



# Characterising the Cytokine and Circulating Immune Cell Response After a Single Bout of Eccentric Stepping Exercise in Healthy Untrained Males

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Received: 28 September 2022 / Accepted: 15 March 2023  
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## Abstract

**Purpose** The mechanisms that underpin exercise-induced muscle damage and recovery are believed to be mediated, in part, by immune cells recruited to the site of injury. The aim of this study was to characterise the effects of muscle damage from bench-stepping on circulating cytokine and immune cell populations post-exercise and during recovery.

**Methods** Ten untrained, healthy male volunteers completed 30 min of bench-stepping exercise to induce muscle damage to the eccentrically exercised leg. Muscle function, muscle pain and soreness were measured before, immediately after and 24, 48 and 72 h after exercise. Plasma creatine kinase, cartilage oligomeric matrix protein, cytokines and circulating immune cell phenotyping were also measured at these timepoints.

**Results** Significant decreases occurred in eccentric, isometric and concentric ( $P=0.018$ ,  $0.047$  and  $0.003$ , respectively) muscle function in eccentrically, but not concentrically, exercised quadriceps post-exercise. Plasma monocyte chemoattractant protein (MCP)-1 concentrations significantly increased immediately after exercise ( $69.0 \pm 5.8$  to  $89.5 \pm 10.0$  pg/mL), then declined to below pre-exercise concentrations ( $58.8 \pm 6.3$  pg/mL) 72 h after exercise. These changes corresponded with the significant decrease of circulating  $CD45^+ CD16^- CD14^+$  monocytes ( $5.8\% \pm 1.5\%$  to  $1.9\% \pm 0.5\%$ ; Pre-exercise vs. 48 h) and increase of  $CD45^+ CD3^+ CD56^-$  T-cells ( $60.5\% \pm 2.2\%$  to  $66.1\% \pm 2.1\%$ ; Pre-exercise vs. 72 h) during recovery.

**Conclusion** Bench-stepping induced muscle damage to the quadriceps, which mediated systemic changes in MCP-1, monocytes and T-cells immediately post-exercise and during recovery. Further research is needed to clarify how modulations in immune subpopulations facilitate muscle recovery and adaptation following muscle damage.

**Keywords** Muscle damage · Concentric · Eccentric · Immune cells · Cytokines

## Abbreviations

ANOVA	Analysis of variance	IFN	Interferon
CK	Creatine kinase	IL	Interleukin
CL	Concentrically exercised leg	MCP-1	Monocyte chemoattractant protein-1
COMP	Cartilage oligomeric matrix protein	MVC	Maximal voluntary contraction
CXCL	C-X-C motif chemokine ligand	PBMC	Peripheral blood mononuclear cells
DOMS	Delayed onset of muscle soreness	SF-MPQ	Short-form McGill pain questionnaire
EL	Eccentrically exercised leg	TNF	Tumour necrosis factor
		VAS	Visual analogue scale

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## Introduction

Unaccustomed and prolonged eccentric exercise can result in muscle damage, characterised by reduced muscle strength and mobility [12], delayed onset of muscle soreness (DOMS) [14] and elevated plasma concentrations of muscle-related proteins [23] persisting for several days. The reduction in muscle function from muscle damage has been largely attributed to the

disruption of the highly arranged sarcomere structure of skeletal muscle [8]. Additionally, muscle damage is proposed to interrupt the excitation–contraction coupling process by reducing sarcoplasmic reticulum  $\text{Ca}^{2+}$  released from the cytosol, lessening the binding of thick and thin filaments during muscle contraction, resulting in the decline in muscle function [37].

Exercise-induced muscle damage also elicits an immune response. Leukocytes accumulate in skeletal muscle following exercise to facilitate muscle repair by removing damaged muscle fibres, and to support muscle regeneration [28]. Research indicates that neutrophils and monocytes are the predominant infiltrating immune cells in skeletal muscle following exercise-induced damage [33]. Infiltrating neutrophils are believed to clear cellular debris from muscle damage through phagocytosis and proteolytic degradation. However, neutrophils can also release high concentrations of reactive oxygen species, further exacerbating muscle damage. Early infiltrating monocytes differentiate into inflammatory macrophages (M1) to clear damaged myofibres. At the early stages of myogenesis, infiltrating monocytes differentiate into more anti-inflammatory macrophages (M2), suggesting the role of M2 macrophages in facilitating skeletal muscle remodelling and repair [31, 33].

Several eccentric exercise protocols have been developed to induce skeletal muscle damage to the knee extensors. One model is bench-stepping [9, 11, 23, 36], which requires participants to step up onto a bench at a constant rate for a defined amount of time. During the exercise, the quadriceps of the leading leg concentrically contracts during the step up, while the quadriceps of the supporting leg eccentrically contracts during the step down. The bench-stepping model has been validated in studies showing it induces muscle damage to the eccentrically exercised quadriceps [23, 36]. To our knowledge, no study has characterised the immune effects of eccentric muscle damage from bench-stepping in untrained individuals immediately after exercise and during recovery. Given the role of immune cells and their mediators in regulating tissue damage and repair, understanding the immunological changes following eccentric exercise-induced muscle damage may provide insight into key immune-related factors that may facilitate muscle recovery from damage.

The aim of this study was to characterise the effects of our muscle damage protocol on plasma cytokine concentrations and circulating immune cell populations immediately after eccentric exercise during recovery. We also determined the effects of our bench-stepping protocol on indices of muscle damage in untrained male participants.

## Materials and Methods

### Study Participants

Ten healthy male volunteers between 21 and 43 years old volunteered to take part in this study. All participants were healthy, were not undergoing any form of exercise training, and were selected for similar fitness characteristics based on the Baecke habitual activity questionnaire [1]. During recruitment, potential participants completed a health screening questionnaire to exclude those who had blood-borne diseases (e.g. hepatitis), high or low blood pressure, chronic conditions (e.g. diabetes, asthma, lupus, arthritis), or bacterial illnesses, or were taking medication that affected blood-clotting properties. Individuals were also excluded from the study if their respective Baecke habitual activity and sports score were above 3 and 3.5, were unable to complete the exercise requirements of the study, had an injury that could be exacerbated by the exercise, or had chronic breathing or heart problems. This study was reviewed and approved by New Zealand's Southern Health and Disability Ethics Committee (20/STH/75, July 2020) and was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12620000565943).

### Study Design

#### Familiarisation Session

Participants who expressed interest and met the inclusion criteria of the study attended a familiarisation session at least one week prior to their trial day. In this session, they were instructed on how to perform the muscle function assessments on an isokinetic dynamometer (Biodex Medical Systems Inc., NY, USA) that they would be required to undergo during their trial and recovery days. Participants were also familiarised to the bench-stepping exercise that they would be required to complete on their trial day.

