CX3CL1 – a macrophage chemoattractant induced by a single bout of exercise in human skeletal muscle

Strömberg A, Olsson K, Dijksterhuis JP, Rullman E, Schulte G, Gustafsson T

1Division of Clinical Physiology, Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden; 2Section of Receptor Biology and Signaling, Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden

Running title: Fractalkine in exercised human skeletal muscle

Corresponding author: Anna Strömberg, anna.stromberg@ki.se, C1:82 Division of Clinical Physiology, Karolinska University Hospital Huddinge, 141 86 Stockholm, Sweden
Abstract

Monocytes/macrophages (MOs/MΦ) are suggested to be crucial for skeletal muscle repair and remodeling. This has been attributed to their pro-angiogenic potential, secretion of growth factors and clearance of tissue debris. Skeletal muscle injury increases the number of MΦ in the tissue, and their importance for muscle regeneration has been supported by studies demonstrating that depletion of MOs/MΦ greatly impairs repair after muscle injury. Whether non-injurious exercise leads to induced expression of chemoattractants for MOs/MΦ is poorly investigated. To this end, we analyzed the expression of CX3CL1 (fractalkine), CCL2 (MCP-1) and CCL22 (MDC) in human skeletal muscle after a bout of exercise, all of which are established MO/MΦ chemotactic factors that are expressed by human myoblasts. Muscle biopsies from the m. vastus lateralis were obtained up to 24 h after 1 h of cycle exercise in healthy individuals and in age-matched non-exercised controls. CX3CL1 increased at both the mRNA and protein level in human skeletal muscle after one bout of exercise. It was not possible to distinguish changes in CCL2 or CCL22 mRNA levels between biopsy vs. exercise effects and the expression of CCL22 was very low. CX3CL1 mainly localized to the skeletal muscle endothelium and it increased in HUVECs stimulated with tissue fluid from exercised muscle. CX3CL1 increased the expression of pro-inflammatory and pro-angiogenic factors in THP-1 monocytes and in human primary myoblasts and myotubes. Altogether this suggests that CX3CL1 participates in cross-talk mechanisms between endothelium and other muscle tissue cells and may promote a shift in the micro-environment towards a more regenerative milieu.

Keywords: Fractalkine, skeletal muscle, endothelium, cross-talk, exercise
Background
A transient increase in inflammatory cells is seen in the muscle tissue after acute muscle damage. These cells aid in the repair process by removing cellular debris and providing a regenerative milieu. In particular, the importance of monocytes (MOs) and macrophages (MΦ) in skeletal muscle repair and remodeling has been demonstrated in several studies (2, 5, 6, 19, 24, 27, 29). Indeed, both MO depletion and selective inhibition of MΦ infiltration lead to severely impaired regeneration following muscle injury in mice (2, 29), which is characterized by fibrosis and delayed satellite cell proliferation (2). Current data provide evidence that the MOs recruited to damaged muscle are pro-inflammatory, and act to phagocytose cellular debris and induce myoblast proliferation (2). These MOs then differentiate into MΦ and switch to an anti-inflammatory phenotype, which promotes myoblast differentiation and angiogenesis (5, 6, 19, 24, 27).

The mechanisms behind skeletal muscle recruitment of MOs and/or of MΦ are just beginning to be discovered. Animal models of muscle injury have established the importance of chemokines expressed in the muscle tissue for the recruitment of MOs/MΦ and muscle regeneration (32, 35). Moreover, a previous study investigating the interaction between human myogenic precursor cells and MOs/MΦ reported that human myoblasts in culture produced the chemokines CX3CL1, CCL22 and CCL2, which selectively attracted MOs (10). Further, these factors were demonstrated to account for a large part of MO chemotaxis and to be expressed by activated human satellite cells in vivo, suggesting that they may be responsible for attracting MOs to human skeletal muscle.

Importantly, since skeletal muscle remodeling occurs also after non-damaging type of exercise, a plausible hypothesis is that such a stimulus also induces the production of inflammatory cell chemoattractants. In support of this hypothesis, it was shown recently
that a prolonged, low-intensity endurance exercise bout in well-trained subjects induced skeletal muscle infiltration of inflammatory cells (22). Furthermore, the number of MΦ increased in mouse skeletal muscle after concentric contractions that did not cause any signs of muscle injury (22, 23). Thus, in the current study the overall aims were to investigate the expression, localization and plausible biological effects of chemoattractants with suggested importance for skeletal muscle remodeling i.e. CX3CL1, CCL2 and CCL22. We hypothesized that a single endurance exercise bout in humans would increase the expression of these chemoattractants, presumably localizing to the satellite cells and muscle fibers. Moreover, based on the knowledge that MOs exhibiting a pro-inflammatory profile arrive first within regenerating muscle, we hypothesized that these chemoattractants could induce a pro-inflammatory phenotype in monocytic cells.

