Exercise Intensity and Lymphocyte Subset Apoptosis

J. W. Navalta1, S. Lyons2, J. Prestes1, S. W. Arnett2, M. Schafer2, G. L. Sobrero2

1 Kinesiology and Nutrition Sciences, University of Nevada, Las Vegas, Las Vegas, United States
2 Kinesiology, Recreation, and Sport, Western Kentucky University, Bowling Green, United States
1 Graduation program on Physical Education, Catholic University of Brasilia, Brasilia, Brazil

Abstract
This investigation assessed the lymphocyte subset response to increasing intensity. Participants completed an exertion test (VO2max), and later performed a 10-min run at 76% VO2max, 5-min at 87%, and run to exhaustion at 100% intensity. Blood was sampled at rest, following each intensity, and 1-h post. Cell concentration, apoptosis (annexin V) and migration (CXCR1) were evaluated in CD4+, CD8+, and CD19+ subsets. Relative data were analyzed using 1-way ANOVA with significance at P<0.05. Absolute changes from rest (Δ baseline) were calculated for exercise conditions. CX3CR1 displayed relative changes 1-h post, (CD8+ Pre = 58%, Post = 68%, 1 h-Post = 37%, P = 0.04) (CD19+ Pre = 1.9%, Post = 3.2%, 1 h-Post = 5.2%, P = 0.02). No relative changes were noted for subsets and annexin V. Absolute changes revealed that CD4+/annexin V+ and CD8+/annexin V+ significantly increased at 76%,(P<0.01). Significant absolute increases were observed in CD4+/CX3CR1 at 87% VO2max and at 87% and 100% VO2max in CD8+/CX3CR1 (P<0.01). Subsets respond differently with intensity with respect to cell count, and markers of apoptosis and cell migration. CD4+ and CD8+ appear to be prone to apoptosis with moderate exercise, but significant increases in migration at higher intensities suggests movement of these cells from the vasculature in postexercise measurements.

Introduction
The general immune response with intense acute physical exercise is an observed increase in lymphocytes followed by a decrease in the postexercise period, termed post-exercise lymphocytopenia [8,26]. Compared to other leukocyte subsets, the relative percentage of circulating lymphocytes has been shown to remain depressed for at least 2h following acute submaximal exercise [20]. It is possible that a portion of the reduction in lymphocytes observed following an exercise bout can be contributed to programmed cell death, or apoptosis. However, the ability of exercise to serve as a sufficient stimulus to induce apoptosis in peripheral blood lymphocytes has been the subject of recent debate. Some investigations have noted a significant increase at the cessation of exhaustive exercise suggesting the involvement of apoptosis [10,12,16], while others have not observed changes in the percent of apoptotic lymphocytes with exercise [22,26,27] suggesting mechanisms other than cell deletion as responsible for the observed lymphocytopenia in the postexercise period.

To our knowledge, our laboratory investigation has been the only one to report the stepwise effect of increasing intensity on lymphocyte apoptosis during an acute bout of exercise [16]. In that study, finger-stick samples were obtained and the morphological method of determining apoptosis was used in subsequent evaluation. We reported that exercise intensities greater than 76% VO2max displayed an incremental increase in the percentage of apoptotic lymphocytes culminating with the greatest values observed at maximal exertion. However, due to the nature of the technique utilized, determining the contribution of specific lymphocyte subsets was impossible. As investigations utilizing a morphological evaluation of apoptosis have generally found significant increases [10,14–16], while those using the early phase biomarker annexin V generally have not observed differences with exercise [22,25], one of our purposes was to replicate our previous study employing the aforementioned annexin V. In addition, lymphocytes are comprised of discreet subsets which have their own specialized function within the immune system (i.e., helper T cells, sup-
pressor T cells, and B cells). It seems intuitive that based on the highly specific nature of these cells, it is likely that each lymphocyte subset may respond to a physiological challenge such as exercise in a different manner, and that subfractions display a differing lymphocytopenia response in the post-exercise period. Cell adhesion molecules in T lymphocytes have been reported to increase with exercise [5,26] however the B-cell response remains uncharacterized. 20 min of moderate-intensity exercise (65–70% VO$_{2\text{max}}$) resulted in a significant increase in L-selectin (CD62L) and CD11a expression in CD4+ helper T cells and CD8+ cytotoxic T cells, and these cell adhesion molecules are associated with leukocyte capture and attachment to the endothelial surface of the blood vessel [5]. Intensive treadmill running (80% VO$_{2\text{max}}$) on a level or downhill grade (~10%) resulted in significant increases in the expression of $\beta_2$ integrin (CD118) and intracellular adhesion molecule-1 (CD54), which are also associated with cellular adhesion of lymphocytes to the endothelium [26]. Our laboratory has recently begun to investigate the effects of exercise on the cellular expression of the transmembrane chemokine hybrid fractalkine receptor CX$_3$CR1 [3], which has been utilized in investigations evaluating pathways regulating leukocyte migration [1,2,7]. We found that repetitive, supramaximal cycle bouts significantly increased the expression of the migration receptor CX$_3$CR1 in CD8+ T lymphocytes, but that the expression of the early phase apoptotic marker annexin V was unaffected [3].