#### Trial Day

This was a single arm, repeated measures study involving ten participants. Five participants were randomly assigned to eccentrically exercise their right leg first and five participants to eccentrically exercise their left leg first. Participants were instructed to avoid strenuous exercise 48 h prior to their scheduled trial day.

On the morning of their trial day, when they first arrived at the facility participants completed the short-form McGill pain questionnaire (SF-MPQ) [20] to rate the present pain of the leg assigned for eccentric exercise. After their first blood

sample collection, participants completed a 5-min warm-up on a Cyclus2 cycle ergometer (RBM elektronik-automation GmbH, Leipzig, Germany) at a work rate of 100 W, followed by measurement of pre-exercise muscle function of both legs. These tests involved participants performing five maximal eccentric, concentric and isometric contractions of their quadriceps, and five maximal concentric contractions of their hamstrings while seated on the isokinetic dynamometer. During these assessments, participants rated the perceived soreness of their exercising leg during the first isometric and eccentric contraction on a visual analogue scale (VAS). Following muscle function assessments, participants completed three 10-min bouts of the bench-stepping exercise to induce quadriceps muscle damage to the eccentrically exercised leg. Immediately after the exercise, a second sample was collected and measures of muscle function and pain/soreness were repeated.

### Recovery Measurements

Participants returned to the clinical facility 24, 48 and 72 h post-exercise. Upon arrival, participants immediately completed the SF-MPQ, followed by donating a venous blood sample. After completing a 5-min warmup on a cycle ergometer, muscle function during five maximal eccentric, concentric and isometric contractions was measured with the Biodex dynamometer on both legs. Participants were instructed to avoid strenuous exercises during the 72-h recovery period. Additionally, participants were requested to maintain their normal diet and avoid consuming dietary supplements and anti-inflammatory and analgesic drugs 24 h before their trial day through to the conclusion of the 72 h exercise recovery timepoint.

### Bench-Stepping Exercise

A modified version of Newham and colleagues' [23] bench-stepping protocol was used; this protocol measurably induces muscle damage to the quadriceps of the eccentrically exercised leg. Briefly, participants completed three 10-min bouts of bench stepping at a cadence of 15 steps per minute, with each bout separated by 2 min of passive rest. The step height was adjusted to 110% of participants' lower leg length (measured from the femoral epicondyle to the floor). During the stepping exercise, the quadriceps of the leading leg concentrically contracted (CL) while stepping up, while stepping down with the same leg ensured that the quadriceps of the contralateral leg contracted eccentrically (EL). Participants were instructed to maintain a consistent speed as they raised and lowered their body to ensure equivalent time under tension during each concentric and eccentric movement. All participants performed the stepping exercise while wearing a vest (Speed Power Stability

Systems, Christchurch, New Zealand) with an additional load equivalent to 15% of their bodyweight.

### Muscle Function Testing

Participants were seated on the Biodex isokinetic dynamometer at a position where the femoral epicondyle of the tested leg was aligned with the machine's axis of rotation. The ankle strap of the machine was positioned 5 cm proximal to the test leg's medial malleolus and the ankle and thigh of the test leg were strapped firmly to the machine to isolate the movement of the quadriceps. The upper body was also secured to the dynamometer with straps across the chest and waist. The range of motion of the test leg was set from full knee flexion to 60° for concentric and eccentric contractions, and fixed at 75° for isometric contractions using the dynamometer's goniometer. Participants were required to perform five maximal eccentric, isometric and concentric contractions with each contraction type separated by 1 min of passive rest. Participants were required to maintain 5 s of maximal effort during each isometric contraction. The torque during concentric contractions was measured at an angular velocity of 30°/s. The absolute peak torque from five maximal voluntary contraction (MVC) for each movement was recorded.

### Subjective Pain Assessments

The SF-MPQ assessed the perceived sensory, affective, and overall pain of the resting eccentrically exercised leg on the exercise trial (pre- and post-bench-stepping) and recovery days. The questionnaire comprised of 15 adjectives, four for affective pain and 11 for sensory pain. Each descriptor was rated on an intensity scale ranging from 0 to 3 (0 = none; 3 = severe) allowing for a maximum total pain score of 45, 12 for affective pain, and 33 for sensory pain. Average pain was also assessed using a 10-cm VAS scale anchored by descriptors "no pain" and "worst possible pain" at the extreme ends of the scale.

Perceived muscle soreness was measured during muscle function assessments before and after the stepping exercise and on the three recovery (24, 48 and 72 h post-exercise) days. Participants rated the severity of soreness of the exercising leg during the first maximal eccentric and isometric contractions on a 5-point Likert scale ranging from 1 (no soreness) to 5 (worst possible pain) [13, 16].

### Blood Sampling

Venous blood samples were collected into heparin vacutainer tubes and centrifuged at 475 g for 10 min at room temperature. The plasma layer was collected for measuring creatine kinase, cartilage oligomeric matrix protein and

cytokine concentrations, aliquoted and stored at  $-80^{\circ}\text{C}$  until analysis.

For isolation of peripheral blood mononuclear cells (PBMCs), the buffy coat interface containing mononuclear cells following centrifugation was collected and the cells were washed with Dulbecco's phosphate-buffered saline (DPBS). Following incubation in erythrocyte lysis buffer (Sigma; Cat. No. R7757), cells were washed and suspended in DPBS for subsequent cell staining.

## Biochemical and Immune Analyses

### Creatine Kinase

Plasma creatine kinase (CK) was measured at a commercial blood testing laboratory (MedLab Central, Palmerston North, New Zealand). Analysis of CK was quantified by measuring the rate of NADH (nicotinamide adenine dinucleotide) formation from an enzymatic reaction using a spectrophotometer.

### Cartilage Oligomeric Matrix Protein

Plasma cartilage oligomeric matrix protein (COMP) was measured using a commercial sandwich enzyme-linked immunosorbent assay kit (Cat. No. RD194080200, BioVendor R&D, Karasek, Czech Republic) according to the manufacturer's instructions. Plasma samples were thawed then diluted 1:50 the kit's dilution buffer prior to analysis. Each sample was analysed in triplicate and plasma COMP concentrations were calculated against the COMP standards provided in the kit. The limit of detection (LOD) of the assay kit is 0.4 ng/mL.