**Methods**

**Ethical approval**
The regional ethical review board in Stockholm, Sweden approved the study. The subjects gave written informed consent before participating in the respective experimental setup. The study conformed to the standards set by the Declaration of Helsinki.

**Exercise and muscle biopsies**
17 male and female moderately active subjects were included in the study and divided into an exercise group (n = 12) and a control group (n = 5). All subjects exercised reported that they exercised 1-3 times per week. The subjects did not use any medications and were non-smokers. Prior to the intervention, their maximal oxygen uptake (VO\(_2\)max) was determined using an incremental cycle ergometer test until exhaustion, with respiratory gases continuously analyzed (Sensor Medics Vmax 229, Intra Medic AB, Bålsta, Sweden). For inclusion to the study, the upper limit for VO\(_2\)max was set to 60 ml/kg/min for men, and 50
ml/kg/min for women. The mean (SD) age, height, weight and VO₂max for the cycling
subjects were 25.9 (3.5) yrs., 178.8 (7.2) cm, 77.9 (10.6) kg and 48.1 (6.6) ml·kg⁻¹·min⁻¹. For
the control subjects the mean (SD) age, height, weight and VO₂max were 24.2 (1.9) yrs.,
176.6 (11.1) cm, 68.8 (6.9) kg and 48.3 (5.8) ml·kg⁻¹·min⁻¹. No significant differences were
found between the groups regarding age, height, weight or VO₂max. The group as a whole
(n=17) had an average (SD) BMI of 23.6 (2.5) kg/m². The average of the males was 24.3 (2.1)
and that of the women was 22.4 (2.9) kg/m². All subjects were Caucasian.

Experimental procedure
The subjects were instructed to abstain from alcohol and exercise for two days before the
test. When arriving at the laboratory, the subjects were randomized to either exercise (8
males and 4 females) or control (3 males and 2 females) groups. The exercise group
performed a 1 h cycling bout consisting of 20 min at a work load corresponding to 50% of
VO₂max and 40 min at a work load corresponding to 65% of VO₂max, while the control group
rested. To measure the perceived effort of the exercising subjects, the Borg scale (rating
exertion on a scale 6-20) was used every 10 min. The following protocol was used to change
load: At 30 min; effort rated < 13, increase by 10 Watts (W). At 40 min; effort rated < 15
increase by 10 W, 17 decrease by 10 W, ≥ 18 decrease by 20 W. At 50 min; effort rated < 16
increase by 10 W, ≥ 18 decrease by 10 W. Muscle biopsies were obtained using the
percutaneous needle biopsy technique from the vastus lateralis muscle of both legs. Before
the 1 h of cycling/resting, one biopsy was obtained from each leg. Biopsies also were
obtained at 30 min, 2 h, 6 h and 24 h after the cycling/resting; the biopsies at 30 min and 6 h
were taken from one leg, while biopsies at 2 h and 24 h were obtained from the other. The
biopsies were immediately frozen in isopentane cooled in liquid nitrogen, and stored at -80
°C. Both groups remained in the laboratory until after the 6h biopsy, and then returned the
morning after for the 24h biopsy. All subjects were given standardized meals the night before, during the day of, and in the morning after the intervention (Table 1).

**Exercise and microdialysate**

Three healthy male subjects with a mean (range) age, height, and weight of 23 (19-26) yrs., 182 (175-190) cm, and 77 (71-85) kg, participated in this part of the study. They did not use any medications and were non-smokers. Subjects performed dynamic constant load one-legged knee extension exercise (60 rpm) in the sitting position using a modified cycle ergometer using the dominant leg (1). One week prior to the experiment, subjects performed a one-legged exercise test with incremental workload: after one min at 60 rpm with 25 W, workload was increased with 5 W every min until exhaustion. Subjects were instructed to abstain from exhaustive exercise during the week prior to the experiment. On the day of the experiment, initial workload was chosen to be 60% of maximal workload (range 25-30 W). After 20 and 40 min, workload was adjusted based on effort assessed with Borg scale. At 20 min; effort rated < 11, increase by 10 W, > 16 decrease by 10 W. At 40 min; effort rated < 13 increase by 5 W, > 16 decrease by 5 W. Two of the subjects worked at 30 W during the whole 1 h bout, while one subject worked at 30 W the first 40 min and then 25 W the last 20 min. All subjects were Caucasian.

