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Apoptosis Does not Contribute to the Blood Lymphocytopenia Observed After Intensive and Downhill Treadmill Running in Humans

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APOPTOSIS DOES NOT CONTRIBUTE TO THE BLOOD LYMPHOCYTOPENIA OBSERVED AFTER INTENSIVE AND DOWNHILL TREADMILL RUNNING IN HUMANS

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The lymphocytopenia that occurs during the recovery stage of exercise may be a result of apoptosis through an increased expression of CD95, a loss of the complement regulatory proteins CD55 and CD59, or both. Trained subjects completed intensive, moderate, and downhill treadmill-running protocols. Blood lymphocytes isolated before, immediately after, 1h after, and 24h after each exercise test were assessed for markers of apoptosis (Annexin-V$^+$, HSP60$^+$), and CD55, CD59, and CD95 expression by flow cytometry. Lymphocytopenia occurred 1h after intensive and downhill running exercise, but no changes in the percentage of Annexin-V+ or HSP60+ lymphocytes were found. Numbers of CD95$^+$, CD55^{dim}, and CD59^{dim} lymphocytes increased immediately after intensive and downhill exercise, which were attributed to the selective mobilization and subsequent efflux of CD8$^+$ and CD56$^+$ lymphocyte subsets. No differences were found between the intensive and downhill protocols. In conclusion, apoptosis of circulating lymphocytes does not appear to contribute to exercise-induced lymphocytopenia.

Keywords: lymphocytosis, CD antigens, muscle damage, heat-shock protein-60, complement regulatory proteins, flow cytometry

INTRODUCTION

Acute bouts of strenuous prolonged submaximal exercise are known to elicit a biphasic perturbation of the blood lymphocyte count. An initial lymphocytosis occurs during and immediately after exercise followed by a rapid lymphocytopenia in the recovery phase after cessation of exercise (Kendall et al. 1990; McCarthy and Dale 1988; Mooren et al. 2002; Simpson et al. 2006; Steensberg et al. 2002). Although it is known that an exercise-induced increase in the secretion of catecholamines and cortisol largely influences the mobilization of lymphocyte subset populations into the blood compartment (Nielsen 2003), the mechanisms underlying the rapid removal of these same cell populations during the recovery phase of exercise are not as well understood.

Apoptosis, or programmed cell death, is a normal physiological function essential for the homeostasis of immunohaemopoietic tissues. For example, apoptosis is critical for curtailing T-lymphocyte clonal expansion following the resolution of viral infections (Ginaldi et al. 2004). The initiation of apoptosis is dependent on a variety of signals, many of which can be modulated by strenuous exercise (Fehrenbach and Northoff 2001; Phaneuf and Leeuwenburgh 2001). Consequently, it has been suggested that apoptosis contributes to the loss of blood lymphocytes after exercise (Mars et al. 1998; Mooren et al. 2002, 2004), possibly via signaling the cell surface death receptor CD95 (Fas/Apo-1; Mooren et al. 2002, 2004), resulting in postexercise lymphocytopenia, which could lead to lowered

immunity in athletes performing frequent and physically demanding training regimens. Unfortunately, studies that have examined the effects of exercise on the extent of blood lymphocyte apoptosis in humans are few and have been inconsistent, thus making it difficult to draw any definitive conclusions (Mars et al. 1998; Mooren et al. 2002, 2004; Steensberg et al. 2002; Wang and Huang 2005).

Heat shock protein 60 (HSP60) is an endogenous protective protein that is found predominantly in the cell mitochondria, although 15%–20% of the total cellular HSP60 content can be located outside the mitochondria (Gupta and Knowlton 2002; Kirchhoff et al. 2002). In the normal cell, the interaction between HSP60 and the proapoptotic gene *bax* is critical in preventing apoptosis (Gupta and Knowlton 2002; Kirchhoff et al. 2002). HSP60 interacts with *bax* within the cytosol of the cell, and the translocation of HSP60 to the cell surface precipitates apoptosis by allowing any unbound *bax* to migrate to the mitochondria, resulting in the release of cytochrome c, activation of caspase 3, and subsequent DNA fragmentation (Gupta and Knowlton 2002; Kirchhoff et al. 2002). In an exercise context, apoptotic lymphocytes have, for the most part, been quantified by fluorescently labeled Annexin-V binding to cell-surface phosphatidylserine exposure (Mooren et al. 2002, 2004; Steensberg et al. 2002; Wang and Huang 2005). No study to date has examined HSP60 expression as a marker of blood lymphocyte apoptosis after exercise.