### Plasma Cytokine

Cytokine concentrations in plasma samples were analysed by bead array using a Biolegend Legendplex™ 13-plex Human Essential Immune Response panel (Cat. No. 740930; San Diego, CA, USA) according to the manufacturer's instructions. Briefly, an aliquot of analyte (plasma sample or standard solution) was added into a 96-well V-bottom plate containing assay buffer and capture beads. After 2 h incubation and washing steps, biotinylated detection antibody was dispensed into all wells and the plate incubated in for 1 h followed by the addition of Streptavidin-phycoerythrin (SA-PE) to all wells for 30 min. After centrifugation, the beads were washed, collected then the simultaneous quantitation of cytokines (interleukin (IL)-4, IL-2, C-X-C motif chemokine ligand (CXCL)-10, IL-1 $\beta$ , tumour necrosis factor (TNF)- $\alpha$ , MCP-1, IL-17A, IL-6, IL-10, interferon (IFN)- $\gamma$ , IL-12p70 and IL-8) in plasma samples was measured using a BD FACSverse™

flow cytometer (BD Biosciences, San Jose, CA, USA). The assay kit's LOD for each of the cytokines in plasma are 0.97 pg/mL (IL-4), 1.81 pg/mL (IL-2), 1.28 pg/mL (CXCL10), 0.65 pg/mL (IL-1 $\beta$ ), 0.88 pg/mL (TNF- $\alpha$ ), 1.45 pg/mL (MCP-1), 2.02 pg/mL (IL-17A), 0.97 pg/mL (IL-6), 0.77 pg/mL (IL-10), 0.76 pg/mL (IFN- $\gamma$ ), 0.77 pg/mL (IL-12p70) and 1.90 pg/mL (IL-8).

## Immune Cell Phenotyping

Isolated PBMCs were stained with Biolegend "zombie NIR" fixable viability dye (Cat. No. 423106; San Diego, CA, USA) according to the manufacturer's instructions for 15 min at  $21^{\circ}\text{C}$ . Cells were then washed in DPBS before being stained with fluorescently labelled cell surface markers at  $4^{\circ}\text{C}$  for 15 min. Biolegend antibodies used were: PE-Fire700 CD45 (Cat. No. 304077); BV605 CD3e (Cat. No. 317444); BV510 CD4 (Cat. No. 3301042); BV650 CD8a (Cat. No. 301042); BV711 CD25 (Cat. No. 302636); APC-Fire750 CD127 (Cat. No. 351350); PerCP CD38 (Cat. No. 356622); BV421 CD68 (Cat. No. 333828); BV570 CD56 (Cat. No. 362540); PE-Cy7 CD16 (Cat. No. 302016); AF488 CD14 (Cat. No. 367130); PE-Cy5 CD15 (Cat. No. 323014); AF647 CD11b (Cat. No. 393110); PE CD11c (Cat. No. 301606); BV750 HLA/DR (Cat. No. 307672). PBMCs were fixed in 4% formalin for 10 min before being resuspended in fluorescence-activated single cell sorting (FACS) buffer and analysed on a Cytex Aurora Spectral 3 laser flow cytometer (Cytex Biosciences, Fremont, CA, USA) with live spectral unmixing. Flow Cytometry Standard (FCS) files were analysed using FlowJo software (BD Biosciences, San Jose, CA, USA) to identify cell phenotype subpopulations.

## Statistical Analyses

Statistical analysis was performed using Genstat V20 (VSNi Ltd, Hemel Hempstead, UK). All data from this study were analysed with analysis of variance (ANOVA). For muscle function measures (isometric, concentric and eccentric contractions) and VAS muscle soreness scores (during eccentric and isometric contractions), the fixed effects included participant, time, exercise mode (EL vs. CL), plus participant  $\times$  exercise mode and time  $\times$  exercise mode interactions. For SF-MPQ scores, creatine kinase, cartilage oligomeric protein, cytokine and immune cell phenotyping measures, the fixed effects were participants and time. Residuals were inspected to ensure the assumptions of ANOVA were valid. Where significant effects were found, least differences were used post hoc to determine which means differed significantly ( $P < 0.05$ ).

## Results

### Physical Characteristics

The variations in the age, height and weight of volunteers recruited to this study are presented in Table 1. Participants recruited to this study were physically untrained and had very similar habitual activity as indicated by the low variation in Work and Sport index scores using the Baecke questionnaire [1].

### Muscle Function

Muscle function assessment showed the significant time effects of the bench-stepping exercise on peak quadriceps eccentric and isometric torque and hamstring concentric torque ( $P=0.018$ ,  $0.047$  and  $0.003$ , respectively). For all three parameters, there was an exercise mode effect (eccentric,  $P=0.006$ ; isometric,  $P=0.017$  and hamstring concentric,  $P=0.003$ ) with changes in muscle function following the bench-stepping exercise mainly observed for EL. Bench-stepping exercise had no significant effect on peak quadriceps concentric torque post-exercise ( $P=0.25$ ) and no significant difference was observed for this parameter between CL and EL ( $P=0.739$ ).

For EL, peak eccentric torque significantly declined ( $P<0.05$ ) immediately after exercise (0 h), and remained significantly lower than pre-exercise peak torque after 72 h post-exercise (Fig. 1A). In contrast, peak eccentric torque for CL remained at pre-exercise levels throughout the 72-h post-exercise period. A significant time  $\times$  exercise mode interaction ( $P=0.013$ ) was also observed so that peak eccentric

torque for EL was significantly lower ( $P<0.05$ ) than CL at all post-exercise timepoints.

An immediate, significant decline ( $P<0.05$ ) in peak quadriceps isometric torque was observed for EL, which recovered to pre-exercise performance by 72 h post-exercise. No significant change from pre-exercise peak isometric torque was observed for CL during the 72-h post-exercise period. Peak isometric torque for EL was significantly lower ( $P<0.05$ ) than for CL at 24 and 48 h post-exercise.

Peak hamstring concentric torque significantly declined ( $P=0.05$ ) immediately post-exercise for EL and remained significantly lower than pre-exercise peak torque at 72 h post-exercise (Fig. 1D). Peak hamstring concentric torque for CL also declined immediately post-exercise ( $P<0.05$ ), but recovered to the equivalent of pre-exercise performance by 24 h post-exercise. In addition, peak hamstring concentric torque for EL was significantly lower ( $P<0.05$ ) at 24 and 72 h post-exercise than that for CL.

### Muscle Soreness

The bench-stepping exercise significantly increased total, sensory and affective pain perceived post-exercise in the eccentrically exercised leg ( $P=0.018$ ,  $0.009$  and  $0.001$ , respectively) compared with pre-exercise scores (Table 2). The total pain scores of the resting eccentrically exercised leg significantly increased immediately after exercise (0 h) and peaked at 48 h post-exercise. Total pain score began to decrease between 48 to 72 h post-exercise, but remained significantly higher than pre-exercise scores at 72 h ( $P<0.05$ ).

When the SF-MPQ descriptors were stratified into the sensory and affective pain subscales, sensory pain scores significantly ( $P<0.05$ ) increased immediately post-exercise and remained elevated at 72 h post-exercise. The affective pain score for EL increased ( $P<0.05$ ) immediately post exercise, before decreasing during recovery so that the mean affective pain score at 72 h post-exercise was similar to pre-exercise scores. While data pertaining to perceived exertion from performing the bench exercise were not collected, a  $1.8 \pm 0.73$  for the *Tiring/Exhausting* scale in the SF-MPQ indicate that participants found the bench-stepping exercise moderately tiring.