**Experimental procedure**

Microdialysate was obtained from the vastus lateralis muscle of both legs after one-legged exercise using CMA 71 High Cut-Off Brain Microdialysis Catheters with a cut-off of 100 kDa (CMA Microdialysis AB, Solna, Sweden). After local anesthesia, the catheters were inserted with a guided cannula and perfused with Ringer’s acetate at a rate of 2µl/min. During the 4 h sampling, the subjects rested in a supine position. The dialysate was collected in vials that were switched every 30 minutes, and then the collected sample was transferred to -80° C.
During the first 30 minutes after insertion, the catheters were infused at a rate of 5 µl /min. The samples collected during the first 60 min post-insertion were not used for the stimulation assay.

**Cell culture experiments**

**Stimulation of HUVECs with microdialysate**

Human umbilical vein endothelial cells (HUVECs), (C-003-5C, Life technologies, Carlsbad, California, USA) were cultured in 24-well plates in Medium 200 (M200) supplemented with low serum growth supplement (LSGS) and 1% Antibiotic-Antimycotic (AbAm) (Life Technologies). When confluent, the cells were stimulated for 2 h with microdialysate diluted 1:2 in Ringer’s acetate. Two wells were used per condition (resting leg, exercised leg), which were pooled in TRIzol (Life Technologies) at cell harvest prior to RNA extraction.

**Stimulation of THP-1 cells with CX3CL1**

THP-1 monocytic cells (Sigma-Aldrich, St. Louis, Missouri, USA) were cultured in RPMI 1640 medium GlutaMAX (Life Technologies) supplemented with 1% AbAm, 10% FBS, 1mM sodium pyruvate and 0.05mM 2-mercaptoethanol. For the stimulation assay, cells were suspended at $1 \times 10^6$ cells/mL in serum-free RPMI medium, and 2 mL were plated in each well in a 6-well plate, with 0, 10 or 100 ng/mL recombinant human CX3CL1 (R&D systems Inc, MN, USA). The rationale behind using 10 ng/mL and 100ng/mL is based on a previous paper where similar dosing was utilized to evaluate the pro-angiogenic effect of CX3CL1 in HUVECs (33). After 5 h, the cell suspensions were centrifuged at 6000 x g, the cell pellets were re-suspended in 1 mL of TRIzol, and kept at -80° C until RNA extraction. The experiment was performed four times in duplicate.
Stimulation of human primary myoblasts and myotubes with CX3CL1

Four healthy, non-smoking subjects (20-31 yrs. old, 2 females, 2 males) were recruited specifically to obtain muscle biopsies for isolation of primary human myoblasts. All subjects were Caucasian. A single biopsy from m. vastus lateralis was obtained from each subject using the percutaneous needle biopsy technique. In brief, the biopsy was digested in 5 ml of 0.25% trypsin 1 mM EDTA at 37°C and 5% CO2 with gentle agitation for 20 min, and the supernatant was diluted in proliferation medium (Dulbecco's modified Eagle's medium (DMEM/F-12) supplemented with 1% AbAm and 20% fetal bovine serum (FBS) (Life Technologies)). Digestion of the slurry was repeated twice. After pre-plating the cells in petri dishes for 20 min, the cells were cultured in T75 flasks (Sarstedt, Stockholm, Sweden), and growth medium was changed every 3rd or 4th day until cells reached 60% confluence. The myoblasts then were trypsinized and seeded in 6-well plates at a density of 80,000 cells per well, in proliferation medium. All cultures had a desmin positivity > 90% analyzed using cytocentrifuged cells. After two days, the medium was changed to low-serum medium (DMEM/F-12 containing 2% FBS, 1% AbAm) and the cells where stimulated with 0, 10 or 100 ng/mL of CX3CL1 for 5 h, with two wells per condition. For myotube experiments, myoblasts in 6-well plates were allowed to reach 90% confluence and then the medium was changed to low-serum medium. After three days, the myotubes were stimulated with CX3CL1 using the same protocol as for the myoblasts. After stimulation, the cells from each condition were pooled in 1 mL of TRIzol and RNA extraction was performed.

Myoblast proliferation assay

Primary human myoblasts were cultured in 96 well plates in DMEM/F-12 containing 20% FBS and 1% AbAm. After two days, the cells received fresh DMEM medium supplemented with 0, 10 or 100ng/mL of CX3CL1, and BrdU was added to all wells. After 20 h, analysis of BrdU
incorporation was performed according to the manufacturer’s instructions (F. Hoffmann-La Roche Ltd, Basel, Schweiz). The experiment was performed four times in triplicate.