The fate of a cell largely is influenced by the activation of complement—a central component of the innate immune system with the ability to lyse bacteria and autologous cells via the formation of the membrane attack complex (MAC; Cole and Morgan 2003). The complement components C5b, C6, C7, C8, and C9 combine to create the MAC, which forms "pores" on the membrane of the target cell, altering its permeability leading to subsequent lysis by colloid osmosis (Koski et al. 1983). Nonspecific binding of complement components to autologous cell membranes can lead to formation of the MAC and subsequent cell lysis (Davies and Lachmann 1993). Normal cells are protected against the lytic effects of complement by regulatory proteins bound to the cell surface. Membrane regulators of complement include decay accelerating factor (DAF; CD55) and MAC inhibiting factor (MACIF; CD59), which are anchored to the lipid cell membrane by glycosylphosphatidyinositol (GPI) anchors. These cell surface glycoproteins protect normal cells from complement-mediated lysis by binding to and dissociating certain components of the complement cascade. CD55 accelerates the decay of C3 convertase enzymes, whereas CD59 inhibits the final stages of MAC assembly by binding to the C5b-8 complex to prevent association with the pore-forming C9 component (Rollins and Sims 1990). It has been reported that blood lymphocytes of patients suffering from Epstein-Barr virus infection, paroxysmal

nocturnal haemoglobinurea, and Sjögren's syndrome show a diminished expression of membrane-bound complement regulatory proteins relative to healthy controls (Cui et al. 2004; Kawano et al. 1997; Tsunoda et al. 2000), with CD59 deficient cells being more susceptible to in vitro apoptosis than their high CD59 expressing counterparts (Tsunoda et al. 2000). The influence that exercise has on lymphocyte membrane expression of complement regulatory proteins has not been studied. A loss of these protective glycoproteins after exercise may leave lymphocytes susceptible to autologous complement attack, resulting in cell death and subsequent lymphocytopenia.

Eccentric exercise protocols such as downhill treadmill running consistently have been used as a model to induce muscle damage and inflammation (Pizza et al. 1995; Smith et al. 1998). In comparison to concentric exercise, eccentric activity has been shown to result in a greater release of immune system modulators such as proinflammatory cytokines, acute phase proteins, and the recruitment of phagocytic cells with the potential to release ROS (Malm et al. 1999; Tidball 1995). These signals have the potential to induce apoptosis and as such lymphocyte apoptosis and changes in cell surface expression of complement regulatory proteins may result from eccentrically biased exercise protocols.

The aims of this study were to assess in trained subjects (1) the relationship between lymphocyte apoptosis and exercise-induced lymphocytopenia, (2) the expression of the cell surface death receptor CD95, and (3) for the first time the investigation of lymphocyte cell surface expression of the GPI-anchored complement regulatory proteins CD55 and CD59 in response to intensive, moderate, and muscle-damaging (downhill running) exercise.

METHODS

Subjects

Eight aerobically trained male subjects volunteered to participate in this study (mean ± SD age: 28.2 ± 5.3 years, height: 176.6 ± 3.8 cm, mass: 70.1 ± 4.8 kg, $\dot{V}O_{2max}$: 63 ± 3 ml·kg^{-1}·min^{-1}). The subjects recruited for all of the experiments described in this article were healthy, actively engaging in endurance-based physical activity, not taking any medication, and were free of any infectious illness for 6 wk prior to their participation in the study. Institutional ethical approval was obtained, and each subject provided written informed consent. All subjects were required to refrain from any strenuous physical activity for 48h before each testing protocol and until the last postexercise blood sample was obtained.

Experimental Design. The maximal oxygen uptake ($\dot{V}O_{2max}$) of all subjects was assessed using an incremental running test on a motorized treadmill (Woodway, ergo ELG 55, Weil am Rhein, Germany) following the protocol described by Simpson et al. (2006). Oxygen uptake (breath by breath) was measured during the test using online gas analysis (CPX MedGraphics, Oldham, UK), and heart rate was recorded every 5s (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 subjects were 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 a downhill treadmill running protocol that required the subjects to run on a –10% gradient at an intensity of 80% $\dot{V}O_{2max}$ for the same duration as the intensive and moderate protocols. The speed of the treadmill during the downhill protocol was adjusted to mimic the same physiological response as the intensive protocol at 80% $\dot{V}O_{2max}$ (controled using heart rate values). All tests were conducted at the same time of day and were performed one week apart.

Intravenous blood samples were collected in 6-ml vacuum tubes containing lithium heparin as an anticoagulant (Becton-Dickinson, Oxford, UK) before, immediately after, 1h after, and 24h after exercise. The methods used in the current study to determine total and differential leukocyte counts and isolate lymphocytes from peripheral blood have been described elsewhere (Simpson et al., 2005, 2006). Whole blood was centrifuged for 10 min at 1000g, and the removed plasma was stored at –80°C until analysis for creatine kinase (CK) and C-reactive protein (CRP).