Significant time ( $P=0.05$ ) and exercise mode effects ( $P=0.006$ ) were observed for muscle soreness (measured during maximal eccentric contraction) (Fig. 2A). Muscle soreness scores significantly increased ( $P<0.05$ ) immediately post-exercise for EL and did not decrease to match pre-exercise scores until 72 h post-exercise. In comparison, CL muscle soreness did not significantly differ ( $P>0.05$ ) from pre-exercise scores at all post-exercise timepoints. There was also a significant time  $\times$  exercise mode interaction ( $P=0.013$ ) for muscle soreness, as

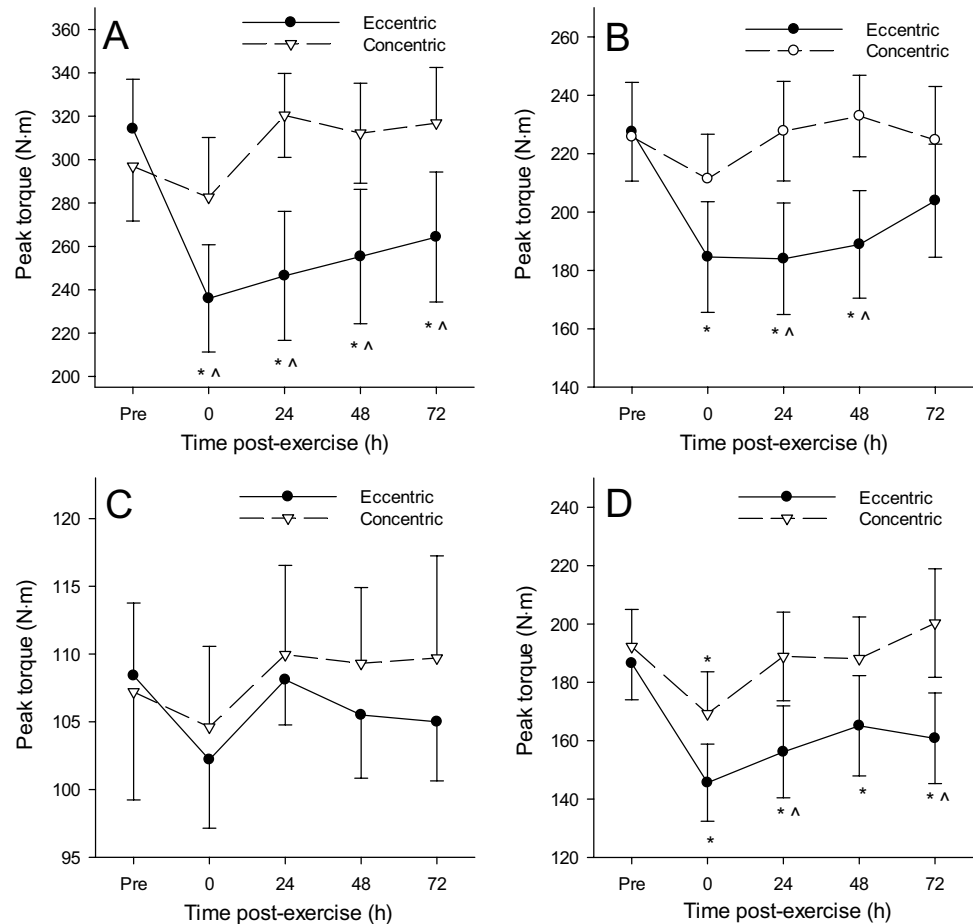
**Table 1** Physical characteristics and habitual activity scores of recruited participants

Variable	Mean $\pm$ SD
Age (years)	31.6 $\pm$ 6.6
Height (cm)	177 $\pm$ 7
Weight (kg)	79.4 $\pm$ 8.3
BMI	25.4 $\pm$ 2.1
Habitual activity	
Work Index	2.4 $\pm$ 0.7
Sport Index	2.8 $\pm$ 0.6
Vest weight (kg)	11.9 $\pm$ 1.2
Bench height (cm)	52.4 $\pm$ 3.6

Physical characteristics and habitual activity scores of recruited participants were determined using anthropometric measures and the Baecke Physical Activity Questionnaire. Vest weight worn by participants was equivalent to 15% of their bodyweight and the bench height was set at 110% of participants' lower leg length. Data are mean  $\pm$  SD from  $n=10$  individuals



**Fig. 1** Maximal voluntary contraction evaluation of the quadriceps after bench-stepping exercise. Peak eccentric (A), isometric (B), concentric (C) torque and hamstring concentric torque (D) were assessed before exercise (pre) and 0, 24, 48 and 72 h after bench-stepping in eccentrically and concentrically exercised muscles. Data are mean  $\pm$  SEM. \* indicates significant difference from pre-exercise (Pre) values ( $P < 0.05$ ). ^ indicates significant difference from concentrically exercised leg ( $P < 0.05$ )



**Table 2** Subjective pain scores in eccentrically exercised quadriceps following the bench-stepping exercise

Pain	Time post-exercise (h)				
	Pre	0	24	48	72
Total pain	1.10 $\pm$ 0.43 <sup>a</sup>	8.60 $\pm$ 1.42 <sup>bc</sup>	9.30 $\pm$ 2.29 <sup>c</sup>	10.0 $\pm$ 2.56 <sup>c</sup>	5.70 $\pm$ 2.01 <sup>b</sup>
Sensory pain	0.80 $\pm$ 0.34 <sup>a</sup>	5.90 $\pm$ 1.09 <sup>b</sup>	7.50 $\pm$ 2.05 <sup>b</sup>	8.30 $\pm$ 2.16 <sup>b</sup>	4.80 $\pm$ 1.76 <sup>b</sup>
Affective pain	0.30 $\pm$ 0.14 <sup>a</sup>	2.70 $\pm$ 0.47 <sup>c</sup>	1.80 $\pm$ 0.37 <sup>bc</sup>	1.70 $\pm$ 0.62 <sup>bc</sup>	0.90 $\pm$ 0.39 <sup>ab</sup>
VAS pain (cm)	0.51 $\pm$ 0.27 <sup>a</sup>	2.93 $\pm$ 0.71 <sup>b</sup>	2.68 $\pm$ 0.64 <sup>b</sup>	3.49 $\pm$ 0.87 <sup>b</sup>	2.18 $\pm$ 0.72 <sup>b</sup>

Sensory, affective and overall pain scores of eccentrically exercised quadriceps were assessed using the short form McGill pain questionnaire before (pre) and 0, 24, 48 and 72 h after the bench-stepping exercise. All values are mean  $\pm$  SEM. Means within each parameter with unlike letters are significantly different ( $P < 0.05$ ). Total pain scores ranging from 0 to 45 (0 = none; 45 = severe), sensory pain scores ranging from 0 to 33 (0 = none; 33 = severe), affective pain scores ranging from 0 to 12 (0 = none; 12 = severe) and Visual analogue scale (VAS) pain ranging from 0 to 10 cm (0 cm = none; 10 cm = severe)