Analysis

Gene expression analysis
Total RNA from skeletal muscle, HUVECs, THP-1 cells, primary human myoblasts and myotubes was prepared by the acid phenol method and quantified by measuring absorbance at 260 nm using NanoDrop 2000 (Thermo Scientific, Göteborg, Sweden). One microgram of RNA was reverse transcribed using the high capacity cDNA reverse transcription kit (Life Technologies). Taqman gene expression assays were purchased from Life Technologies, and GAPDH was used as endogenous control. The following assays were used: CX3CL1 (Hs00171086_m1), CCL2 (Hs00234140_m1), CCL22 (Hs01574247_m1), GAPDH (Hs02758991_g1), IL8 (Hs00174103_m1), IL6 (Hs00174103_m1), VEGF-A (Hs00900055_m1), TGF-beta (Hs00998133_m1), TNF-α (Hs99999043_m1), IL10 (Hs00961622_m1), MMP9 (Hs00234579_m1), Myostatin (Hs00976237_m1), iNOS (Hs01075529_m1), MyoD (Hs02330075_g1) and Myogenin (Hs01072232_m1). Gene expression levels were determined using the $2^{-\Delta\DeltaCT}$ method, relating mRNA changes as ratio to the house-keeping gene.

ELISA
15 mg of frozen muscle from exercise (n=6) and control (n=3) subjects was homogenized in 0.1 M potassium phosphate buffer containing 0.05% bovine serum albumin (BSA) and cOmplete protease inhibitor cocktail tablets (one tablet per 10 mL buffer; F. Hoffmann-La Roche Ltd, Basel, Switzerland). 40 μl of buffer was used per mg of muscle. Homogenization was performed on ice using glass homogenizers, and the homogenates then were rotated
for 1 h at 4°C. The homogenates then were centrifuged for 10 min at 1500 x g in 4°C, and subsequently stored at -80°C until analysis. The protein concentration was measured using the Bradford assay. The muscle homogenate was analyzed for CX3CL1 protein concentration by ELISA following the manufacturer’s instructions (R&D systems Inc.).

**Immunofluorescence**

The frozen muscle biopsies were cut into 5 μm sections and placed on Superfrost/Plus microscope slides (Thermo Scientific). The sections were fixed for 10 min in 4% phosphate buffered formaldehyde, and then washed 3 min in phosphate buffered saline (PBS), 3 min in 0.3% Triton-X/PBS and 3 min in PBS. The tissue sections were blocked in 4% FBS/PBS for 30 min, and then incubated with the primary antibody overnight at 4°C in a humid chamber. The antibodies used were: goat anti-human CX3CL1 at 1:250 (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), mouse anti-human CD31 at 1:300, (Dako Sweden AB, Stockholm, Sweden), mouse anti-human CD56 (Clone MY31, BD Biosciences, Franklin lakes, NJ, USA) at 1:20, mouse anti-human Caveolin-3 (A-3) at 1:500 (Santa Cruz Biotechnology Inc.), goat anti-human IL8 at 1:20 (Abcam, Cambridge, UK) and rabbit anti-human Collagen-4 at 1:1000 (Rockland Inc., Limerick, PA, USA). After washing in PBS, sections were incubated with the donkey anti-mouse Alexa568 antibody or the donkey anti-rabbit Alexa568 antibody at 1:800, and the donkey anti-goat Alexa488 at 1:800 (Life Technologies) for 60 min in room temperature, washed again and mounted in ProLong Gold antifade reagent (Life Technologies) containing the DNA stain DAPI. Both primary and secondary antibodies were diluted in 1% FBS/PBS. Fluorescent signals (Fig. 2A-D) were detected with a Zeiss LSM710 laser scanning microscope in combination with the ZEN2011 software (Zeiss, Jena, Germany) using the laser lines 405 (DAPI), 488 (Alexa488) and 561 (Alexa568) for fluorescence excitation. Image rendering (Fig. 2B-D) was done with Bitplane Imaris software using the
volume tool. A Leica DMLA microscope equipped with a Leica DFC 450 digital camera (Leica Microsystems AB, Sweden) was used to obtain conventional fluorescent images (Fig. 2E-G, 3D-G) using an oil immersion objective with magnification 63×1.25.

**Statistics**
The effect of exercise on gene and protein levels in human skeletal muscle was analyzed using two-way repeated measures ANOVA with the factors condition (exercise and control) x time (pre, post 30, post 2h, post 6h and post 24h). The Holm-Sidak all pairwise comparison was used as post-hoc test to locate the points of interaction. The effect of CX3CL1 stimulation on gene expression in myoblasts, myotubes and THP-1 cells was analyzed using one-way repeated measures ANOVA, with the Holm-Sidak all pairwise comparison used as post-hoc test. Gene expression in HUVECs was analyzed using a two-tailed t-test. These statistical analyses were performed using SigmaPlot 13.0 (Systat Software Inc., San Jose, CA, USA). The mRNA data from muscle biopsies was not normally distributed as assessed by the Shapiro-Wilks test. To address this, the Mauchly test for sphericity together with the Greenhouse-Geisser correction was utilized to correct the degrees of freedom for the F-distribution in the two-way repeated measures ANOVA. This analysis was performed using SPSS v.22 (SPSS, Chicago, IL, USA). Differences were considered statistically significant at P < 0.05.