Detection of Lymphocyte Apoptosis and Necrosis. Annexin-V-Fluos labeling solution (Roche Diagnostics, Mannheim, Germany) was prepared by adding 20 µl of Annexin-V Fluos labeling reagent and 20 µl propidium iodide solution to 1 ml H-HEPES incubation buffer for the detection of apoptotic and necrotic cells, respectively. Isolated lymphocytes (1×10^6) were stained with 100 µl Annexin-V-Fluos labeling solution and incubated at room temperature for 15 min before resuspension in 0.5 ml H-HEPES incubation buffer. Analysis was conducted by 2-color flow cytometry. As flow cytometry involves the analysis of cell populations based on their light scatter properties, it is possible that the morphological shape changes that occur during apoptosis could alter the scatter properties of the cells. To ensure that apoptotic cells would still be detected in the analytical gate of the flow cytometer, blood lymphocytes treated with the apoptosis-inducing drug camptothecin were used in the assay as a positive control.

Detection of Cell Surface CD95 (Fas/Apo-1) Expression. The lymphocyte expression of CD95 was assessed using direct immunofluorescence assays. FITC-conjugated mouse IgG1 antihuman Fas monoclonal antibody (mAb) was diluted with phosphate buffered saline + 1% bovine serum albumin (PBS-BSA) to a working ratio of 1:50. Isolated lymphocytes (0.5×10^6) were labeled with 10 µl of the diluted antibody or an equal concentration of mouse IgG1 isotype control for 45 min at room temperature. After incubation, lymphocytes were resuspended in 0.5 ml PBS-BSA and analyzed by one color flow cytometry. Both mAbs were purchased from Sigma (Dorset, UK).

Detection of Cell Surface CD55, CD59, and HSP60 Expression. Lymphocyte cell surface expression of CD55, CD59, and HSP60 was assessed using indirect immunfluorescence assays. All antibodies were titrated to determine optimal conditions for analysis by flow cytometry. Isolated lymphocytes (0.5×10^6) were incubated for 45 mins at room temperature with the following primary mAbs: CD55 (NL120; Fifth International Leukocyte Typing Workshop, Boston, USA), CD59 (313.710; made by Dr. Robin Fraser, Scottish National Blood Transfusion Service and characterized by Dr. Keith Guy, Napier University, Edinburgh, UK) and HSP60 (BOB78; provided by Dr. Jim Ross, University of Edinburgh, Edinburgh, UK). The antiphycoerythrin 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 antimouse IgG secondary antibody (Sigma, Dorset, UK) for 30 mins. After incubation, cells were washed twice, resuspended in 0.5 ml PBS-BSA, and analyzed by one-color flow cytometry.

Analysis of Cell Surface Glycoproteins on Lymphocyte Subsets. A further eight subjects with similar physical 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 analyze the effects of exercise on the lymphocyte subset expression of cell surface glycoproteins. Each subject completed an initial assessment of $\dot{V}O_{2max}$, following the same protocol as the previous subjects. One week later, subjects completed the intensive treadmill running protocol at 80% $\dot{V}O_{2max}$ until exhaustion only (mean running time: 40.0 ± 19.4 min). Intravenous blood samples were obtained before, immediately after, and 1h after completion of the intensive treadmill running protocol.

The expression of CD55, CD59 and CD95 on $CD3^+$, $CD4^+$, $CD8^+$, and $CD56^+$ lymphocyte subset populations was determined using direct immunofluorescence assays. Isolated lymphocytes (0.5×10^6) were incubated for 45 mins at room temperature, with one of the following FITC

conjugated mAbs: CD55 IgG2a (BD Pharmingen, CA, USA), CD59 IgG2a (Immunotools, Friesoythe, Germany), or CD95 IgG1 (Sigma, Dorset, UK), and one of the following PE conjugated mAbs: CD3 IgG1, CD4 IgG1, CD8 IgG2a, and CD56 IgG1 (all purchased from Immunotools, Friesoythe, Germany). 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 analyzed by two-color flow cytometry. All mAbs used previously were titrated to determine optimal conditions for analysis by flow cytometry.

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 following methods previously described (Simpson et al. 2006).

Biochemical Analysis. The total CK activity of plasma was determined using a standard laboratory kit in accordance with the manufacturer's instructions (Instrumentation Laboratory, MA, USA). Plasma CRP activity was determined by ELISA following methods previously described (Simpson et al. 2005).

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. The assumption of sphericity for the ANOVA was tested using Mauchley's method, and any violation of the assumption was corrected for using the Huynh-Feldt method. Statistical significance was accepted at $p < 0.05$.