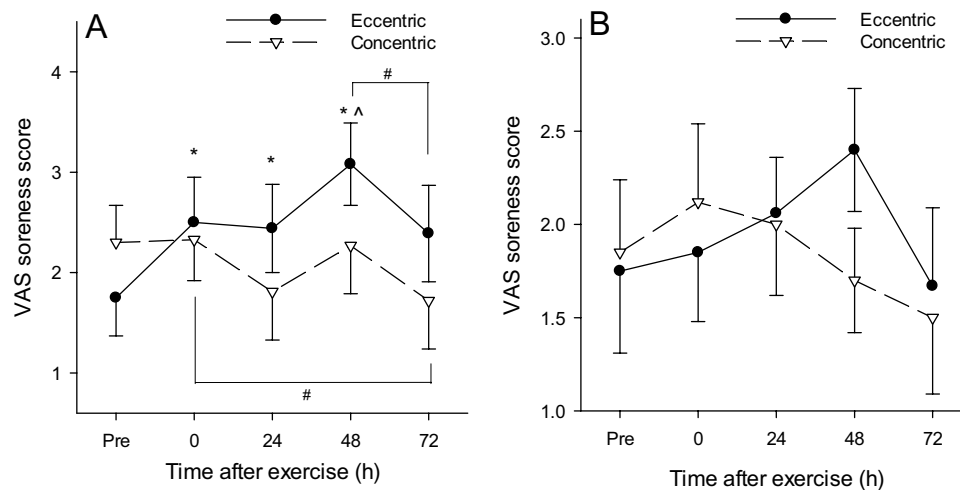
indicated by significantly higher ( $P < 0.05$ ) mean scores for EL than for CL at 48 h post-exercise.

In comparison to soreness measured during maximal eccentric muscle contractions, no significant time ( $P = 0.46$ ) or exercise mode ( $P = 0.12$ ) effects were found for when soreness was measured during maximal isometric quadriceps contraction (Fig. 2B). Mean pain scores for EL and CL did not significantly differ ( $P > 0.05$ ) from

pre-exercise scores during maximal isometric contraction at any post-exercise timepoints.

### Plasma Cytokine and Tissue Damage Biomarkers

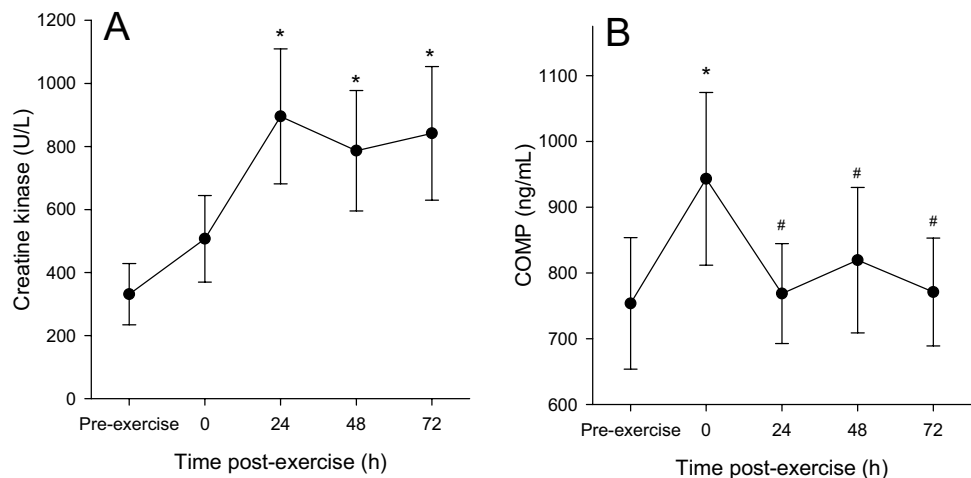
The bench-stepping exercise immediately increased post-exercise CK concentrations ( $P = 0.041$ ), which remained significantly higher 24, 48 and 72 h post-exercise relative



**Fig. 2** Muscle soreness scores during eccentric (A) and isometric (B) maximal voluntary contraction assessments of eccentrically and concentrically exercised quadriceps muscles before (pre) and 0, 24, 48 and 72 h after the bench-stepping exercise. Data are means  $\pm$  SEM. \* indicates significant difference from pre-exercise (Pre) scores ( $P < 0.05$ ).

# indicates significant difference between measurement timepoints ( $P < 0.05$ ). ^ indicates significant difference from concentrically exercised leg ( $P < 0.05$ ). VAS = visual analogue score, ranging from 0 to 5 (0 = no soreness; 5 = worst possible pain)

**Fig. 3** Creatine kinase (A) and cartilage oligomeric matrix protein (COMP) (B) concentrations in plasma pre-exercise and 0, 24, 48 and 72 h after the bench-stepping exercise. Data are means  $\pm$  SEM. \* indicates significant difference from pre-exercise concentration ( $P < 0.05$ ). # indicates significant difference from 0-h concentration ( $P < 0.05$ )



to pre-exercise concentrations (Fig. 3A). Circulating COMP significantly ( $P < 0.05$ ) increased immediately post-exercise (0 h), before returning to pre-exercise concentrations by 24 h post-exercise (Fig. 3B).

Plasma MCP-1 significantly increased ( $P < 0.05$ ) immediately post-exercise (0 h), then declined to pre-exercise concentrations by 24 h post-exercise (Table 3). The concentration of MCP-1 continued to decrease so that by 72 h, it was significantly lower than pre-exercise values.

Although not statistically significant ( $P = 0.077$ ), plasma IFN- $\gamma$  concentrations showed a trend ( $P = 0.077$ ) to increase post-exercise, peaked 48 h post-exercise, then returned to pre-exercise concentrations by 72 h post-exercise. No significant changes in the plasma concentrations

of the other 11 cytokines were detected following the bench-stepping exercise.