**Results**

**Increase in CX3CL1 mRNA and protein in human skeletal muscle with exercise**
The mRNA level of CX3CL1 increased in the skeletal muscle tissue at 30 min post exercise compared to pre levels. No change in CX3CL1 mRNA levels was seen in the control group (Fig. 1A, interaction between exercise and control P = 0.002). For the CCL2 and CCL22 mRNA levels, no significant interaction was found between the groups (Fig. 1C and 1D, P = 0.48 and...
P = 0.6 respectively). No significant difference was seen between males vs females in the mRNA levels of CX3CL1, MDC or MCP-1. The expression levels of CCL22 were low with a mean CT value of 33.2 in the pre biopsies in cycling and 32.8 in control subjects at cDNA dilution 1:10 corresponding to 18 ng RNA per well. Since no changes were seen between exercise and rest in the gene expression of CCL2 and CCL22, the remainder of the study focused on CX3CL1. The protein level of CX3CL1 in the muscle homogenate was increased at 2 h post exercise and returned to resting levels at 24 h post. The mean (SD) value expressed as pg CX3CL1/µg protein was 0.07 (0.04), 0.12 (0.06), 0.21 (0.11), 0.13 (0.04), 0.09 (0.08) in the exercise subjects. No change in CX3CL1 protein levels was seen in the control group (Figure 1B, interaction between exercise and control, P = 0.04).

**CX3CL1 is expressed on endothelium in human skeletal muscle**

To visualize the localization of CX3CL1, immunofluorescent staining for CX3CL1 was performed on muscle sections (Fig 2A). Confocal imaging showed CX3CL1 colocalized with the endothelial cell marker CD31, and in addition was detectable within endothelial cell nuclei (Fig. 2B-D). Faint CX3CL1 staining also was associated with muscle fibers, next to the endothelium, and the endomysium, which could represent shed CX3CL1. The staining level of CX3CL1 was slightly increased 2 h post exercise compared to pre levels (Fig. 2E-F). Satellite cells showed no or very weak CX3CL1 staining (Fig. 2G). To investigate whether factors in the skeletal muscle tissue fluid could induce the expression of CX3CL1 in endothelial cells, HUVECs were stimulated with microdialysate obtained from exercised muscle. The level of CX3CL1 mRNA increased 1.9-fold in HUVECs stimulated with exercise microdialysate compared with microdialysate from the resting leg, suggesting that at least part of the induction in CX3CL1 may be due to factors increasing in the muscle interstitial fluid with exercise (Fig. 2H, P = 0.02).
CX3CL1 induces factors involved in skeletal muscle remodeling in human monocytic and myogenic cells

THP-1 monocytic cells were stimulated with CX3CL1 to assess whether CX3CL1 could modulate their inflammatory profile. The expression of pro-inflammatory TNF-α (P = 0.005) and IL6 (P = 0.02) and anti-inflammatory IL10 (P = 0.01) were increased significantly in cells treated with 100 ng/mL CX3CL1, and there was a trend towards an increased level of VEGF-A (P = 0.08). For MMP9 and TGF-β, the ANOVA detected a significant treatment effect, which however could not be located with the post-hoc test (Fig. 3A). iNOS was not expressed in any condition.

To further investigate plausible biological effects of CX3CL1, the expression of factors involved in muscle remodeling was measured in primary human myoblasts and myotubes following CX3CL1 stimulation. In myoblasts, the expression of IL6 was increased in cells treated with 100 ng/mL of CX3CL1 (P = 0.02), and there was a trend towards increased CCL2 (P = 0.07) and IL8 (P = 0.1). 100 ng/mL CX3CL1 decreased the level of MyoD (P = 0.04), but did not alter Myogenin (P = 0.47) or VEGF-A (P = 0.69). Myostatin (P = 0.14) and MMP9 (P=0.11) exhibited a tendency to increase, although MMP9 was virtually not expressed with an average CT-value of 35.4 at cDNA dilution 1:5 corresponding to 36 ng RNA per well (Fig. 3B). Myotubes increased their expression of IL8 (P = 0.008), IL6 (P < 0.001), MMP9 (P = 0.03) and CCL2 (P = 0.003) at 100 ng/mL while no change was seen for VEGF-A (P = 0.78), Myostatin (P = 0.55), MyoD (P = 0.75) or Myogenin (P = 0.15) (Fig. 3C). Since IL8 was highly induced in myotubes with CX3CL1 treatment, immunofluorescent staining of IL8 was performed in the skeletal muscle tissue before and after exercise. The staining for IL8 in skeletal muscle sections was increased at 2h post exercise compared to pre levels (Fig 3D-G).