RESULTS

All subjects successfully completed each of the three treadmill running protocols. Changes in blood leukocyte counts, lymphocyte counts, plasma CK activity, and CRP concentration are presented in Table 1. The blood lymphocyte count increased immediately after all three treadmill-running protocols, with the extent of lymphocytosis being more pronounced after the two protocols conducted at 80% $\dot{V}O_{2max}$ (intensive and downhill). Lymphocytopenia was observed 1h after exercise for the intensive and downhill protocols only, before returning to baseline levels 24h later.

Table 1. Total Blood Leukocyte Counts, Lymphocyte Counts, Plasma Creatine Kinase (CK) Activity, and c-reactive Protein (CRP) Concentration in Response to Intensive, Moderate and Downhill Treadmill Running (mean ± SD)

	Pre	Post	1h Post	24h Post
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 ($IU . l^{-1}$)	141 ± 47	184 ± 69	184 ± 37	218 ± 143
CRP ($mg . l^{-1}$)	0.40 ± 0.32	0.41 ± 0.32	0.37 ± 0.24	0.40 ± 0.3
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 ($IU . l^{-1}$)	167 ± 113	167 ± 49	145 ± 52	152 ± 108
CRP ($mg . l^{-1}$)	0.37 ± 0.30	0.42 ± 0.16	0.37 ± 0.40	0.29 ± 0.32
Downhill 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*
CRP ($mg . l^{-1}$)	0.36 ± 0.32	0.41 ± 0.16	0.38 ± 0.40	0.70 ± 0.30*

*Indicates statistically significant difference from preexercise values ($p < 0.05$)

Plasma CK activity and CRP concentration increased 24h after the downhill protocol only ($p < 0.05$).

Apoptotic lymphocytes in response to exercise were identified as being Annexin-V^+/PI^- or $HSP60^+$ (Figure 1). Only very low levels of apoptotic lymphocytes were found in the bloodstream, and these did not change in response to exercise ($p > 0.05$). Staining the cells with PI revealed no necrotic lymphocytes to be present in the bloodstream (data not shown). The number and percentage ($p < 0.05$) of blood lymphocytes expressing CD95 increased immediately after the intensive and downhill treadmill running protocols (Figure 2), followed by a reduction in the number of $CD95^+$ lymphocytes 1h later ($p < 0.05$).

Analysis by flow cytometry revealed that ~98% of all lymphocytes were positive for the complement regulatory proteins CD55 and CD59 before and after all three protocols. Two distinct cell populations with strikingly different fluorescent intensities ("dim" or "bright") for CD55 and CD59 expression, however, were observed, and electronic markers were placed over the CD55 and CD59 "dim" populations on the flow cytometry histogram. Significant increases ($p < 0.01$) in the number and percentage of $CD55^{dim}$ and $CD59^{dim}$ lymphocytes were observed immediately postexercise for the intensive and downhill protocols only (Figure 3). The number of $CD55^{dim}$ and $CD59^{dim}$ lymphocytes fell below the

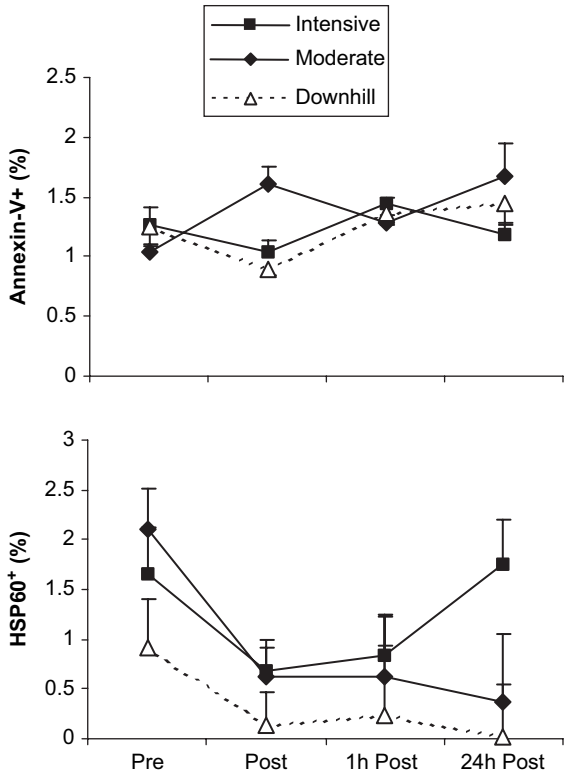

Figure 1. Cells undergoing apoptosis were assessed by Annexin-V binding to cell surface phosphotydylserine exposure and HSP60 expression. No statistically significant changes in the percentage or total number (data not shown) of apoptotic lymphocytes were found in response to intensive, moderate, or downhill treadmill-running ($p > 0.05$).

preexercise values 1h later for all treadmill running protocols ($p < 0.01$), before returning to basal levels 24h after.

