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ORIGINAL ARTICLE

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The effects of intensive, moderate and downhill treadmill running on human blood lymphocytes expressing the adhesion/activation molecules CD54 (ICAM-1), CD18 (β_2 integrin) and CD53

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Abstract This study examined the effects of intensive, moderate and downhill treadmill running on blood lymphocyte expression of adhesion/activation (AA) molecules. Trained subjects completed three treadmill-running protocols of identical duration: (1) an intensive protocol at 80% $\dot{V}_{O_2max}$ to volitional exhaustion, (2) a moderate protocol at 60% $\dot{V}_{O_2max}$ and (3) a −10% downhill (eccentric) protocol at 80% $\dot{V}_{O_2max}$. Blood samples were taken before, immediately after, 1 and 24 h after exercise. Isolated lymphocytes were assessed for expression of the AA molecules CD54, CD18 and CD53 by flow cytometry. Lymphocyte counts increased immediately after all running protocols. Lymphocytopenia was observed 1 h after the intensive and eccentric protocols only. Plasma creatine kinase increased 24 h after the downhill protocol only. Increases in the number and percentage of $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ lymphocytes were observed immediately after the intensive and eccentric protocols, with the numbers falling below pre-exercise values at 1 h post-exercise for all protocols. No differences were found between the intensive protocol and the eccentric protocol at the same relative intensity. Analysis of lymphocyte subsets showed that the total number of $CD3^+$, $CD4^+$, $CD8^+$ and $CD56^+$ lymphocytes increased after the intensive protocol before falling below pre-exercise values at 1 h post-exercise. A relatively greater mobilisation of $CD56^+$ and $CD8^+$ cells accounts for the changes in $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ cell populations. Lymphocytes that enter and exit the circulation following exercise express high levels of AA molecules, which may mediate extravasation and post-exercise lymphocytopenia. This effect appears to be influenced by exercise intensity and not muscle damage.

Keywords Lymphocytosis · Lymphocytopenia · CD antigens · Muscle damage · Flow cytometry

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Introduction

Arduous periods of training and competition often leave elite athletes susceptible to upper respiratory tract infections (URTI) (Nieman 1994; Peters 1997). Alterations in the number and function of leukocytes in the blood compartment after exercise may predispose athletes to infection. Lymphocytes comprise about 30% of the total leukocytes in blood and are fundamental to anti-microbial resistance and adaptive immunity. They recognise microorganisms, produce antibodies, kill virally infected cells and memorise previous infections. Endurance-based exercise is known to elicit an initial increase in the total number of blood lymphocytes, followed by a rapid lymphocytopenia in the recovery phase after cessation of exercise (Mooren et al. 2002; Nieman et al. 1995; Pizza et al. 1995; Smith et al. 1998; Steensberg et al. 2002), with this effect being more pronounced for the $CD8^+$ T-cell and $CD3^-/CD56^+$ natural killer (NK)-cell populations (Gannon et al. 2001; Nieman et al. 1995). It has been proposed that the decline in numbers and function of blood lymphocytes during the recovery phase of exercise leaves the host athlete more vulnerable to bacterial and viral infections (Pedersen and Ullum 1994). Equally, the mobilisation and movement of large numbers of cells from peripheral lymphoid tissues in response to exercise may perturb resistance to infection. The mechanisms underlying increased levels of lymphocyte trafficking induced by exercise are not fully elucidated.

The initial rise in blood lymphocyte numbers in response to exercise is a reflection of cell mobilisation from peripheral lymphoid organs, which may be mediated by increased levels of catecholamines (Gabriel and Kindermann 1998; Hay and Andrade 1998). However, the mechanisms underlying the fall in blood lymphocyte counts during the recovery phase of exercise are not understood. Some previous studies have suggested that this is due to cell death by apoptosis (Mars et al. 1998). However, this is equivocal; more recent research has found only very low levels of apoptotic cells in the bloodstream after exercise (Mooren et al. 2002; Steensberg et al. 2002). It is possible that exercise-induced lymphocytopenia reflects a mobilisation of blood lymphocytes to other body compartments. The process of exercise-induced leukocyte mobilisation, like normal cell trafficking, is dependent on the expression of cell surface adhesion and activation (AA) molecules and their interaction with their corresponding ligands. AA molecules allow lymphocytes to recognise and bind to endothelial cells, facilitating extravasation and subsequent migration of cells into the tissues or lymphoid organs (Gabriel and Kindermann 1998; Jalkanen and Salmi 1999). The interaction of lymphocytes with endothelial cells is a multistep process beginning with a weak and transient contact mediated by selectins and their ligands (Vestweber and Blanks 1999). Firmer adhesion, mediated by lymphocyte expression of integrins and molecules of the immunoglobulin (Ig) superfamily, paves the way for migration of cells across the endothelium (Hubbard and Rothlein 2000; Jalkanen and Salmi 1999). Previous studies of exercise-induced alterations of lymphocyte AA molecules have focused mainly on L-selectin (CD62L) and the β_2 integrins (CD18/CD11a/CD11b heterodimers) (Hong et al. 2005; Jordan et al. 1997; Kurokawa et al. 1995; Nielsen and Lyberg 2004; Shephard 2003; van Eeden et al. 1999). Following exercise, the total number of lymphocytes expressing CD62L has been shown to decrease (Nielsen and Lyberg 2004; van Eeden et al. 1999), whereas the number of cells expressing integrin can decline or increase (Gannon et al. 2001; Jilma et al. 1997; Nielsen and Lyberg 2004), depending on exercise modality and/or intensity.

The effects of exercise on lymphocyte membrane expression of the AA molecules CD54 (ICAM-1) and CD53 have received little or no research attention. CD54 belongs to the Ig superfamily and is an important molecule for the firm adhesion of lymphocytes to activated endothelium during activation and extravasation (Hubbard and Rothlein 2000). Levels of soluble CD54 increase in plasma after exercise (Jilma et al. 1997; Nielsen and Lyberg 2004; Rehman et al. 1997), but data reporting its expression on the surface of blood lymphocytes in response to exercise are scarce (Goebel and Mills 2000; Perez et al. 2001). CD53 is a 35–42 kDa type II glycoprotein belonging to the transmembrane-4 superfamily and is expressed on all peripheral blood leukocytes. Its functions include signal transduction and cell activation/adhesion (Cao et al. 1997). Potential alterations of lymphocyte CD53 expression following exercise have not been studied.

Although the expression of some AA molecules on the surface of lymphocytes has been examined during and immediately after exercise, data showing AA molecule expression during periods of lymphocytopenia are lacking. It is important to determine whether the mechanisms underlying exercise-induced lymphocytosis and subsequent lymphocytopenia are influenced by membrane bound AA molecules. Given that this phenomenon appears to be related to exercise intensity (Kendall et al. 1990; Mooren et al. 2002), the phenotypic characteristics of lymphocytes that enter and exit the circulation under different intensities of exercise should be examined. Further, both infectious and non-infectious processes of inflammation can alter the adhesion of leukocytes to activated endothelium (Gabriel and Kindermann 1998). Eccentric exercise protocols, such as downhill running, have consistently been used in a laboratory setting to induce muscle damage and inflammation (Pizza et al. 1995; Schwane et al. 1983; Smith et al. 1998). It has been reported that a greater lymphocytosis, but a similar extent of lymphocytopenia, occurs after downhill running relative to level running (Pizza et al. 1995). Whether or not signals produced as a result of exercise-induced muscle damage will alter AA molecule expression on lymphocytes and stimulate their preferential homing to sites of inflammation is unclear.

The aim of this study was to examine peripheral blood lymphocyte membrane expression of CD54 (ICAM-1), CD18 (β_2 integrin) and the tetraspan molecule CD53 in response to intensive, moderate and eccentric exercise. It was hypothesised that the total number of lymphocytes expressing these AA molecules would increase immediately after exercise before falling below pre-exercise values during the recovery period, and that this effect would be related to exercise intensity. A model of eccentric exercise at a defined intensity was also used to examine the influence of exercise-induced muscle damage on blood lymphocytes.