The localization was not conclusive but mainly was found in the interstitium, associated with
muscle fibers and endothelium. Further, due to the decreased myoblast expression of MyoD
with CX3CL1 stimulation, a proliferation assay was performed. However, stimulation of
human myoblasts with CX3CL1 did not affect myoblast proliferation rate as measured by
BrdU incorporation (Fig. 4, P = 0.47).

Discussion
The key observations of the current study were 1) CX3CL1 is increased in human skeletal
muscle at the mRNA and protein level after one bout of endurance exercise, 2) CX3CL1
expression is localized mainly to the skeletal muscle endothelium and increases in HUVECs
stimulated with tissue fluid from exercised muscle, 3) when cultured in the presence of
CX3CL1, THP-1 monocytes and human primary myoblasts and myotubes increased their
expression of pro-inflammatory, pro-angiogenic and chemotactic factors.

Following acute muscle injury, muscle regeneration is largely dependent on the recruitment
of MOs and MΦ to create a regenerative milieu. While this is well established in the setting
of muscle injury, less is known regarding recruitment of MOs/MΦ to muscle in response to
physiological stimuli associated with muscle remodeling processes such as exercise. The
importance of several chemokines for the recruitment of MOs and MΦ to muscle has been
demonstrated in both rodents (32, 35) and in human cell models (10). In the latter, CX3CL1,
CCL22 and CCL2 were described to be important MO/MΦ chemoattractants produced by
human muscle cells (10). In the present study, an increase in CX3CL1 at both the mRNA and
protein level was observed in the exercised human skeletal muscle, demonstrating that the
production of MO/MΦ-recruiting chemokines is increased following a single bout of exercise
in humans. In contrast with CX3CL1, no significant interaction was found between the
exercise and control groups for changes in CCL2 or CCL22. Because of this, it was not
possible to distinguish biopsy versus exercise effects, and changes in CCL2 or CCL22 in the
exercised muscle could not be evaluated.

Although the cellular source underlying the production of chemokines in muscle tissue is
unknown, cultured human satellite cells can produce chemo-attractants (including CX3CL1,
CCL22 and CCL2) that selectively attract MOs and amplify the chemotactic activity of MΦ
(10). This finding indicates a secretory role for the satellite cell and suggests that satellite
cells may be one source of muscle tissue-derived chemokines that contributes to the
recruitment of MOs/MΦ. Based on these findings, we hypothesized CX3CL1 to be localized
to the skeletal muscle fibers and/or satellite cells of human muscle tissue (10). However, in
contrast to our hypothesis, our immunostaining showed a clear localization of CX3CL1 to the
endothelium with only a weak staining visible on the muscle fiber membranes. Thus, the
induced expression of CX3CL1 in the exercised skeletal muscle presumably reflects an
increase in the endothelium. Endothelial expression of CX3CL1 is known to be induced by the
pro-inflammatory factors TNF-α, IFN-gamma and IL1 (4, 18), factors demonstrated to be
released from human myogenic cells (3, 7, 28). In fact, the increased expression of CX3CL1 in
HUVECs stimulated with microdialysate from exercised skeletal muscle supports the concept
that a soluble factor increases in the interstitial milieu of the exercising muscle, leading to
the induction of endothelial CX3CL1 expression. However, to our knowledge, due to
technical challenges (high levels of macro-proteins and globulins), no proteomic analysis of
microdialysate such as mass spectrometry have succeeded as of now, why the specific
exercise induced factors causing the increase in endothelial CX3CL1 was not determined.

CX3CL1 exists in two forms; anchored to the membrane where it functions as an adhesion
molecule, or shed as a soluble chemoattractant; both participate in the described biological
effects of CX3CL1. Thus, in addition to inducing migration, CX3CL1 enhances the adhesion of MOs to endothelium (9, 16). In the regenerative process, the MOs that first arrive within the damaged muscle are reported to have a pro-inflammatory phenotype and express TNF-α (2, 13, 34). Indeed, in the present study, CX3CL1 stimulation of THP-1 monocytes resulted in a strong increase of the factors TNF-α, IL6 and MMP9, all markers of a pro-inflammatory profile, together with an increase in the anti-inflammatory factor IL10. Interestingly, both IL6 and TNF-α also are known to stimulate proliferation of satellite cells (21, 30); thus, this could be one indirect mechanism by which CX3CL1 acts as a mitogen for muscle cells. Altogether, in addition to serving as a chemokine for MOs/MΦ, this indicates that CX3CL1 may modulate the phenotype of MΦ residing in the skeletal muscle tissue.