The total number of blood lymphocyte subsets expressing CD55, CD59, and CD95 are shown in Table 2. Total numbers of $CD3^+$, $CD8^+$, and $CD56^+$ lymphocytes increased immediately postexercise, with all lymphocyte subset numbers falling below the preexercise values 1h later ($p < 0.05$). The observed changes in cell number were more pronounced for the $CD8^+$ and the $CD56^+$ cells than for the other lymphocyte subsets, resulting in an alteration of the lymphocyte subset distribution following the exercise protocol.

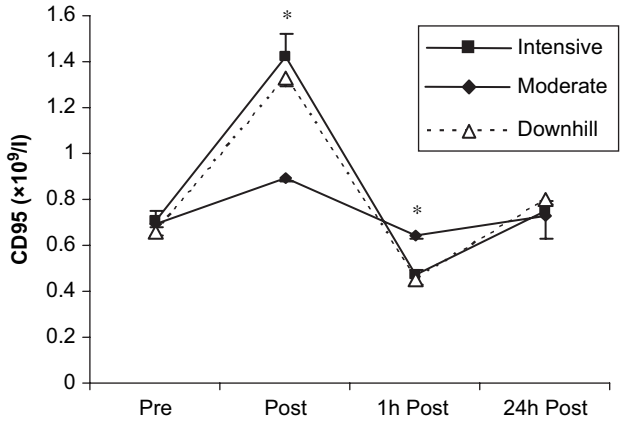

Figure 2. The total number of blood lymphocytes expressing the cell surface death receptor CD95 (Fas/Apo-1) before and after intensive, moderate, and downhill treadmill-running (mean ± SE). *Indicates statistically significant difference from the preexercise value for the intensive and downhill treadmill-running protocols only ($p < 0.01$).

The numbers of lymphocyte subsets expressing the measured cell surface antigens in response to the intensive treadmill running protocol are shown in Figure 4. The numbers of $CD3^+$, $CD4^+$, $CD8^+$, and $CD56^+$ lymphocytes expressing $CD59^{dim}$ were found to increase immediately after the exercise protocol ($p < 0.05$), with the numbers of $CD59^{dim}/CD56^+$ cells falling below the preexercise values 1h later ($p < 0.05$). The numbers of $CD55^{dim}/CD8^+$ and $CD55^{dim}/CD56^+$ lymphocytes also increased immediately after exercise before falling below the preexercise values 1h later ($p < 0.01$). The numbers of $CD95^+/CD3^+$, $CD95^+/CD8^+$, and $CD95^+/CD56^+$ lymphocytes increased immediately postexercise, with the numbers of $CD95^+/CD56^+$ cells falling below the preexercise values 1h later ($p < 0.05$).

The percentage of all $CD3^+$, $CD4^+$, $CD8^+$, and $CD56^+$ lymphocytes expressing the cell surface death receptor CD95 and low levels of the complement regulatory proteins CD55 and CD59 (i.e., "dim") are shown in Figure 5. The percentage of all $CD3^+$, $CD4^+$, and $CD8^+$ populations expressing $CD55^{dim}$ and $CD59^{dim}$ increased immediately postexercise ($p<0.01$), whereas no changes were found for the $CD56^+$ cell population ($p>0.05$). No changes in the percentage of all $CD3^+$, $CD4^+$, and $CD8^+$ cells expressing $CD95^+$ were found after the exercise protocol. An increase in the percentage of all $CD56^+$ cells expressing $CD95^+$ was found at 1h postexercise.