Materials and methods

Subjects

All eight subjects who volunteered for this study were male, aerobically trained and actively engaged in endurance-based physical activity (mean ± SD age: 28.2 ± 5.3 years, height: 176.6 ± 3.8 cm, mass: 70.1 ± 4.8 kg, $\dot{V}_{O_{2max}}$ 62.5 ± 3.3 ml kg^{-1} min^{-1}). At the time of the study, all subjects were healthy, not taking any medication including non-steroidal anti-inflammatory drugs and were free of any infectious illness for 6 week prior to their participation in the study. Written informed consent was obtained from each subject and the institution granted ethical approval. Subjects were required to refrain from any strenuous physical activity for 48 h before and 24 h after each testing protocol.

Experimental design

Subjects reported to the laboratory for an initial assessment of $\dot{V}_{O_2max}$ by completing an incremental running protocol on a motorised treadmill (Woodway, ergo ELG 55, Weil am Rhein, Germany). The $\dot{V}_{O_2max}$ test began with the subject running on a level gradient at a speed of 10 km h^{-1}. The speed of the treadmill was increased by 3 km h^{-1} every 3 min until a maximum speed of 16 km h^{-1}or 19 km h^{-1} was attained. After 3 min running at the maximum speed, the inclination of the treadmill was increased by 2.5% every minute until volitional exhaustion. Oxygen uptake (breath by breath) was measured during the test using online gas analysis (CPX MedGraphics, Oldham, UK) and heart rate was recorded every 5 s (S610, Polar Electro, Kempele, Finland).

One week after the initial $\dot{V}_{O_2max}$ assessment, subjects completed an intensive treadmill-running protocol. The treadmill remained at a level gradient (0%) and was set at a speed corresponding to 80% $\dot{V}_{O_2max}$. The subject was asked to maintain this pace until volitional exhaustion (mean running time: 33.8 ± 12.3 min). The moderate treadmill-running protocol required the subject to run on a level gradient at a speed corresponding to 60% $\dot{V}_{O_2max}$ for the same duration attained during the intensive protocol. The final test consisted of an eccentric treadmill-running protocol that required the subjects to run down a −10% gradient at an intensity of 80% $\dot{V}_{O_2max}$ for the same duration as the intensive and moderate protocols. It has been shown previously that $\dot{V}_{O_2max}$ and heart rate is lower during downhill treadmill running (−10% gradient) relative to level running (0% gradient) at the same treadmill velocity (Schwane et al. 1983). Therefore, in order to compare the effects of a level running protocol to a downhill running protocol without discrepancies in exercise intensity, the speed of the treadmill was adjusted to mimic the same physiological response as the intensive protocol at 80% $\dot{V}_{O_2max}$ (controlled by the heart-rate response). All tests were conducted at the same time of day and a period of one week was interspersed between each test. Subjects consumed water ad libitum during the treadmill-running protocols in order to maintain hydration status.

For all treadmill-running protocols, intravenous blood samples were collected in 6-ml vacuum tubes containing lithium heparin as an anticoagulant (Becton-Dickinson, Oxford, UK) at pre, immediately post, 1 h post and 24 h post-exercise. Whole blood was mixed (1:20) with white blood cell stain diluent (crystal violet in 10% acetic acid) and total leukocyte counts were estimated manually using a haemocytometer. Blood smears were stained with a Romanowsky stain (Raymond A. Lamb, London, UK) to determine differential leukocyte counts. For each sample, at least 300 cells were counted for the determination of total and differential leukocyte populations and an individual who was not aware of the sample identity performed the cell counts. Whole blood was centrifuged for 10 min at 1,000*g* and the removed plasma was stored at −80°C until analysis for creatine kinase (CK) activity.

Lymphocyte isolation procedure

Whole blood anticoagulated with lithium heparin was mixed with an equal volume of 0.9% NaCl. A 6 ml volume of the diluted blood was then carefully layered over 3 ml Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged at 800*g* at room temperature for 30 min. The distinct band formed by the lymphocytes after centrifugation at the sample/medium interface was carefully removed. Cells were washed twice for 10 min at 250*g*, firstly with 0.9% NaCl, followed by PBS + 1% bovine serum albumin + 0.02% sodium azide (PBS-BSA). Staining the cells with trypan blue revealed ~98% viability for all samples.

Measurement of lymphocyte cell surface CD54, CD18 and CD53 expression

Lymphocyte cell surface expression of CD54, CD18 and CD53 was assessed using indirect immunofluorescence assays. All antibodies were titrated to determine optimal conditions for analysis by flow cytometry. Isolated lymphocytes (0.5×10^6) were incubated for 45 min at room temperature with the following primary monoclonal antibodies (mAbs): CD18 (HIG. 125; Guy and Ross, Unpublished), CD53 (NL33; Fifth International Leukocyte Typing Workshop, Boston, USA) and CD54 (ICAM-1) (Sigma, Dorset, UK). The anti-phycoerythrin mAb 2A4 (Guy et al. 1988) and CD44 (F10; European Collection of Animal Cell Cultures, Salisbury, UK) were used in each assay as negative and positive controls, respectively. Cells were washed twice in PBS-BSA and incubated at room temperature with an FITC conjugated $F(ab')_2$ fraction of sheep anti-mouse IgG secondary antibody (Sigma, Dorset, UK) for 30 min. Cells were washed twice and analysed by one-colour flow cytometry.

Measurement of lymphocyte subset cell surface CD54, CD18 and CD53 expression

For this part of the study, a further eight aerobically trained male subjects, with similar physiological characteristics to those described earlier (mean ± SD age: 28.5 ± 6.8 years, height: 175.7 ± 3.6 cm, mass: 67.3 ± 5.1 kg, $\dot{V}_{O_2max}$ 63.6 ± 3.2 ml kg^{-1} min^{-1}), were recruited to analyse the effects of exercise on the lymphocyte subset expression of AA molecules. As before, all subjects were healthy, not taking any medication including non-steroidal anti-inflammatory drugs and were free of any infectious illness for 6 week prior to their participation in the study. Each subject completed

an initial assessment of $\dot{V}_{O_2max}$ following the same protocol as the previous subjects. One week later, each subject completed the intensive treadmill-running protocol at 80% $\dot{V}_{O_2max}$ until exhaustion only (mean running time: 40.0 ± 19.4 min), with blood samples being obtained before, immediately after and 1 h after exercise.

Lymphocyte subsets were identified by their cell surface expression $CD3^+$, $CD4^+$, $CD8^+$ and $CD56^+$. Isolated lymphocytes (0.5×10^6) were incubated for 45 min at room temperature with the following FITC conjugated mAbs: CD54, CD18 and CD53 and PE conjugated mAbs: CD3, CD4, CD8 and CD56. The CD8 mAb was IgG2a isotype and all other mAbs were IgG1. Appropriate FITC and PE conjugated isotype controls were used in each assay to account for background binding of IgG1 and IgG2a. After incubation, lymphocytes were resuspended in 0.5 ml PBS-BSA and analysed by two-colour flow cytometry. All mAbs used for the analysis of AA molecules on lymphocyte subsets were purchased form Immunotools (Friesoythe, Germany) except the CD53 mAb, which was purchased from BD Pharmingen (California, USA).