### Immune Cell Phenotyping

Significant changes in circulating “classical” CD45<sup>+</sup> CD16<sup>-</sup> CD14<sup>+</sup> monocytes and total CD45<sup>+</sup> CD3<sup>+</sup> CD56<sup>-</sup> T-cell population ( $P = 0.030$  and  $0.038$ , respectively) were observed up to 72 h following the bench-stepping exercise (Fig. 4). CD45<sup>+</sup> CD16<sup>-</sup> CD14<sup>+</sup> monocyte cell counts declined post-exercise so that CD45<sup>+</sup> CD16<sup>-</sup> CD14<sup>+</sup> monocyte counts at 48 and 72 h post-exercise were significantly lower ( $P < 0.05$ ) than pre-exercise and 0 h post-exercise numbers. Conversely,

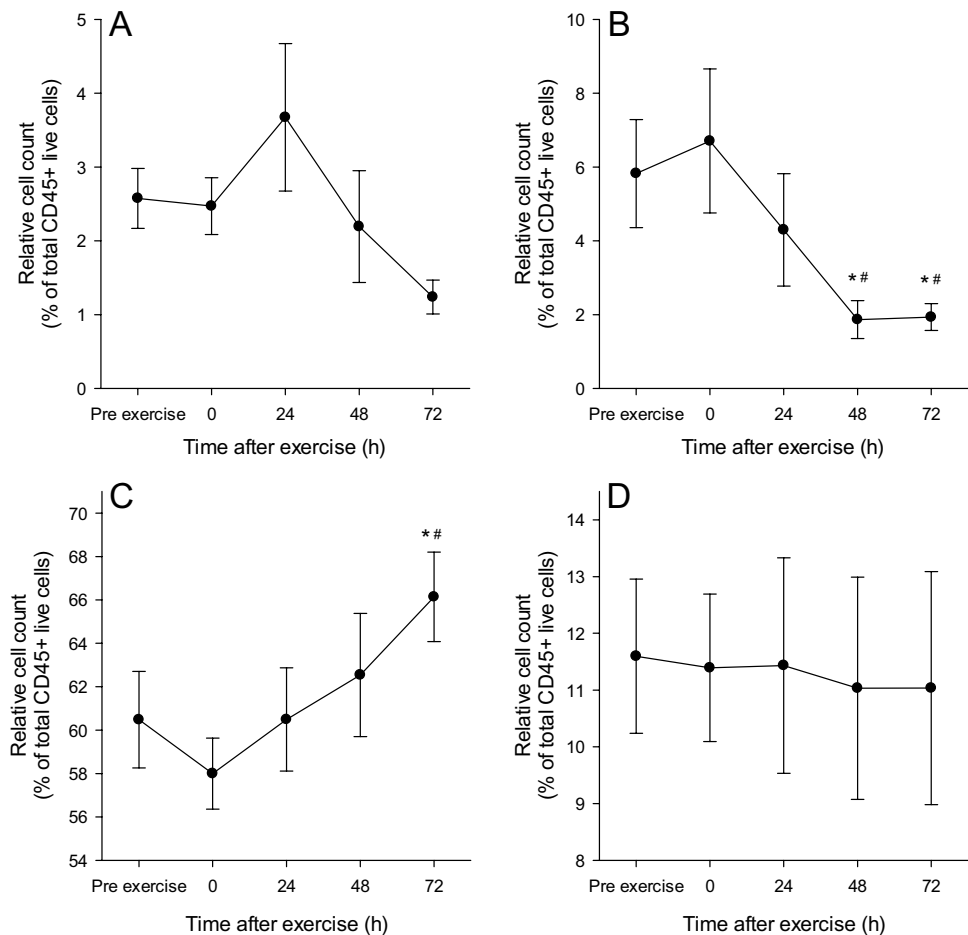
**Table 3** Plasma cytokine concentrations (pg/mL) following the bench-stepping exercise

Cytokine	Time post-exercise (h)					Time effect ( <i>P</i> value)
	Pre-exercise	0	24	48	72	
IL-4	6.12 ± 1.95	5.72 ± 1.95	6.14 ± 2.15	8.24 ± 1.92	5.56 ± 2.02	0.117
IL-2	3.60 ± 0.94	3.46 ± 0.86	3.47 ± 0.92	4.08 ± 0.89	3.28 ± 0.85	0.434
CXCL10	52.7 ± 11.5	51.4 ± 14.4	72.5 ± 13.6	49.7 ± 5.3	45.1 ± 6.1	0.101
IL-1 $\beta$	5.21 ± 1.39	5.59 ± 1.50	5.58 ± 1.56	4.88 ± 1.28	5.05 ± 1.30	0.553
TNF- $\alpha$	3.45 ± 1.37	3.35 ± 1.37	3.65 ± 1.49	3.66 ± 1.26	3.82 ± 1.58	0.419
MCP-1	69.0 ± 5.8 <sup>b</sup>	89.5 ± 10.0 <sup>c</sup>	73.7 ± 8.8 <sup>b</sup>	60.8 ± 5.7 <sup>ab</sup>	58.8 ± 6.3 <sup>a</sup>	0.001
IL-17A	1.56 ± 0.26	1.32 ± 0.22	1.56 ± 0.26	2.39 ± 0.34	1.37 ± 0.15	0.099
IL-6	2.46 ± 0.55	2.70 ± 0.54	2.91 ± 0.76	4.62 ± 0.85	2.11 ± 0.50	0.124
IL-10	1.96 ± 0.37	1.69 ± 0.31	1.91 ± 0.33	3.11 ± 0.50	1.52 ± 0.14	0.100
IFN- $\gamma$	11.1 ± 3.3	8.3 ± 2.6	10.8 ± 3.1	22.0 ± 4.7	7.3 ± 2.1	0.077
IL-12p70	1.23 ± 0.22	1.04 ± 0.19	1.25 ± 0.30	1.93 ± 0.33	0.87 ± 0.09	0.137
IL-8	2.35 ± 0.43	2.29 ± 0.52	2.41 ± 0.56	2.95 ± 0.38	1.87 ± 0.25	0.407

Cytokine concentrations in plasma samples pre-exercise and 0, 24, 48 and 72 h after the bench-stepping exercise. Data are means  $\pm$  SEM. ANOVA of data for each cytokine was conducted to determine significant time effects. Means for cytokines with unlike letters are significantly different ( $P < 0.05$ )

*IL* interleukin, *CXCL* C-X-C motif chemokine ligand, *MCP-1* monocyte chemoattractant protein-1, *TNF* tumour necrosis factor, *IFN* interferon

**Fig. 4** Relative counts of circulating CD45<sup>+</sup>CD14<sup>-</sup>CD16<sup>+</sup> neutrophils (A), CD45<sup>+</sup>CD16<sup>-</sup>CD14<sup>+</sup> monocytes (B), CD45<sup>+</sup>CD3<sup>+</sup>CD56<sup>-</sup> T-cells (C) and CD45<sup>+</sup>CD3<sup>-</sup>CD56<sup>+</sup> Natural Killer cells (D) pre-exercise and 0, 24, 48 and 72 h after the bench-stepping exercise. Data are means  $\pm$  SEM. \* indicates significant difference from pre-exercise cell count ( $P < 0.05$ ). # indicates significant difference from 0-h cell count





CD45<sup>+</sup> CD3<sup>+</sup> CD56<sup>-</sup> T-cell counts increased post-exercise so that T-cell counts at 72 h post-exercise were significantly higher ( $P < 0.05$ ) than pre-exercise and 0 h post-exercise numbers. There was no change over time in the balance between CD8a<sup>+</sup> cytotoxic, CD4<sup>+</sup> Th helper or Treg T cell subpopulations. The bench-stepping exercise had no significant effects on post-exercise CD45<sup>+</sup> CD14<sup>-</sup> CD16<sup>+</sup> neutrophil or CD45<sup>+</sup> CD3<sup>-</sup> CD56<sup>+</sup> Natural Killer cell counts.