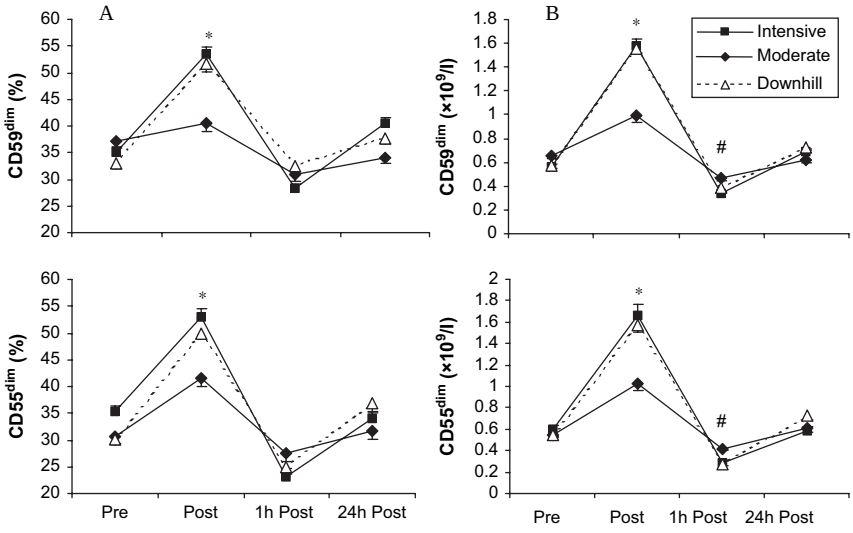

Figure 3. Changes in the percentage (A) and total number (B) of lymphocytes expressing low levels (i.e., "dim") of the complement regulatory proteins CD55 (DAF) and CD59 (MACIF) in response to intensive, moderate, and downhill treadmill-running (mean ± SE). *Indicates statistically a significant difference from preexercise for the intensive and downhill protocols only ($p < 0.01$). #Indicates statistically significant difference from preexercise for all protocols ($p < 0.01$).

Table 2. Total Lymphocyte and Lymphocyte Subset Counts in Response to the Intensive Treadmill-Running Protocol ($\times 10^9 . l^{-1}$)

	Pre	Post	Post 1h
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 preexercise values: *$p < 0.05$, **$p < 0.01$.

DISCUSSION

This study examined the effects of intensive, moderate, and downhill treadmill running on the extent of blood lymphocyte apoptosis and lymphocyte

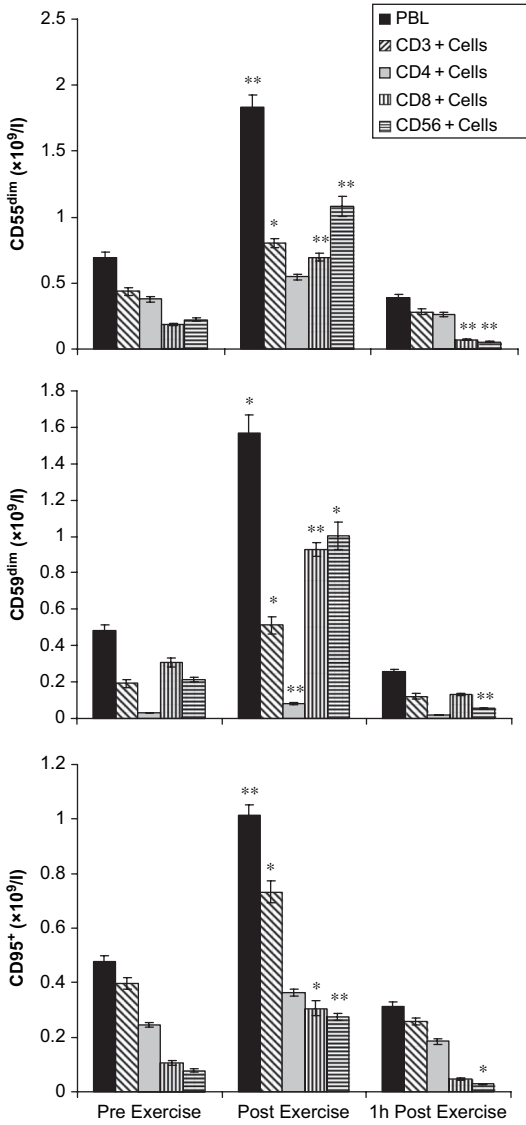

Figure 4. The total number of all peripheral blood lymphocytes (PBL) and $CD3_+$, $CD4_+$, $CD8_+$, and $CD56_+$ lymphocyte subsets expressing the complement regulatory proteins CD55 $(DAF)^{dim}$, CD59 $(MACIF)^{dim}$, and the cell surface death receptor CD95 (Fas/Apo-1) in response to an intensive treadmill-running protocol at 80% $\dot{V}O_{2max}$ to volitional exhaustion. Values are mean ±SE. Statistically significant differences from the preexercise value indicated by $^*(p < 0.05)$ and $^{**}(p < 0.01)$.