Flow cytometry

All lymphocyte phenotype analysis was conducted on a flow cytometer (FACSCalibur, BD Biosciences, San Jose, California) equipped with a 15 mW argon ion laser emitting light at a fixed wavelength of 488 nm. Fluorescent signals were collected in logarithmic mode (4 decade logarithmic amplifier) and cell numbers per channel in linear mode. An electronic gate was placed around the lymphocyte population in the flow cytometry forward and side scatter mode and 10,000 of the gated events were acquired for analysis. The purity of the gated population was ~98% lymphocytes as assessed by CD14 (monocyte marker) and CD66 (granulocyte marker) exclusion (Immunotools, Friesoythe, Germany). FITC fluorescence was detected in the FL1 filter centred at 530 nm with a 30 nm half-peak bandpass. PE fluorescence was detected in the FL2 filter centred at 578 nm with a 28 nm bandpass. During two-colour analysis, any overlapping of the two emission spectra was adjusted using electronic colour compensation. The fluorescent amplifier of the FL1 and the FL2 detector filter (for two-colour analysis) was adjusted to ensure that the negative cell population (lymphocytes incubated with the 2A4 anti-phycoerythrin antibody or the appropriate isotype control) appeared in the first logarithmic decade. An electronic marker was placed at the limit of the negative control to quantify the percentage of lymphocytes and lymphocyte subsets that were positive and negative for each cell surface antigen. Absolute numbers of lymphocytes positive for each cell surface antigen were determined by multiplying the percentage values by the corresponding total lymphocyte count.

Plasma creatine kinase activity

The total CK activity of plasma was determined as a marker of muscle damage using a standard laboratory kit in accordance with the manufacturer's instructions (Instrumentation Laboratory, MA, USA).

Statistical analysis

All results are presented as the mean ± SE unless stated otherwise. A two-way repeated measures ANOVA with Bonferroni's post-hoc test was used to detect differences over time and between trials. For the single protocol design, a one-way repeated measures ANOVA with Bonferroni's post-hoc test was used to detect changes over time. Statistical significance was accepted at $P < 0.05$.

Results

All subjects successfully completed each of the three treadmill-running protocols. Changes in the total leukocyte count, lymphocyte count and plasma CK activity are presented in Table 1. Changes in blood lymphocyte counts followed an anticipated pattern, with an initial lymphocytosis occurring immediately after all three treadmill-running protocols ($P < 0.01$). The two treadmill protocols conducted at 80% $\dot{V}_{O_2max}$ (intensive and eccentric) showed the most marked lymphocytosis. Lymphocytopenia was observed 1 h after the intensive and eccentric protocols only ($P < 0.01$), with the total lymphocyte count returning to baseline levels at 24 h post-exercise for all three protocols. The eccentric protocol was the only treadmill test to induce muscle damage, as indicated by the elevated plasma CK activity observed at 24 h post-exercise.

Data showing the numbers and the percentage of lymphocytes expressing defined AA molecules are presented in Fig. 1. Significant increases in the number and percentage of CD54 expressing lymphocytes were observed immediately post-exercise for the intensive and eccentric protocols only ($P < 0.01$). The numbers of $CD54^+$ lymphocytes fell below baseline values at 1 h post-exercise for all three protocols ($P < 0.01$). Both the numbers and percentage of $CD54^+$ lymphocytes returned to basal levels at 24 h post-exercise for all three protocols. Representative flow cytometry histograms showing the log fluorescent intensity of lymphocyte CD54 expression relative to negative and positive controls are shown in Fig. 2.

Analysis by flow cytometry revealed that ~98% of all lymphocytes were $CD18^+$ and $CD53^+$ before and after all three protocols. However, two distinct cell populations with strikingly different fluorescent intensities ("dim" or "bright") of CD18 (Fig. 3) and CD53 (Fig. 4) expression were observed. An electronic gate

Table 1 Total leukocyte counts, lymphocyte counts and plasma creatine kinase (CK) activity after intensive, moderate and eccentric treadmill running

	Pre	Post	Post 1 h	Post 24 h
Intensive protocol				
Leukocytes ($\times 10^9$ l^{-1})	5.1 ± 1.4	8.6 ± 2.4*	7.0 ± 3.1*	5.7 ± 1.4
Lymphocytes ($\times 10^9$ l^{-1})	1.6 ± 0.4	3.0 ± 1*	1.2 ± 0.2*	1.7 ± 0.3
CK (IU/l)	141 ± 47	184 ± 69	184 ± 37	218 ± 143
Moderate protocol				
Leukocytes ($\times 10^9$ l^{-1})	6.0 ± 1	7.2 ± 1.6*	6.9 ± 2*	6.2 ± 1
Lymphocytes ($\times 10^9$ l^{-1})	1.8 ± 0.3	2.4 ± 0.5*	1.5 ± 0.2	1.9 ± 0.3
CK (IU/l)	167 ± 113	167 ± 49	145 ± 52	152 ± 108
Eccentric protocol				
Leukocytes ($\times 10^9$ l^{-1})	6.6 ± 1.9	11.8 ± 7*	7.7 ± 2.7*	6.8 ± 1.6
Lymphocytes ($\times 10^9$ l^{-1})	1.8 ± 0.5	3.1 ± 0.7*	1.2 ± 0.5*	1.9 ± 0.4
CK (IU l^{-1})	96 ± 48	139 ± 57	204 ± 114	778 ± 377*

Values are mean ± SD
*Indicates statistically significant difference from pre-exercise values ($P < 0.05$)

was placed over the "bright" populations on the flow cytometry histogram, and the effects of exercise on the numbers and percentage of $CD18^{bright}$ and $CD53^{bright}$ lymphocytes were analysed (Fig. 1). Statistically significant increases in the numbers and percentage of $CD18^{bright}$ and $CD53^{bright}$ lymphocytes were observed