CX3CL1 has further been suggested to have a beneficial role in muscle regeneration through a direct effect on myogenic cells (31). Therefore, the effect of CX3CL1 on primary human myoblasts and myotubes was investigated. CX3CL1 stimulation of primary human myoblasts and myotubes resulted in a massive induction of IL8, CCL2 and IL6. These all are known pro-angiogenic factors and chemotactic mediators for MOs/MΦ, and may thereby serve to further increase recruitment of MOs/MΦ into the muscle tissue. Interestingly, an increase in IL8 with exercise could also be demonstrated in muscle sections, with staining localized to myofibers and endothelium. In myotubes, CX3CL1 stimulation increased the level of MMP9, which has been proposed to affect collagen turnover in skeletal muscle (11, 15, 25), stimulate angiogenesis and regulate the bioavailability of various growth factors by proteolytic cleavage of extracellular matrix molecules (14, 15, 20). This observed induction of MMP9 expression especially is interesting since this factor recently was shown to increase immediately after a single bout of exercise (26). We could not detect an effect of CX3CL1 on
the proliferation of human primary myoblasts. However, the decrease in MyoD together with the trend towards a decrease in Myostatin suggests that CX3CL1 may influence myoblast growth. In summary, CX3CL1 induced the monocytic and myogenic expression of several factors known to increase in human skeletal muscle with exercise, suggesting a role for CX3CL1 in the adaptive response following an exercise bout.

We are well aware that there are other factors induced with exercise, with a biological function that in part depends on their chemoattractant property (17). The main aim of this study, however, was not to analyze the level of all putative chemokines but to characterize chemokines shown in ex vivo human muscle models to have importance for cross-talk between muscle cells and MOs/MΦ. The current finding is supported by a recent study by Catoire et al in which exercise-induced myokines with chemoattractant properties were identified in a more unbiased strategy by use of microarray analysis (8). Even though their analysis employed a low fold-change cut-off value, their results support exercise induced expression of genes encoding chemoattractants. In fact, both CX3CL1 and CCL2 were major factors in their identified networks, and both factors increased at mRNA levels in skeletal muscle tissue, which was followed by increased plasma protein levels. The latter supports secretion of these factors from the skeletal muscle tissue. In our study, we could not detect any difference in CCL2 expression between exercise and controls when all time points were included in the analysis. However, if only the time points pre and 30 min post were compared, similar to the study by Catoire et al, an increase of CCL2 with exercise relative to controls also was observed in the present study. Thus, the present study indirectly supports an exercise-induced expression of CCL2, but to accurately establish changes in the expression of this factor, future studies should consider a design that does not utilize repetitive biopsy sampling. It is concluded that 1) CX3CL1 increases in human skeletal muscle
with exercise and is mainly localized to the endothelium; 2) exercise-induced factors in the skeletal muscle participate in this regulation; and 3) CX3CL1 increases the expression of pro-inflammatory and chemotactic factors in MOs as well as expression of pro-inflammatory, pro-angiogenic and chemotactic factors in myogenic cells.

Perspectives and significance

The present findings suggest a CX3CL1-dependent bidirectional signaling between endothelium and cells in the muscle tissue with exercise. This is in line with previous data demonstrating a cross talk between endothelial cells, MOs and myogenic cells in skeletal muscle remodeling (10, 12). Our results corroborate earlier studies demonstrating that non-injurious exercise promotes inflammatory processes in the skeletal muscle, and suggest that these are part of the physiological adaptation to increased physical activity. However, it is unclear whether a larger induction of inflammatory processes translates to a greater adaptation to exercise, or whether it could be deleterious to the remodeling process. This question is of importance in understanding the skeletal muscle adaptation in young versus elderly which can have a chronic low grade inflammation, as well as in the context of non-steroidal anti-inflammatory drugs that are known to be used to a high degree by both recreational and professional athletes.

Authors’ contribution

AS: Designed the study, collected and analyzed the material, interpreted the data and drafted the manuscript. KO: Participated in the acquisition of data and helped drafting the manuscript. ER: Designed and conducted the microdialysis experiment. JPD: Performed the confocal imaging. GS: Helped with confocal imaging and drafting the manuscript. TG:
Conceived the study, contributed to the design and coordination of the study and drafted the manuscript.

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Disclosures
No conflicts of interest are declared by the authors.