## Discussion

Results from this study demonstrated that 30 min of bench-stepping exercise resulted in muscle damage in eccentrically, but not concentrically, exercised legs in untrained male volunteers. Post-exercise muscle function measures indicated that bench-stepping induced muscle damage to the quadriceps and hamstring of the eccentrically exercised leg. Eccentric-exercise induced muscle damage in EL was further confirmed by the occurrence of DOMS and increasing plasma creatine kinase concentrations that persisted for up to 72 h after exercise. The changes in physiological and biological markers of muscle damage were associated with decreased circulating monocytes and increased T cells. To our knowledge, these findings are the first to report the dynamic changes in immune cells following bench-stepping-induced muscle damage. How these immune cell population changes interact with skeletal muscle repair following exercise-induced muscle damage is unclear, and further research to investigate these interactions is required.

The reduction in muscle function following exercise is a common indicator of muscle damage. Previous bench-stepping studies on similar cohorts reported decrements in post-exercise peak isometric torque ranging from 12% to 20% in eccentrically exercised quadriceps that recovered to pre-exercise peak torque after 48 h of recovery [9, 11, 23, 36]. Consistent with these findings, the peak isometric torque of EL in this study declined by 18% following bench-stepping, and did not recover to pre-exercise values until 72 h post-exercise. Differences in the bench-stepping protocol imposed on volunteers (i.e. exercise time, additional load during exercise) could influence the magnitude of muscle damage sustained from the exercise, resulting in a more pronounced reduction in muscle function post-exercise.

Results from this study highlight the contrasting effect of repeated eccentric and concentric contractions have on muscle function. Peak eccentric, isometric and concentric torque significantly declined in EL immediately after exercise, persisting for at least 48 h. In comparison, concentric contractions had no significant impact on post-exercise muscle function. Although hamstring concentric torque was significantly reduced immediately after exercise, this had recovered to pre-exercise values within 24 h, suggesting

that this loss in muscle function was due to muscle fatigue rather than damage. These findings are consistent with prior reports [9, 36] showing a temporal decline in quadriceps muscle function following eccentric, but not concentric, exercise. Additionally, our findings concur with studies [9, 11] that reported no significant DOMS in the concentrically exercised quadriceps following bench-stepping.

Exercise-induced muscle damage is typically accompanied by muscle soreness that lasts for several days after exercise. While the mechanisms underpinning the phenomenon of exercise-induced DOMS is unclear, several hypotheses implicate the role of mechanical and biochemical factors in the development and dissipation of DOMS post-exercise [3, 17]. Among these biochemical factors are the inflammatory mediators bradykinin [22] and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) [30] that are upregulated following exercise-induced muscle damage by infiltrating immune cells or muscle tissue. The post-exercise upregulation of these pain mediators has been proposed to sensitise afferent receptors in skeletal muscle resulting in DOMS. Circulating bradykinin and PGE<sub>2</sub> concentrations was not measured in this study and the contribution of these immune activation markers on DOMS following bench stepping is warrants further investigation.

The severity of DOMS in the resting EL was evaluated with the SF-MPQ comprised of 15 pain descriptors to give composite affective, sensory and total pain scores. Additionally, the average pain of the resting EL was assessed with a VAS scale. Muscle soreness (total and average pain) findings in this study aligned with previous research that report soreness peaking at 48 h post-exercise then dissipating to pre-exercise scores after three [35, 36] and five [24] days of recovery in eccentrically exercised quadriceps. Our findings also revealed that affective pain peaked 24 h post exercise, while sensory pain trends were consistent with total and average pain. These observations suggest that muscle soreness experienced at various post-exercise timepoints may be characterised by different dimensions of pain, with sensory pain predominantly contributing to soreness associated with exercise-induced DOMS. No significant DOMS was observed in the quadriceps following concentric exercise. This is consistent with previous findings [9, 11] and further confirms that concentric exercise has minimal or no effect in inducing muscle damage during bench-stepping.

Muscle soreness during maximal eccentric contractions immediately increased after exercise and peaked at 48 h post exercise in the EL but not in the CL. In comparison, soreness during maximal isometric contractions was not affected by the bench stepping exercise in both EL and CL. Taken together, these findings indicate that the impact of muscle damage on DOMS during exercise is dependent on the mode of exercise with greater soreness reported during eccentric contractions.

The disruption of the myofibrillar structure following eccentric exercise results in the release of CK into circulation, making it an indirect biomarker for muscle damage. Previous studies have reported bench-stepping exercises to induce dynamic changes in plasma CK, peaking 48–72 h post-exercise then returning to baseline by seven days of recovery [11, 23, 36]. Our results showed a significant increase in CK 24 h post-exercise which remained elevated for the remainder of the study, further establishing the effectiveness of the bench-stepping protocol in inducing muscle damage.

Cartilage oligomeric protein (COMP) is a glycoprotein that is found in ligaments, tendons and cartilage [21] and stabilises collagen in mature cartilage. It is a biomarker for knee joint and cartilage degeneration and is utilised to screen for those with osteo- and rheumatoid arthritis [34]. Given the impact of exercise on joints, COMP has been used as a biomarker to determine how various modes of exercise affects the cartilage of healthy adults. Ultra-marathon running (200 km), for example, resulted in elevated serum COMP that persisted for several days, only subsiding to pre-race concentrations five days after the event [15]. In comparison, shorter and less strenuous bouts of exercise such as drop jumps [25], shorter running distances (10–42 km) [15] and the bench-stepping performed in this study resulted in the immediate rise in plasma COMP, which returned to pre-exercise concentrations within 48 h. Kim and colleagues [15] proposed that the acute transient increase in plasma COMP may not be due to cartilage damage. This is because moderate exercise and physical tasks (occupations that require lifting heavy loads) elicited similar transitory trends in COMP, when cartilage damage does not occur [4]. The transient increase in COMP detected following bench-stepping may be due to the exercise-induced efflux of COMP from the synovial fluid of joints into the bloodstream, which subsides upon the termination of the exercise.

Exercise-induced muscle damage has been reported to induce a dynamic inflammatory response, resulting in a temporary increase in circulating cytokine concentrations post-exercise. Although the nature of the bench-stepping exercise requires eccentric and concentric movements of the quadriceps, analysis of muscle biopsies has shown disparately higher cytokine gene expression in eccentrically exercised quadriceps [10]. Previous muscle damage studies have reported the temporary increase in plasma MCP-1, peaking within 6 h post-exercise then returning to pre-exercise concentrations by 24 h post-exercise [27, 38]. Similar trends in plasma IL-6 [27] have been reported following repeated maximal voluntary quadriceps contractions. Of the several cytokines measured, only MCP-1 was affected by the exercise intervention in this study: significantly increasing immediately post-exercise, then progressively declining thereafter to below pre-exercise concentrations

following 72 h of recovery. The minimal changes in circulating cytokines suggest two possible scenarios: either the bench-stepping protocol utilised in this study induced a negligible inflammatory response, despite inducing muscle damage, or the changes recorded were minimal because blood collections were not taken at early post-exercise timepoints when transient increases in inflammatory cytokines have been reported to occur.