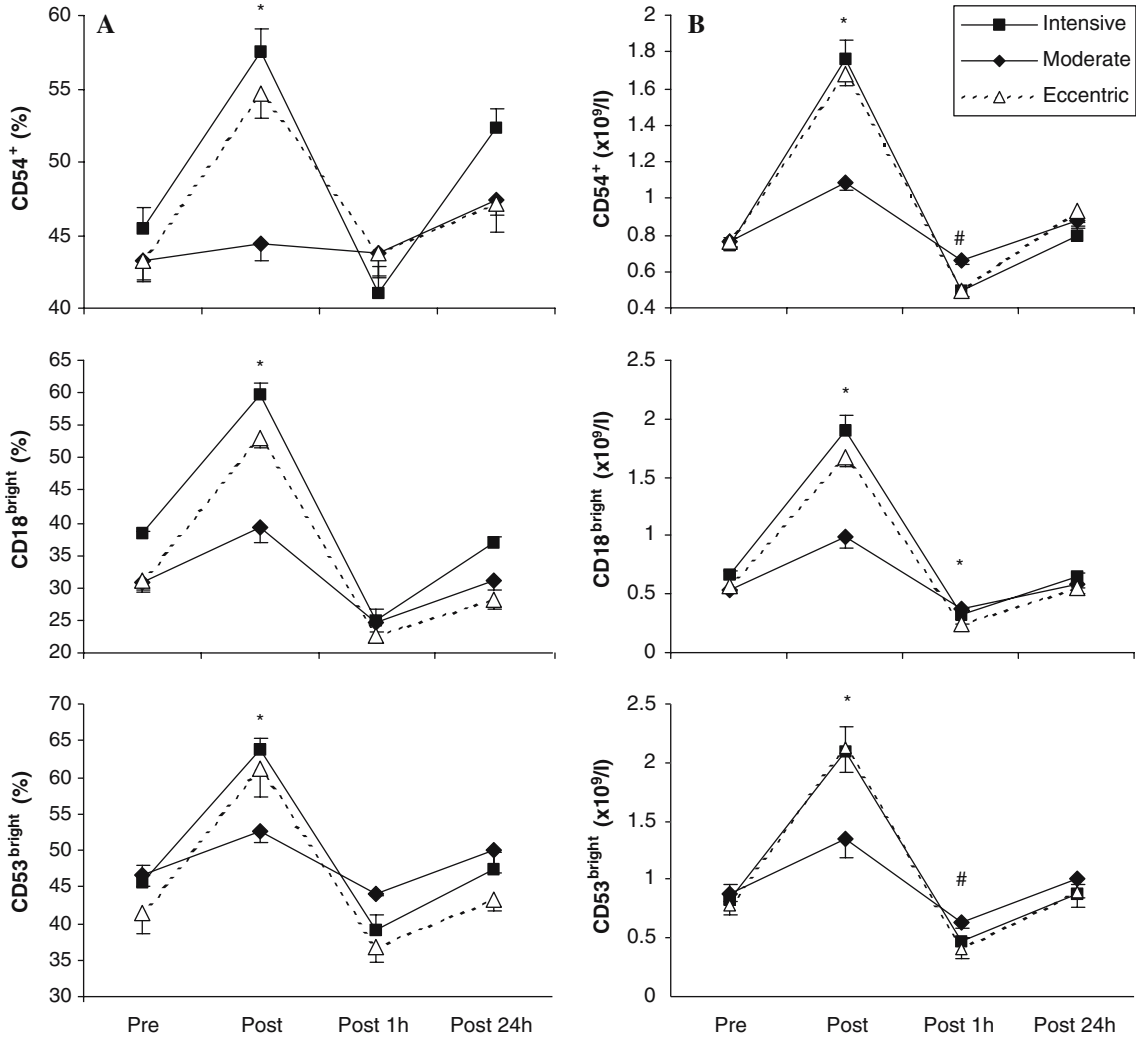

Fig. 1 The percentage (**a**) and total number (**b**) of the cell surface adhesion/activation (AA) molecules CD54 $(ICAM\text{-}1)^+$, CD18 ($\beta 2$ $integrin)^{bright}$ and $CD53^{bright}$ on peripheral blood lymphocytes before and after three treadmill-running protocols (mean ± SE). The intensive protocol was conducted on a level gradient at 80% $\dot{V}_{O_{2max}}$ the moderate protocol on a level gradient at 60% $\dot{V}_{O_{2max}}$ and the eccentric protocol on a −10% gradient at 80% $\dot{V}_{O_{2max}}$. Each subject ran for the same duration during all three protocols. * Indicates statistically significant difference from pre exercise for the intensive and eccentric protocols only ($P < 0.01$). # Indicates statistically significant difference from pre-exercise for all protocols ($P < 0.01$)

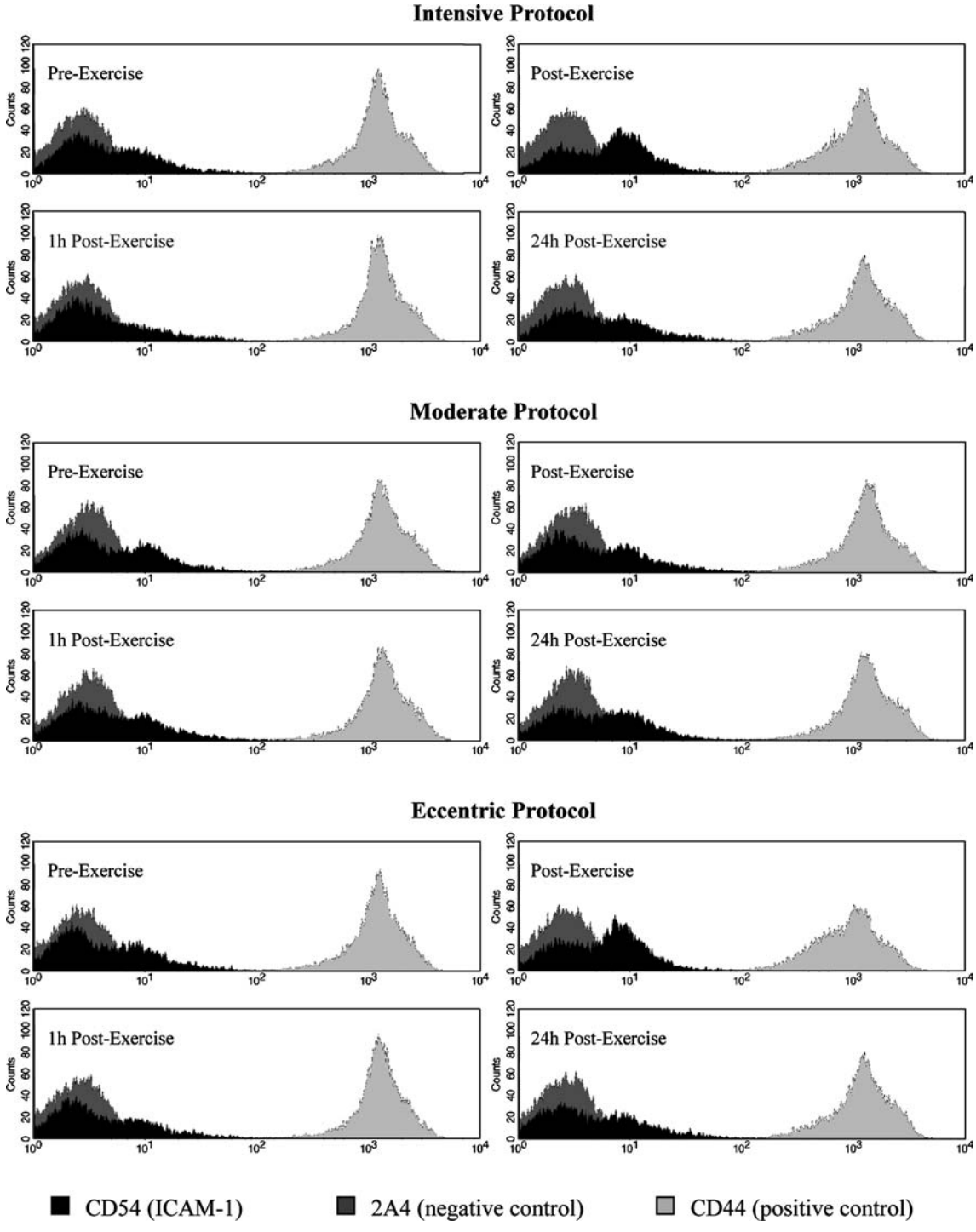

Fig. 2 Representative flow cytometry histograms for Subject 2 showing the lymphocyte cell surface expression of CD54 (ICAM-1) relative to negative and positive controls before, immediately after, 1 h after and 24 h after the intensive, moderate and eccentric treadmill-running protocols. The horizontal axis of each histogram shows the fluorescent intensity on a logarithmic scale and the vertical axis shows the relative cell counts/channel on a linear scale

immediately after the intensive and eccentric treadmill-running protocols ($P < 0.01$). These protocols also resulted in the numbers, but not the percentage, of $CD18^{bright}$ and $CD53^{bright}$ lymphocytes to fall below baseline values at 1 h post-exercise ($P < 0.01$) before returning to baseline levels at 24 h post-exercise. The moderate protocol resulted in the numbers of $CD53^{bright}$ lymphocytes to fall below the pre-exercise values at 1 h post-exercise ($P < 0.01$) but no statistically significant alterations in the numbers or