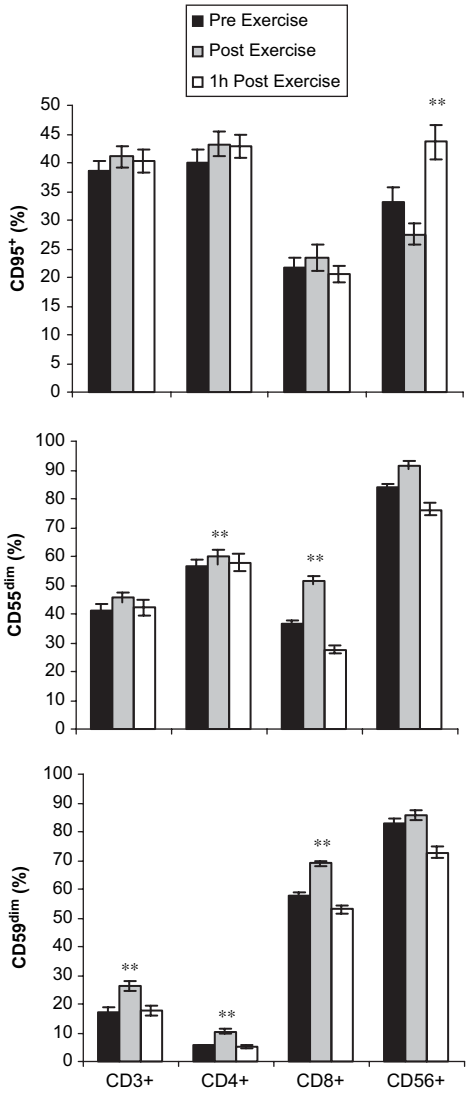

Figure 5. The percentage of all CD3+, CD4+, CD8+, and CD56+ blood lymphocyte subsets expressing the cell surface complement regulatory proteins CD55 (DAF)^{dim} and CD59 (MACIF)^{dim} and the cell surface death receptor CD95 (Fas/Apo-1)$^{+}$ before and after an intensive treadmill-running protocol at 80% $\dot{V}O_{2max}$ to volitional exhaustion. Values are mean ±SE. Statistically significant differences from the preexercise value indicated by $^{**}(p < 0.01)$.

cell surface expression of the GPI-anchored complement regulatory proteins CD55 (DAF) and CD59 (MACIF) and the cell surface death receptor CD95 (Fas/Apo-1) in trained subjects. The novel aspects of this study follow: (1) the examination of blood lymphocyte apoptosis after muscle-damaging (downhill running) exercise; (2) the use of HSP60 cell membrane expression as a marker of lymphocyte apoptosis after exercise; and (3) the examination of CD55 and CD59 membrane expression on blood lymphocytes in response to exercise. Despite observing a significant reduction in the total blood lymphocyte count 1h after the intensive and downhill treadmill running protocols, no evidence of lymphocyte apoptosis was found. Using three different modalities of exercise, therefore, we have found no evidence to suggest that apoptosis affecting blood lymphocytes contributes to exercise-induced lymphocytopenia. Changes in lymphocyte expression of CD95 and complement regulatory proteins are largely influenced by the preferential mobilization and subsequent removal of $CD8^+$ cells and $CD56^+$ cells from the blood compartment. As such, it is not likely that the expression of these cell surface glycoproteins are being altered at the cellular level in response to exercise.

The influence of exercise intensity on blood lymphocytosis and lymphocytopenia has been shown previously (Kendall et al. 1990; Mooren et al. 2002; Simpson et al. 2006). Mooren et al. (2002) reported a greater lymphocytosis and subsequent lymphocytopenia following exhaustive treadmill running at 80% $\dot{V}O_{2max}$ in comparison to a moderate protocol conducted at 60% $\dot{V}O_{2max}$. An increase in the percentage of apoptotic lymphocytes was found only after the exhaustive protocol (Mooren et al. 2002). It is important to note, however, that the values for apoptotic cells reported by Mooren et al. (2002) were very small and were unlikely to account for the large numbers of lymphocytes typically lost from the bloodstream during the recovery phase of exercise. Indeed, a lymphocytopenia occurred after the moderate protocol despite no increase in the level of apoptosis (Mooren et al. 2002). In contrast, using comparable exercise protocols, the current study found no change in the percentage or total number of apoptotic lymphocytes after exercise. This discrepancy may be attributable to the fitness levels of the respective subjects as the participants in the current study had a mean $\dot{V}O_{2max}$ value of 63 $ml \cdot kg^{-1} \cdot min^{-1}$, whereas the subjects used in the study by Mooren et al. (2002) had a mean $\dot{V}O_{2max}$ value of 50 $ml \cdot kg^{-1} \cdot min^{-1}$. Indeed, Mooren et al. (2004) recently showed that untrained subjects are more susceptible to lymphocyte apoptosis following prolonged exercise than their more aerobically trained counterparts. Furthermore, Umegaki et al. (1998) documented slight chromosomal damage in blood lymphocytes of untrained but not trained subjects following 30 mins of treadmill running at 85% $\dot{V}O_{2max}$.