References


Figure legends

Figure 1. (A, C, D) Fold-change of CX3CL1, CCL2 and CCL22 mRNA and (B) CX3CL1 protein levels in human vastus lateralis muscle up to 24 h after 1h of either cycling (Exercise) or rest (Control). Values for CX3CL1, CCL2 and CCL22 mRNA are mean (SD) for 12 exercise subjects and 5 control subjects. Values for CX3CL1 protein are mean (SD) for 6 exercise subjects and 3 control subjects. C x T, a significant interaction between condition (C) and time (T), P < 0.05. *** = P < 0.001 vs Pre.

Figure 2. (A) Immunofluorescent staining of muscle section from human vastus lateralis, displaying CX3CL1 (green) and caveolin-3 for muscle fiber membranes (red), with DAPI as nuclear stain (blue). Size bar = 50 µm. (B, C, D) Confocal micrographs after image analysis with the Imaris software display CX3CL1 (green) together with the endothelial marker CD31 (red) and DAPI (blue). Size bars = 3 µm. (E, F) Muscle sections stained for CX3CL1 (green) and Caveolin-3 (red). DAPI (blue) used as nuclear stain. Size bars = 10 µm. (E) before exercise and (F) 2h post exercise. (G) Muscle section 2h post exercise stained for CX3CL1 (green) and the satellite cell marker CD56 (red), with DAPI as nuclear stain (blue). (H) CX3CL1 mRNA levels (mean (SD), n = 3) in HUVECs stimulated with microdialysate obtained at rest and after exercise, * P < 0.05.

Figure 3. (A) Fold-change of mRNA expression in THP-1 cells stimulated with 0 (Ctrl), 10 or 100 ng/mL CX3CL1 (n=4), * = p < 0.05, 100 vs 10 ng/mL and Ctrl. For MMP9 and TGF-beta, the ANOVA located a difference between treatments, although the post-hoc test could not isolate any difference. (B, C) Fold-change of mRNA expression in primary human myoblasts and myotubes stimulated with 0 (Ctrl), 10 or 100 ng/mL CX3CL1 (n=4). * = p < 0.05, 100 ng/mL vs 10 ng/mL and Ctrl. # = p < 0.05, 100 ng/mL vs Ctrl. ** = p < 0.01, 100 ng/mL vs 10 ng/mL and Ctrl. *** p < 0.001 100 ng/mL vs 10 ng/mL and Ctrl. A logarithmic scale was used for the Y axis of all graphs, and the values are presented as mean (SD). (D-G) Muscle sections stained for IL8 (green) together with Collagen-4 (red) for the basal lamina. DAPI (blue) was used as nuclear stain. Size bars = 10 µm. (D,E) before exercise, (F,G) 6 h post exercise.
Figure 4. BrdU proliferation assay of myoblasts stimulated with 0 (Ctrl), 10 or 100 ng/mL CX3CL1 (mean (SD), n=4).

Table legend

Table 1. All subjects, exercise and control, were provided the same diet during the two days of the experiment. Water was given ad libitum.
<table>
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<th>Time</th>
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| Evening meal (x 2 days) | Drink yoghurt 350 ml – 245 kcal, 1.75 g fat, 45 g carbohydrates, 10.5 g protein, 0.3 g salt | 245 kcal, 1.75 g fat, 45 g carbohydrates, 10.5 g protein, 0.3 g salt. 
1 banana | The subjects were given the food the day before the experiment, and instructed to have the evening meal at 8 pm after their dinner. |
|              | Breakfast (x 2 days)           | 200 g fruit yoghurt - 160 kcal, 4 g fat, 26 g carbohydrates, 6.6 g protein, 0.2 g salt. 
Kellogg’ s corn flakes one serving – 108 kcal, 2.2 g fat, 22 g carbohydrates, 0.8 g protein, 0.1 g salt. 
Orange juice 200 g – 90 kcal, < 0.5 g fat, 18 g carbohydrates, 2 g protein, < 0.1 g salt. 
½ cheese sandwich 38 g – 127 kcal, 5.5 g fat, 14 g carbohydrates, 5 g protein, 0.7 g salt. | The subjects were instructed to eat the breakfast at home at least 1h before arriving to the laboratory. |
| Lunch (x 1 day) | Pasta salad with chicken 380 g – kcal not stated. 39% of contents is pasta with pesto, 18% chicken filet, 16% seasonal salad, 8% cherry tomato, 8% beans in rape seed oil, 5% white cabbage | Pasta salad with chicken 380 g – kcal not stated. 39% of contents is pasta with pesto, 18% chicken filet, 16% seasonal salad, 8% cherry tomato, 8% beans in rape seed oil, 5% white cabbage | The subjects had lunch after the 2h post biopsy. |
| Fruit (x 1 day) | 1 apple  
1 banana | 1 apple  
1 banana | The subjects had the fruits after the 30 min post biopsy. |