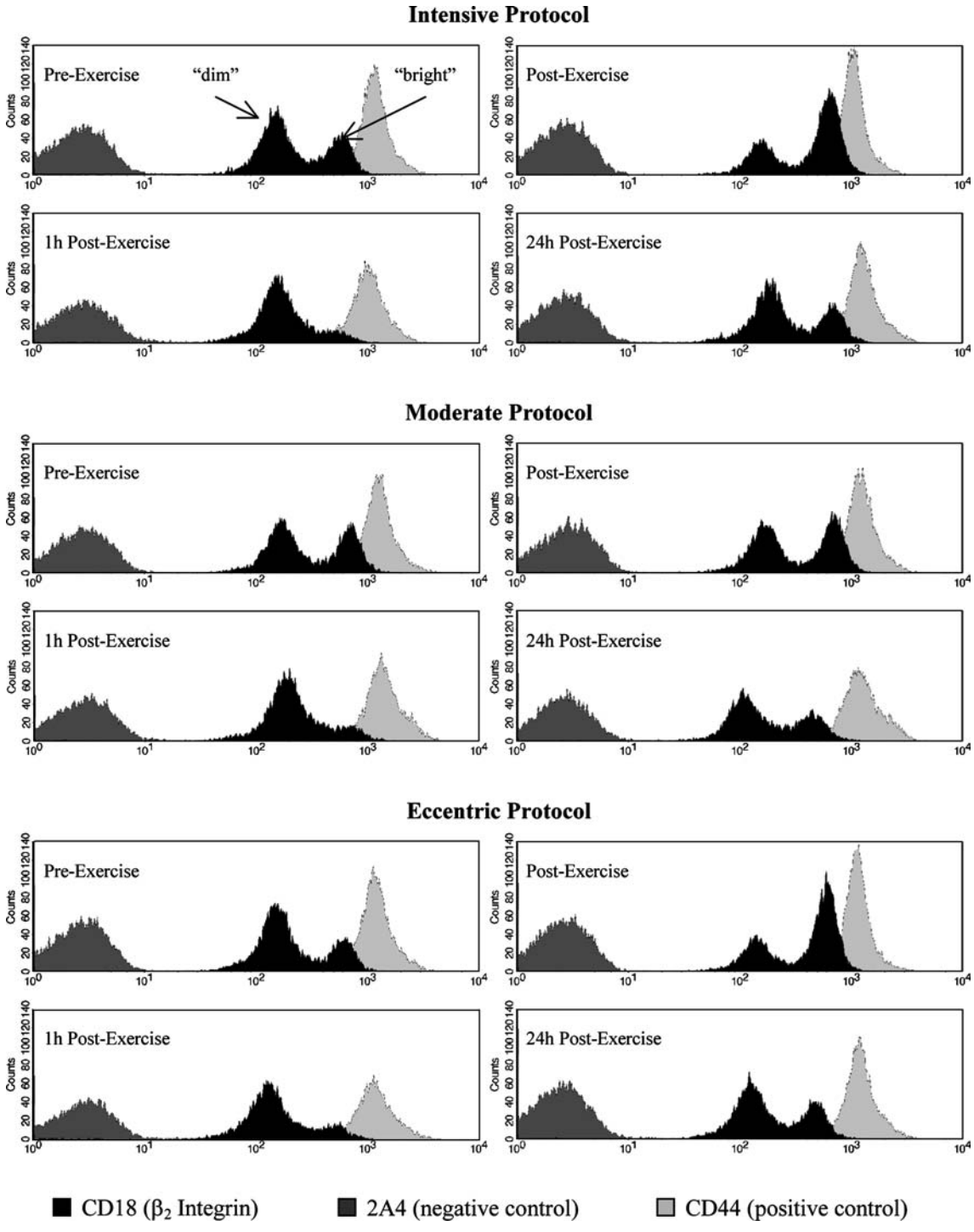

Fig. 3 Representative flow cytometry histograms for Subject 6 showing the lymphocyte cell surface expression of CD18 (β_2 Integrin) relative to negative and positive controls before, immediately after, 1 h after and 24 h after the intensive, moderate and eccentric treadmill-running protocols. The horizontal axis of each histogram shows the fluorescent intensity on a logarithmic scale and the vertical axis shows the relative cell counts/channel on a linear scale. ~98% of all lymphocytes were found to be positive for CD18 expression, with two distinct cell populations with strikingly different fluorescent intensities (termed "dim" and "bright") being observed

percentage of $CD18^{bright}$ lymphocytes were observed at any time point following this protocol ($P > 0.05$). Representative flow cytometry histograms showing the log fluorescent intensity of CD18 and CD53 relative to negative and positive controls are shown in Figs. 3 and 4, respectively.

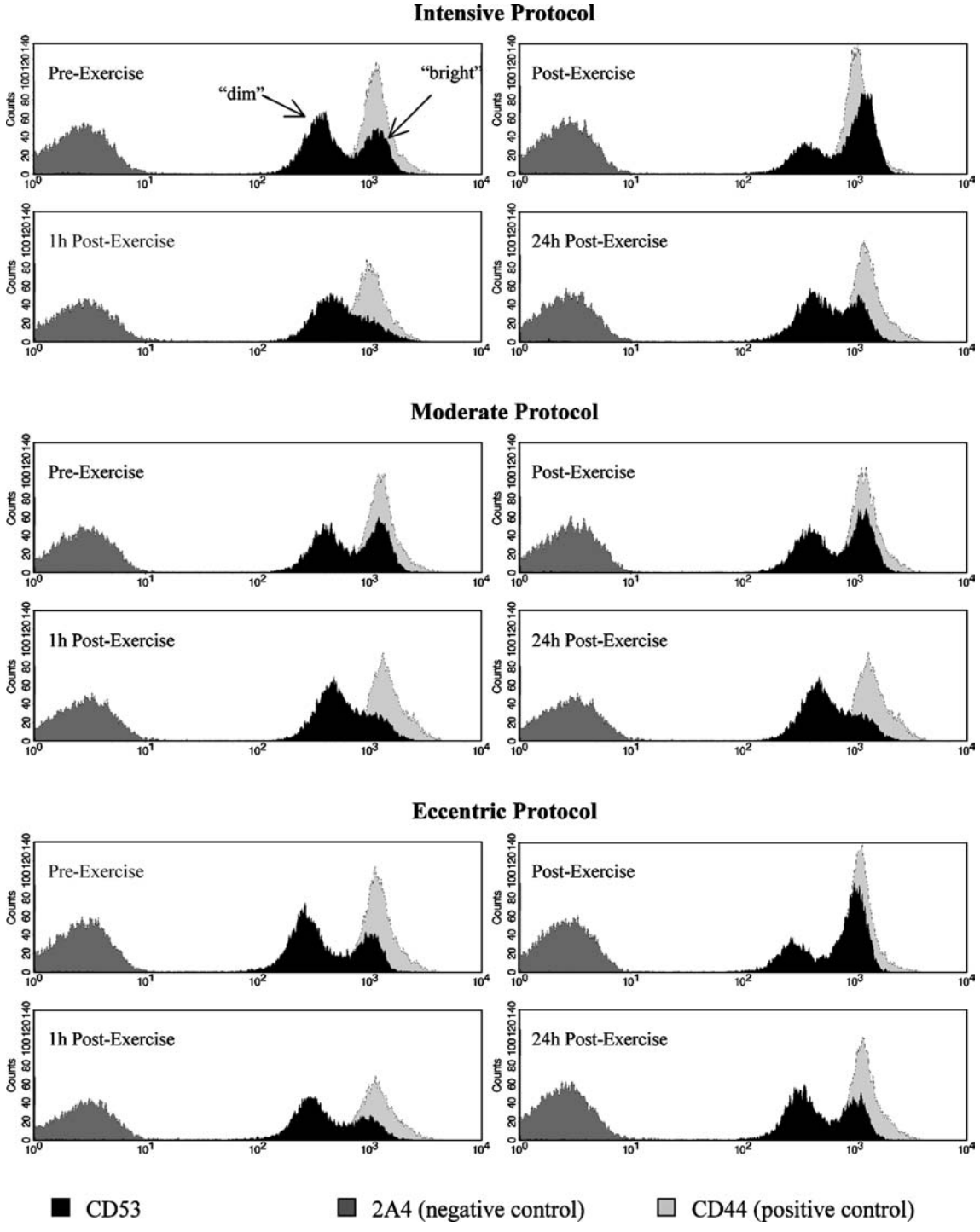

Fig. 4 Representative flow cytometry histograms for Subject 6 showing the lymphocyte cell surface expression of CD53 relative to negative and positive controls before, immediately after, 1 h after and 24 h after the intensive, moderate and eccentric treadmill-running protocols. The horizontal axis of each histogram shows the fluorescent intensity on a logarithmic scale and the vertical axis shows the relative cell counts/channel on a linear scale. ~98% of all lymphocytes were found to be positive for CD53 expression, with two distinct cell populations with strikingly different fluorescent intensities (termed "dim" and "bright") being observed