Despite many reports of exercise-induced DNA damage to blood leukocytes (Mars et al. 1998; Umegaki et al. 1998; Mastaloudis et al. 2004; Wang and Huang 2005) results from human studies examining the effects of exercise on blood lymphocyte apoptosis have been equivocal (Mars et al. 1998; Mooren et al. 2002; 2004; Steensberg et al. 2002; Wang and Huang 2005). Steensberg et al. (2002) reported no change in the total number of apoptotic blood lymphocytes isolated from trained subjects following 2.5 h of treadmill running at 75% $\dot{V}O_{2max}$. Conversely, using the TUNEL method, Mars et al. (1998) reported up to 95% of lymphocytes to be apoptotic immediately after and during the recovery phase of a maximal treadmill running protocol. These results, however, must be interpreted with caution due to the small sample size ($n=3$) and the high variation of apoptotic lymphocytes among subjects even in the preexercise values (Mars et al. 1998).

Other studies using human subjects that have examined the effects of exercise on blood lymphocyte apoptosis have assessed for phosphatidylserine exposure on the cell surface using Annexin-V (Mooren et al. 2002, 2004; Steensberg et al. 2002; Wang and Huang 2005). In addition to this method, we also examined cell surface expression of HSP60 as a marker of apoptosis (Gupta and Knowlton 2002; Kirchhoff et al. 2002). Similar to the Annexin-V method, only very small levels of lymphocytes expressing HSP60 were found in the bloodstream, and these did not change in response to any of the treadmill running protocols. In support of this, Fehrenbach et al. (2000) found no discernible HSP60 expression on the surface of lymphocytes before or after a half-marathon race. Despite the high-intensity treadmill protocols used in the present study eliciting a lymphocytopenia during the recovery phase of exercise, the lack of an increase in Annexin-V^+ or $HSP60^+$ lymphocytes after exercise suggests that apoptosis affecting blood lymphocytes does not contribute to exercise-induced lymphocytopenia.

Lymphocytes expressing CD95 and low levels of CD55 and CD59 were mobilized into the blood compartment after the high-intensity exercise protocols, suggesting that these cells may be more susceptible to apoptosis and complement-mediated cell lysis than the lymphocytes in the blood at preexercise. Increases in lymphocyte CD95 expression after exercise has been reported previously (Mooren et al. 2002, 2004); however, it is difficult to determine if these changes are occurring on a per cell basis due to the changes in lymphocyte number and subset distribution that occur in response to exercise. Consistent with previous observations, it was found that the $CD8^+$ and $CD56^+$ lymphocyte subsets expressed both CD55 and CD59 at lower intensities than $CD3^+$ and $CD4^+$ lymphocytes (Nagakura et al. 1993; Solomon et al. 1995; Cui et al. 2004). As the relative change in number of $CD8^+$ and $CD56^+$ cells after exercise was greater than the change in $CD3^+$ and $CD4^+$ cells, the decreased expression

of CD55 and CD59 on the total lymphocyte population was expected due to the preferential mobilization of CD8+ and CD56+ cells by exercise. Increases in the percentage of all $CD4^+$ and $CD8^+$ lymphocytes expressing low levels of CD55 and CD59 that occurred after intensive exercise, however, may indicate a loss of GPI-linked proteins at the cellular level or heterogeneity in complement regulatory protein expression between blood resident and exercise-induced mobilized $CD8^+$ and $CD4^+$ lymphocytes. Future research should attempt to clarify if GPI-linked proteins are lost from lymphocytes on a per-cell basis following intensive exercise as this could have important implications for the immune health of elite athletes.

The increased number of $CD95^+$ lymphocytes after exercise was accounted for by an increase in the $CD95^+/CD8^+$ and $CD95^+/CD56^+$ cells, whereas the numbers of $CD95^+/CD4^+$ lymphocytes did not change. Furthermore, no changes in the percentage of all $CD3^+$, $CD4^+$, or $CD8^+$ lymphocytes expressing CD95 were found in response to exercise. Therefore, no evidence was found in this study to suggest that CD95 expression was being altered at the cellular level.

Despite evidence of muscle damage and a modest increase in plasma CRP activity after the downhill running protocol, no apoptotic blood lymphocytes were found. Furthermore, changes in the cell surface expression of CD55, CD59, and CD95 were not different from the non-muscle-damaging protocol of the same relative intensity and duration. This suggests that changes in blood lymphocyte populations expressing these cell surface glycoproteins are influenced by the intensity of exercise and not muscle damage.

In conclusion, apoptosis affecting blood lymphocytes did not contribute to the lymphocytopenia associated with the high-intensity and muscle-damaging exercise protocols used in this study, even when there was evidence of an inflammatory-like immune response. Furthermore, changes in lymphocyte expression of the cell surface death receptor CD95 do not appear to be occurring at the cellular level. As such, mechanisms other than cell death by apoptosis are likely to be responsible for the widely reported exercise-induced lymphocytopenia. An extravasation of particular lymphocyte subsets to other body compartments could be responsible (Simpson et al. 2006), and future studies should endeavor to identify the homing destination of blood lymphocytes after exercise.

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