Monocyte chemoattractant protein-1 is a potent chemokine that regulates the migration and infiltration of monocytes to damaged tissue, where they differentiate into macrophages to clear damaged myofibres and facilitate skeletal muscle remodelling and repair [2]. The integrated roles of MCP-1 and monocytes in regulating muscle recovery following muscle damage have been demonstrated in both pre-clinical and clinical studies. Mice lacking MCP-1 (MCP-1<sup>-/-</sup>) had reduced monocyte recruitment to skeletal muscle following ischemic injury, which corresponded with impaired muscle regeneration [29]. Increased skeletal muscle MCP-1 protein and gene expression following eccentric muscle damage in adult human males was associated with increased infiltration of macrophages into skeletal muscle [6, 10]. Exercise studies have also shown that exercise-induced increases in plasma MCP-1 correspond with post-exercise circulating monocytosis and neutrophilia [18, 27]. In this study, we did not observe significant increases in circulating classical CD16<sup>-</sup> CD14<sup>+</sup> monocytes, despite the increase in plasma MCP-1 immediately post-exercise. It is plausible that the bench-stepping exercise induced monocytosis, but was not detected due to lack of measurements at critical timepoints early post-exercise where previous studies have reported monocytosis to occur. The progressive decline of plasma MCP-1 concentrations during exercise recovery corresponded with the post-exercise reduction in circulating monocytes. Further research is needed to clarify the implications of reduced circulating CD45<sup>+</sup> CD16<sup>-</sup> CD14 monocytes on the reparative process of skeletal muscle following exercise-induced damage.

Findings from previous research indicate that the exercise-induced infiltration of T cells in skeletal muscle is dependent on the duration and intensity of the exercise. For example, completing an ultra-endurance event resulted in T cell infiltration [19], while a single bout of muscle-damaging exercise did not trigger a T cell response [18]. Contrary to these findings, we measured a significant increase in circulating CD3<sup>+</sup> T lymphocytes 72 h post-exercise. It is possible that the bench-stepping exercise in this study may have induced a greater degree of muscle damage than other muscle damage models, resulting in the delayed increase in circulating T cell numbers. The post-exercise T-cell response in this study may also be explained by the greater exercise stress induced by the bench-stepping protocol. The bench-stepping exercise requires significant more cardiovascular

and metabolic demands than traditional eccentric protocols. As such, this model may be closer to a high-intensity run (albeit eccentric and concentric work split between legs instead of each leg performing both), which has been reported to mediate a transient increase in circulating T-cell populations [26].

Although the role of T cells in facilitating muscle recovery following exercise-induced muscle damage is unclear, two hypotheses have been proposed. Firstly, infiltrating T cells may upregulate MCP-1 secretion in skeletal muscle and resident macrophages, attracting monocytes to the site of injury, which consequently initiate satellite cell proliferation and muscle regeneration [39]. The second hypothesis is that recruited T cells develop a memory from being exposed to the immunological milieu of exercise-induced muscle damage, and consequently mediate a swifter immune response upon the onset of the same exercise several days following muscle damage [7]. Eccentric muscle damage increases major histocompatibility-complex proteins in skeletal muscle, which are recognised by infiltrating T cells. Upon recovery from muscle damage, the remnant of T cells in skeletal muscle are primed to respond to subsequent exercise bouts, resulting in less damage than in the initial bout. Further research is required further understand the mechanisms by which T cells facilitate muscle repair and adaptation following exercise-induced muscle damage.

Despite the clear effect of the bench stepping protocol in inducing muscle damage and immune activation, the current study has some limitations. Firstly, the relevance of these findings may only be applicable to males. Although unaccustomed exercise induces a similar degree of muscle damage in men and women, muscle soreness and exercise-induced inflammatory responses have been reported to be attenuated in women compared with men [5, 32]. The effect of bench stepping on measures of muscle damage and immune parameters may also be limited to untrained individuals unaccustomed to the bench stepping exercise. Due to the capacity of skeletal muscle to rapidly adapt to unaccustomed exercise, the effects of exercise on muscle damage and immune activation observed in this study may be blunted if the exercise is repeated following recovery from the initial muscle-damaging bout [6]. As previously highlighted, future trials investigating immunological changes following exercise-induced muscle damage should incorporate measurements within 12 h post-exercise, as this is when transient increases in cytokines and circulating immune cells are likely to occur [18, 27].

In conclusion, the reduced post-exercise muscle function, DOMS and increased plasma creatine kinase concentrations confirm that the bench-stepping protocol applied in this study induced muscle damage in the quadriceps of untrained male participants following eccentric exercise. With the exception of MCP-1, bench-stepping had no

effect on circulating cytokine concentrations at any of the timepoints measured. Bench-stepping induced a delayed decrease in circulating monocytes which coincided with the increase in circulating T cells following muscle damage. It is possible that the T-cell response following muscle damage may be useful in facilitating muscle adaptation and protecting from declines in muscle function following subsequent sessions of the same exercise.

**Acknowledgements** The authors thank the individuals within the wider Palmerston North community who agreed to participate in this study. We also thank Duncan Hedderley for the statistical analysis of the data.

**Author Contributions** DL, MB and MRM conceived and designed the research. DL, NN and GMS facilitated the recruitment of participants and running the exercise trial. OMS, NN and NSB contributed to the analysis of biological samples collected in this study. DL, MB and OMS analysed and interpreted the physiological and immune-related data. DL wrote the manuscript. All authors have read and approved the manuscript.

**Funding** Open Access funding enabled and organized by CAUL and its Member Institutions. This work was funded under the “Musseling-up 2.0: Greenshell™ mussels for inflammation, metabolism and muscular skeletal function” programme of the High Value Nutrition National Science Challenge for New Zealand and Sanford Limited.

**Data Availability** The data that support the findings of this study are not openly available due to them containing information that could compromise research participant privacy/consent. They are available from the corresponding author upon reasonable request.

## Declarations

**Conflict of interest** On behalf of all authors, the corresponding author states that there is no conflict of interest.

**Ethics Approval** All methods and procedures were reviewed and approved by New Zealand’s Southern Health and Disability Ethics Committee (20/STH/75).

**Consent to Participate** All recruited participants voluntarily signed an informed consent form to participate in this study.

**Consent to Publish** The authors affirm that human research participants provided informed consent for publication of data presented in this study.

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