The total numbers of lymphocytes and lymphocyte subset counts in response to the intensive treadmill-running protocol for the second group of subjects are presented in Table 2. As before, the total lymphocyte count increased to statistically significant levels immediately after exercise ($P < 0.01$) before falling below the pre-exercise value at 1 h post-exercise ($P < 0.05$). The total number of $CD3^+$, $CD8^+$ and $CD56^+$ lymphocytes were found to increase immediately post-exercise. At 1 h post-exercise, the total numbers of $CD3^+$, $CD4^+$,

Table 2 Total lymphocyte and lymphocyte subset counts in response to the intensive treadmill-running protocol ($\times 10^9$ l^{-1})

	Pre	Post	Post 1 h
Lymphocytes	1.5 ± 0.4	3.2 ± 1.3*	1.0 ± 0.4*
$CD3^+$ Cells	1.1 ± 0.3	1.9 ± 0.8*	0.7 ± 0.3*
$CD4^+$ Cells	0.7 ± 0.2	1.0 ± 0.5	0.5 ± 0.3*
$CD8^+$ Cells	0.5 ± 0.2	1.4 ± 0.6**	0.3 ± 0.1*
$CD56^+$ Cells	0.3 ± 0.1	1.2 ± 0.7**	0.07 ± 0.03**

Values are mean ± SD
Statistically significant difference from pre-exercise values: *$P < 0.05$, **$P < 0.01$

$CD8^+$ and $CD56^+$ were lower than the pre-exercise value. The changes in cell numbers were more pronounced for the $CD8^+$ cells and the $CD56^+$ cells than for the other lymphocyte subsets, resulting in an alteration of the lymphocyte subset distribution following the exercise protocol.

For the analysis of CD18 and CD53 expression, an electronic gate was placed around the lymphocyte subset populations that were expressing CD18 and CD53 at "bright" fluorescent intensities. The numbers and percentage of all lymphocytes expressing the cell surface antigens $CD54^+$, $CD54^+/CD3^+$, $CD54^+/CD4^+$, $CD54^+/CD8^+$, $CD54^+/CD56^+$, $CD18^{bright}$, $CD18^{bright}/CD3^+$, $CD18^{bright}/CD4^+$, $CD18^{bright}/CD8^+$, $CD18^{bright}/CD56^+$, $CD53^{bright}$, $CD53^{bright}/CD3^+$, $CD53^{bright}/CD4^+$, $CD53^{bright}/CD8^+$ and $CD53^{bright}/CD56^+$ in response to the intensive treadmill-running protocol are presented in Fig. 5. Consistent with the results obtained in response to the intensive protocol with the first group of subjects, significant increases in the numbers and percentage of all lymphocytes expressing $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ were observed immediately after this protocol ($P < 0.01$). The numbers of $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ and the percentage of all $CD53^{bright}$ lymphocytes were found to be lower than the pre-exercise value at 1 h post-exercise ($P < 0.05$). The numbers of all lymphocyte subsets expressing $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ were found to increase immediately after the exercise protocol, but there was a relatively greater increase in the numbers of $CD8^+$ cells and $CD56^+$ cells expressing these AA molecules. In addition, the percentage of all lymphocytes expressing $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ and co-expressing the subset markers $CD8^+$ and $CD56^+$ were found to increase immediately after the exercise protocol, whereas no change was observed in the expression of these AA molecules on the $CD4^+$ subset population. At 1 h post-exercise, the numbers of $CD3^+$, $CD8^+$ and $CD56^+$ cells expressing $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ were found to fall below the pre-exercise value, whereas the numbers of $CD4^+$ cells expressing these AA molecules did not change ($P > 0.05$).

The percentage of all $CD3^+$, $CD4^+$, $CD8^+$ and $CD56^+$ lymphocyte subsets expressing $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ are shown in Fig. 6. The $CD8^+$ and $CD56^+$ cells had a greater expression of $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ in comparison to the $CD3^+$ and $CD4^+$ cell populations at all sampling time points before and after exercise. The percentage of all $CD3^+$, $CD4^+$ and $CD8^+$ cell populations expressing $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ increased immediately post-exercise ($P < 0.01$), whereas no changes were found for the $CD56^+$ cell population ($P > 0.05$). The percentage of all $CD8^+$ cells expressing $CD18^{bright}$ and $CD53^{bright}$ fell below the pre-exercise values at 1 h post-exercise.

Discussion

This study examined the effects of intensive, moderate and muscle-damaging exercise on peripheral blood lymphocytes expressing the AA molecules CD54 (ICAM-1), CD18 (β_2 integrin) and, for the first time, the tetraspan molecule CD53 in relation to exercise-induced lymphocytosis and lymphocytopenia. The influence of exercise intensity on lymphocytosis and lymphocytopenia has been shown previously (Kendall et al. 1990; Mooren et al. 2002). The results of the present study suggest that lymphocytes that enter and subsequently exit the bloodstream in response to exercise express high levels of the cell surface AA molecules CD54, CD18 and CD53 and that their mobilisation into the bloodstream is influenced by the intensity of exercise and does not appear to be related to exercise-induced muscle damage. The expression of these AA molecules may govern the rapid circulatory trafficking of lymphocyte subset populations during and after exercise. Indeed, following the intensive protocol, a greater mobilisation and subsequent removal from the bloodstream of $CD8^+$ and $CD56^+$ lymphocyte subset populations was observed in comparison to the $CD4^+$ lymphocyte population. The $CD8^+$ and $CD56^+$ lymphocyte subset populations were also found to have a far greater level of cell surface expression of CD54, CD18 and CD53 than the $CD4^+$ and the total $CD3^+$ T-cell population. Changes in the numbers and distribution of AA positive lymphocytes were found to be transient, as these values returned to baseline levels 24 h after all three treadmill-running protocols.

Lymphocytes released into the bloodstream during exercise express high levels of CD18 (β_2 integrin) and the extent of their mobilisation is influenced by the intensity of exercise. This finding is supported by studies that have examined other subunits of β_2 integrin on the surface of other leukocyte populations. CD11b associates with CD18 to form a heterodimer known as Mac-1, which facilitates the activation and extravasation of blood lymphocytes (Lee and Corry 2004). Jordan et al. (1999) reported that the CD11b expression on granulocytes increased immediately after an incremental maximal treadmill test and a competitive marathon race but not after a 3 h run at a moderate intensity. This suggests that the release of granulocytes expressing CD11b into

