane (Fig. 7g). Such fibres were observed in the exercised biopsies from five subjects after the second exercise bout. These deformed skeletal muscle fibres were probably formed following the damage caused by the first bout of exercise. According to the examination by transmission electron microscopy it seemed likely that also cell types other than macrophages were involved in processes around these fibres (Fig. 12).

Skeletal muscle fibre injury in human biceps brachii muscle after eccentric exercise

In the present study, eight of total 23 subjects showed skeletal muscle fibre injury detected at light microscopy level as dystrophin negative fibres and infiltration of CD68 positive cells at 4 or 7 days after the first exercise bout. These subjects belonged to the categories moderate (5 subjects of 10 showed dystrophin negative fibres) and severe (3 subjects of 7 showed dystrophin negative fibres) effects of eccentric exercise (Suppl. Fig. 14A). In similar arm exercise Stauber et al. (1990) have observed necrotic fibres in biopsies from biceps brachii muscle, but biopsies were obtained only at 2 days after the eccentric exercise. In general necrotic fibres are very seldom observed in exercised human skeletal muscles. One reason for this is probably; whether the biopsy is obtained from the damaged part of muscle. Eccentric exercise with the knee-extensors is more frequently used damaging exercise protocol than the current protocol with elbow flexors. In these knee-extensor experiments biopsies are often obtained from vastus lateralis muscle, whereas the most affected muscle seems to be rectus femoris muscle (Prior et al. 2001; Paulsen et al. 2010b). Muscle damage after eccentric exercise has been shown to be more pronounced in elbow flexors than in knee extensors (Chen et al. 2011). In addition to that it has been shown by magnetic resonance and ultrasound imaging that brachialis muscle is more affected by eccentric exercise than biceps brachii (Nosaka and Sakamoto 2001). Histological finding from the present study showed deformed skeletal muscle fibres in five subjects in the moderate and severe categories after the

second bout of exercise. However these deformed fibres were most likely regenerating fibres after the first bout of exercise rather than affected by the second exercise bout. According to our knowledge this has not been reported earlier in exercised induced human skeletal muscles. In addition, the results from the present study demonstrated that decrease in muscle force generating capacity can be used as an indirect indicator for severity of skeletal muscle damage (Suppl. Fig. 14). This was shown as enhanced response in non-invasive measurements CK (Fig. 2) and accumulation of leucocytes detected by scintigraphy (Fig. 3) as well as by observation of injured skeletal muscle fibres in muscle biopsies in categories moderate and severe effects of eccentric exercise (Suppl. Fig. 14). According to our knowledge this has not been demonstrated earlier. The current results that exactly the same amount of eccentric muscle contractions causes variable responses among subjects, can be applied for example for monitoring recovery of muscle before the second bout of exercise. If the muscle force generating capacity has not totally recovered to the baseline level, it is most likely that muscle is undergoing regeneration, which makes it difficult interpret the actual repeated bout effect.

Summary

In summary, the results from the present study suggest that the monoclonal CD66b antibody is suitable for localization of neutrophils in human skeletal muscle. The number of CD66b positive cells was very low and there was no uniform pattern regarding how CD66b positive cells were distributed between exercised and non-exercised biopsies or between different time points. However, unusually high numbers of CD66b positive cells may indicate non-exercised related trauma for example from previous muscle biopsy. Furthermore, the current results may indicate that neutrophils are not involved in exercise induced skeletal muscle fibre injury. The widely used macrophage marker CD68 showed reactivity against more cell types than monocytes/macrophages in human skeletal muscle and therefore cannot be applied for a quantitative value of inflammatory cells. In addition to monocytes/macrophages, which were observed in damaged skeletal muscle fibres in large numbers, satellite cells and fibroblast-like cells were observed to be CD68 positive. The number of CD68 positive cells per 100 fibres seemed to be two to three times higher than corresponding number of CD16 positive cells. The CD16 positive cells may represent an important population of macrophages in skeletal muscle degeneration and regeneration. Further research is needed to study the occurrence of CD16 positive cells in skeletal muscle, and especially, which portion of these tissue macrophages that

is overlapping with CD68 expressing cells. Furthermore, the present results strengthen previous observations suggesting that muscle force-generating capacity after single bout of eccentric exercise is a good indirect indicator of muscle damage in humans and that skeletal muscle fibre injury and prolonged regeneration process could be one of the reasons for impaired peripheral muscle function after high force eccentric exercise. Finally, individual differences in the exercise responses, such as muscle force, creatine kinase, skeletal muscle fibre injury and leucocyte infiltration, varies greatly, therefore it is critical to acknowledge these variations when designing an experiment and drawing conclusions from average data.

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