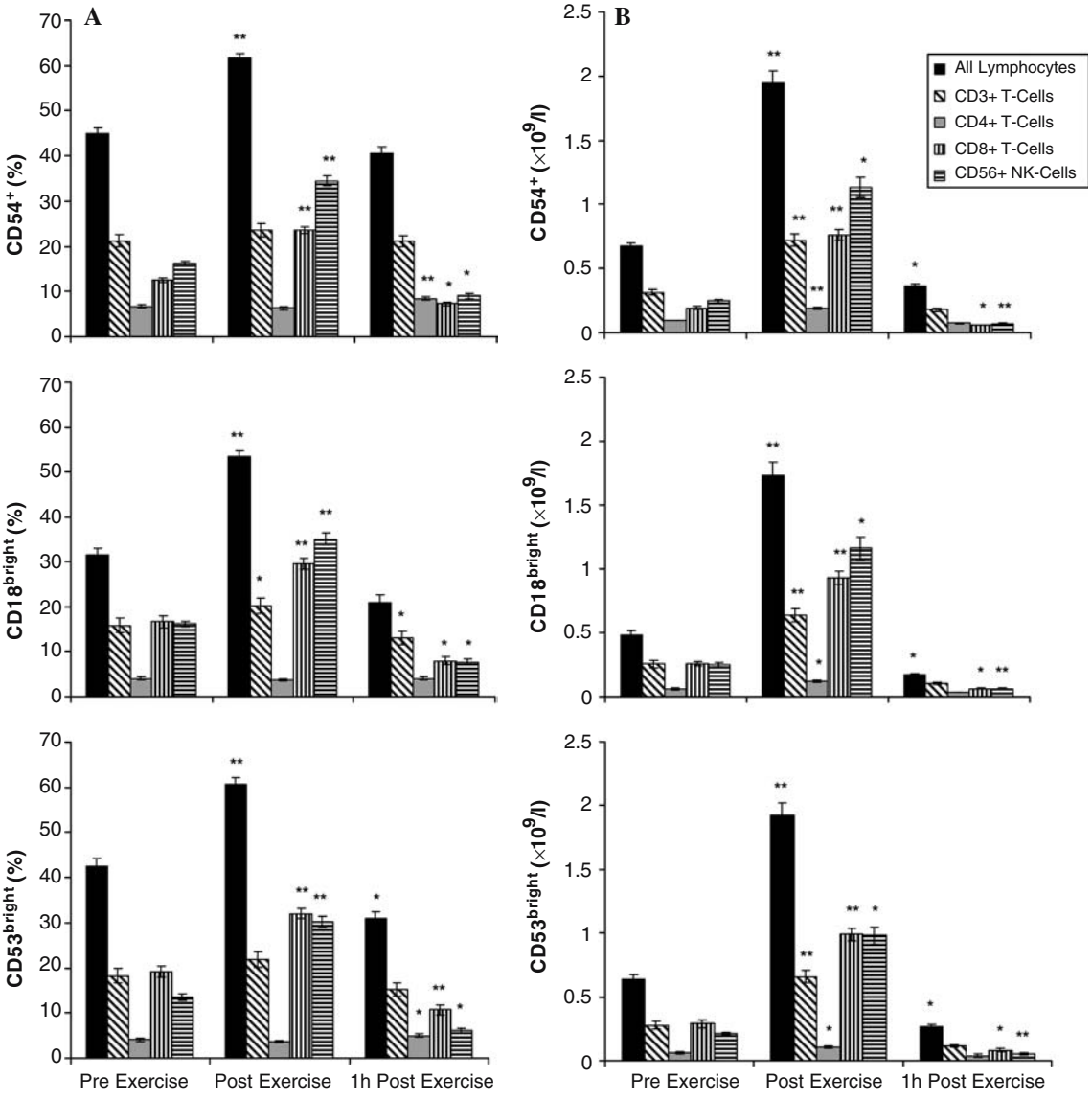

Fig. 5 The percentage of all peripheral blood lymphocytes (**a**) and total number of lymphocytes (**b**) expressing the cell surface AA molecules CD54 (ICAM-1)$^{+}$, CD18 (β2 integrin)^{bright} and CD53^{bright} and the lymphocyte subset markers CD3, CD4, CD8 and CD56 in response to an intensive treadmill-running protocol at 80% $\dot{V}_{O_{2max}}$ to volitional exhaustion. Values are mean ± SE. Statistically significant differences from the pre-exercise value indicated by *($P < 0.05$) and **($P < 0.01$)

the bloodstream during exercise may be influenced by the intensity of exercise performed. Further, following competitive endurance events such as marathon running, lymphocyte expression of CD11b has been reported to increase (Nielsen and Lyberg 2004).

The present study found no change in the numbers or percentage of lymphocytes expressing CD18^{bright} immediately after the moderate treadmill-running protocol, whereas Jordan et al. (1997) previously reported that the expression of CD18 on lymphocytes is decreased after 3 h running at a moderate intensity. This discrepancy may be related to the large differences in exercise duration between the two studies. As such, the trafficking of blood lymphocytes expressing high levels of CD18 may vary during exercise depending on the volume (intensity × duration) of exercise performed. Lymphocytes are reported to travel at a speed of 0.5–5 mm s^{-1} in the small blood vessels where extravasation occurs (Jalkanen and Salmi 1999). Due to the high velocity of blood flow associated with increases in cardiac output, the speed of lymphocyte travel in some tissues is likely to increase markedly during exercise, making it more difficult for the cells to bind to their corresponding ligands on the endothelium in a shear resistant manner. Therefore, even lymphocytes that express high levels of CD18 may not be capable of leaving the bloodstream during high-intensity exercise protocols, and cessation of exercise may be required before their demargination can take place. In contrast, during moderate intensity exercise, the velocity of blood flow may be slow enough to allow

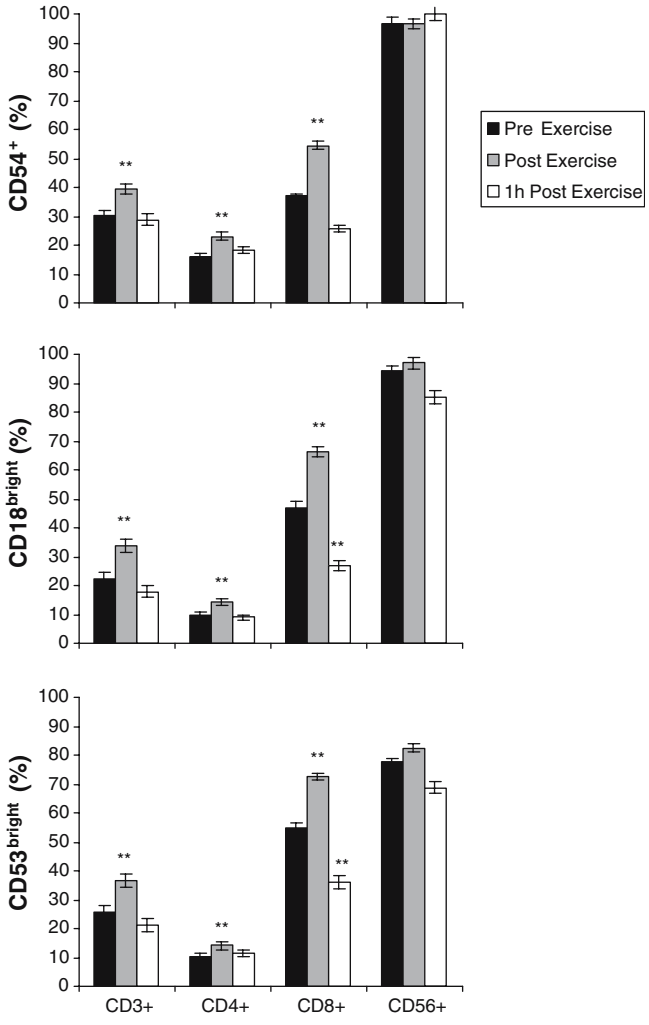

Fig. 6 The percentage of all $CD3^+$, $CD4^+$, $CD8^+$ and $CD56^+$ blood lymphocyte subsets expressing the cell surface AA molecules CD54 $(ICAM\text{-}1)^+$, CD18 ($\beta 2$ integrin)^{bright} and $CD53^{bright}$ before and after an intensive treadmill-running protocol at 80% $\dot{V}_{O_{2max}}$ to volitional exhaustion. Values are mean ± SE. Statistically significant differences from the pre-exercise value indicated by *($P < 0.05$) and **($P < 0.01$)

$CD18^{bright}$ lymphocytes to adhere firmly to the vessel walls and their subsequent demargination could take place during the exercise protocol itself. However, as the numbers and percentage of $CD18^{bright}$ lymphocytes did not change immediately after the moderate protocol in the present study, any movement of these cells out of the bloodstream may only occur during moderate intensity exercise if the protocol is of a longer duration. This may explain why Jordan et al. (1997) reported a decreased lymphocyte expression of CD18 immediately post-exercise. It is not known, however, if a lymphocytopenia occurred immediately after this protocol, as the total lymphocyte counts were not reported (Jordan et al. 1997). Although it is suggested that an increase in the rate of blood flow could affect the ability of lymphocytes to extravasate from the bloodstream during exercise, it would depend on where these cells were destined to migrate. For instance, blood flow rate through the capillaries is reduced in some tissues during exercise where vasoconstriction occurs such as the gut, the liver and the kidneys.

In addition to the mobilisation of $CD18^{bright}$ lymphocytes immediately after intensive exercise, both treadmill-running protocols conducted at an intensity of 80% $\dot{V}_{O_{2max}}$ also resulted in an increase in the numbers and percentage of $CD54^+$ and $CD53^{bright}$ lymphocytes, whereas no change was found after the moderate protocol. An increase in $CD54^+$ lymphocytes following exercise has been reported previously. Goebel and Mills (2000) and Kuhlwein et al. (2001) found that the total number of $CD54^+$ lymphocytes increased after ergometry based cycling for 15 to 18 min at a workload corresponding to 75% of the estimated $\dot{V}_{O_{2max}}$ Perez et al. (2001) reported an increase in the total number of $CD54^+$ cells in response to 10×2 min periods of high-intensity cycle ergometry and a 90-min-soccer practice in a group of children aged 9 to 15 years. In the present study, a significant reduction in the numbers of $CD54^+$ and $CD53^{bright}$ lymphocytes was observed at 1 h post-exercise following all three treadmill-running protocols, suggesting that even moderate exercise is enough to elicit a lymphocytopenia of $CD54^+$ and $CD53^{bright}$ blood lymphocytes. As a corresponding reduction in the total blood lymphocyte count was only observed after the two protocols conducted at 80% $\dot{V}O_{2max}$, a lymphocyte population, i.e. $CD54^-$ and/or $CD53^{dim}$ may leave the bloodstream after intensive but not moderate exercise. Therefore, as lymphocyte populations with a $CD54^-$ and/or $CD53^{dim}$ phenotype may not be as efficient at leaving the bloodstream, their subsequent demargination following acute bouts of exercise may require physiological signals that facilitate extravasation in an exercise-intensity dependent manner, such as cortisol or epinephrine (Gabriel and Kindermann 1998). In addition, intensive but not moderate exercise may increase the expression of other AA molecules that facilitate extravasation on these lymphocyte populations.

In contrast to the present data, the only study to measure lymphocyte CD54 expression during the recovery phase of exercise found no lymphocytopenia and no reduction in the numbers of $CD54^+$ lymphocytes (Goebel and Mills 2000). In addition to the discrepancies in exercise modality, intensity, duration and subject fitness levels, sampling at 15 min post-exercise in the study conducted by Goebel and Mills (2000) may have been too soon to document post-exercise lymphocytopenia. Hong et al. (2005) recently reported that the demargination of selected lymphocyte subsets, and lymphocyte subsets expressing the AA molecule CD62L, was attenuated in trained versus untrained subjects exercising at the same relative intensity.

The exercise-induced alterations of $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ blood lymphocytes reported in this study may reflect changes in AA molecule expression on a per cell basis and/or a preferential trafficking of specific lymphocyte subset populations

expressing high levels of AA molecules. Lymphocytes are a heterogeneous group of cells that are identified by their cell surface expression of distinct glycoproteins. In response to the intensive treadmill-running protocol, all lymphocyte subsets were mobilised into the bloodstream immediately after exercise, but the relative proportion of each particular subset was markedly altered. In comparison to the $CD3^+$ and $CD4^+$ cell populations, greater relative numbers of $CD8^+$ and $CD56^+$ cells were mobilised and subsequently removed from the bloodstream in response to the intensive exercise protocol. The $CD8^+$ and $CD56^+$ cells were also found to express greater levels of CD54, $CD18^{bright}$ and $CD53^{bright}$ than the $CD3^+$ and the $CD4^+$ cell populations. Therefore, alterations of the $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ cell populations observed in the total lymphocyte pool is likely to reflect an altered distribution of lymphocyte subset populations in response to exercise, rather than an increased expression of AA molecules on a per cell basis.

In an attempt to gain a better understanding as to how exercise effects the AA molecule expression on specific lymphocyte subset populations, the effects of intensive exercise on the percentage of all $CD3^+$, $CD4^+$, $CD8^+$ and $CD56^+$ lymphocytes expressing AA molecules was analysed. Increases in the percentages of all $CD4^+$ and $CD8^+$ cells expressing $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ were found immediately after the intensive exercise protocol. However, it is not known from this observation whether this increase in AA molecule expression reflects alterations on a per cell basis or some heterogeneity between the exercise-induced mobilised and blood resident $CD4^+$ and $CD8^+$ cells. The percentage of all $CD56^+$ cells expressing $CD54^+$, $CD18^{bright}$ and $CD53^{bright}$ did not change in response to exercise. This suggests that the AA molecules expressed on $CD56^+$ cells were not altered on a per cell basis and that the $CD56^+$ cells that were mobilised into the bloodstream following exercise are of a similar phenotype to the blood resident $CD56^+$ cells.

Although likely to be influenced by expression of β_2 adrenergic receptors and an alteration of AA molecules (Gabriel and Kindermann 1998; Hay and Andrade 1998), the origin and destination of mobilised lymphocytes during and after exercise has not been established. Lymphocytes may enter the circulation from the lymph nodes or secondary lymphoid organs such as the spleen, before migrating to the tissues or returning to their pre-exercise destinations (Gabriel and Kindermann 1998; Hay and Andrade 1998). It has been suggested previously that reductions in the blood lymphocyte count after exercise may reflect an infiltration of these cells into damaged skeletal muscle (Pizza et al. 1995). To our knowledge, we have examined for the first time the effects of downhill treadmill running on lymphocytes expressing AA molecules. This protocol was included to induce skeletal muscle damage in an attempt to gain an insight to the destination of peripheral blood lymphocytes during periods of exercise-induced lymphocytopenia. If lymphocytes were destined to migrate towards damaged muscle then it might be postulated that the alteration of cell surface AA molecules, lymphocytosis and subsequent lymphocytopenia would be different to that observed after a non muscle-damaging protocol at the same relative intensity and duration. Pizza et al. (1995) previously reported a greater lymphocytosis but a similar extent of lymphocytopenia following downhill relative to level running. The downhill treadmill-running protocol in the present study induced muscle damage, evident by the increases in plasma CK activity; however, there were no differences in total lymphocyte counts or expression of AA molecules in comparison to the non-muscle damaging protocol at the same relative intensity and duration. It is therefore unlikely that exercise-induced lymphocytopenia reflects an infiltration of blood lymphocytes into damaged skeletal muscle. Indeed, using multiple biopsies, Malm et al. (2000) reported that eccentric cycling exercise did not result in T-cell infiltration in human skeletal muscle. More invasive procedures may be required in order to fully elucidate the destination of AA positive lymphocytes following exercise and are unlikely to be possible in a human model. Infusing radiolabelled AA positive lymphocytes into animals following exercise to establish their sequestration in the tissues would prove illuminating.

This study examined the effects of intensive, moderate and downhill treadmill running on peripheral blood lymphocyte counts, and analysed cellular expression of the AA molecules CD54, CD18 and, for the first time, the tetraspan molecule CD53. The expression of lymphocyte AA molecules and the interaction with their ligands on the vascular wall facilitates the extravasation of blood lymphocytes to areas of infection or inflammation. As the lymphocyte populations that are redistributed during and after exercise have a high AA molecule phenotype, particularly the $CD8^+$ and $CD56^+$ cell populations, post-exercise lymphocytopenia is likely to reflect an extravasation of specific lymphocyte populations out of the bloodstream. In conclusion, the results of this study suggest that the population of lymphocytes that enter and subsequently exit the bloodstream in response to an acute bout of exercise express high levels of the cell surface AA molecules CD54, CD18 and CD53. This effect appears to be influenced by the intensity of exercise and not related to exercise-induced muscle damage. Future research should investigate the homing destination of AA positive blood lymphocytes following exercise.

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