To our knowledge, our previous morphological study [16] is the only investigation to have assessed lymphocyte apoptosis with increasing levels of exercise intensity, as well as into the post-exercise period. Based on the previous literature, it is apparent that the post-exercise lymphocytopenia response could be attributed to cellular migration from the vasculature, deletion of lymphocytes via apoptosis, or a combination of these mechanisms induced prior to the cessation of exercise. Currently, the contribution of exercise-induced cell death and/or cellular migration is unknown in lymphocyte subfractions. We hypothesize that there will be a differential contribution between the interplay of lymphocyte apoptosis (measured via annexin V) and extravasation into the lymphoid pools (measured by CX$_3$CR1 expression) for specific lymphocyte subfractions (CD4+, CD8+, and CD19+) with increasing levels of exercise intensity. Therefore, the purpose of this investigation was to determine how increasing levels of exercise intensity modulated markers of apoptosis and migration in the primary lymphocyte subsets.

Methods

Subjects

Participants consisted of males (N=12) with mean height = 178.3 ± 1.4 cm (SEM), weight = 89.3 ± 5.0 kg, age = 32 ± 1 years, and VO$_{2\text{max}}$ = 50.4 ± 1.8 ml kg$^{-1}$ min$^{-1}$. Prior to participation, subjects completed the Modified AHA/ACSM Health/Fitness Facility Preparticipation Screening Questionnaire. Participants were excluded from the study if reporting one or more of the history, symptoms, or other health issues categories; or if reporting 2 or more of the the cardiovascular risk factors. In addition, at the time of the investigation all subjects participated in regular exercise (1–2 h per session, at least 3–4 days per week). All participants voluntarily provided written informed consent, which was approved by the University’s Human Subjects Review Board. In addition, this investigation meets ethical standards established for sport and exercise science research [4].

Protocol

Participants reported to the laboratory on 2 occasions. During the first session, a test for aerobic capacity was completed (VO$_{2\text{max}}$). All participants were classified as “Good” or “Very Good” according to their aerobic capacity. Upon arriving at the Exercise Physiology Laboratory, resting measurements of height and weight were taken, and the metabolic analysis system (Vista Mini-CPX, Vacu-Med, Ventura, CA) was calibrated. Participants began the exercise period with a standardized warm-up of walking on the treadmill (T9700HRT, Vision Fitness, Lake Mills, WI) for 3 min at 80.5 m min$^{-1}$ (3 mph), and each subsequent stage was 3 min in length. The speed was then increased to 134.1 km min$^{-1}$ (5 mph), and then to 160.9 km min$^{-1}$ (6 mph). At this point, speed was held constant while percent grade was increased 2% with each successive stage until exhaustion. VO$_{2\text{max}}$ was assumed to have been achieved when 2 of the following criteria were met: volitional fatigue, an increase in workload with no increase in VO$_2$, RER greater than 1.15, heart rate within 10 b min$^{-1}$ of the estimated maximum for age.

The relative percentage of VO$_{2\text{max}}$ for each stage of the test was determined as (stage VO$_2$/VO$_{2\text{max}}$) × 100. Once these were calculated for each participant, common exercise intensities were established within ±5%. For our group of participants, the common exercise intensities corresponded to 76%, 87%, and maximal (i.e., 100%). The speed and grade combinations that corresponded to 76% and 87% VO$_{2\text{max}}$ were then determined for each participant. On the next visit to the laboratory at least one week later, after completing the standardized warm-up, participants ran for 10-min at 76% VO$_{2\text{max}}$ followed by a 5-min rest period. Next, a 5-min run was performed at 87% VO$_{2\text{max}}$ again followed by a 5-min rest. Finally, a run to exhaustion was performed as speed and grade were adjusted as described previously for the VO$_{2\text{max}}$ test (i.e., 100% VO$_{2\text{max}}$). The total time spent at this stage was 3.04 ± 0.92 min.

Blood processing

All flow cytometry antibodies and buffers were obtained from e-Bioscience (San Diego, CA) unless noted otherwise. Blood was sampled at rest, following the 76% and 87% runs, immediately after exercise (i.e., 100% VO$_{2\text{max}}$), and 1-h post exercise. Whole blood was obtained via finger-stick into heparinized capillary tubes (Globe Scientific Inc, Paramus, NJ) and processed as described previously [13]. Briefly, whole blood was added to panels containing staining buffer and tittered concentrations of antibodies including CD3, CD4, CD8, CD19, annexin V and CX$_3$CR1 (BioLegend, San Diego, CA) and incubated for 30 min in the dark at room temperature. Following this, samples were centrifuged at 3 000 × g for 10 min and then incubated at room temperature in red blood cell lysis buffer for 10 min before PBS (Sigma-Aldrich, St. Louis, MO) was added. Samples were centrifuged at 3 000 × g for 10 min, decanted, and then evaluated by flow cytometry (Accuri C6, Ann Arbor, MI).

Statistical analysis

Data for relative measures were analyzed using 1-way analysis of variance with significance accepted at the P ≤ 0.05 level (IBM SPSS 18.0, Somers, NY). Post hoc evaluations were completed using the Least Significance Difference test when appropriate. Absolute changes from rest (Δ baseline) values were calculated according to the following formula: [(measure-baseline) - baseline$^{-1}$] × 100. As we expected the absolute change from rest with regard to apoptotic and migratory markers to be similar to...
the change in cell volume, the $\chi^2$ test was utilized with statistical
significance accepted at the $P \leq 0.05$ level. Based on our previous
investigation [16], a large effect size was anticipated, but to use
a conservative approach, sample size was calculated using a
medium effect size corresponding to a 25% relative increase in
postexercise apoptosis. To detect this increase in lymphocyte
apoptosis, it was determined that a minimum of, at least, 8 sub-
jects was necessary.

Results

\[\text{CD4+}\]

CD4+ cells displayed an increase in cell count with exercise.
While slight changes were noted at the 76% and 87% intensities,
a significant increase was noted only at exhaustion (i.e., 100%
$V_{O2,\text{max}}$) ($P = 0.05$, \textit{Fig. 1}). No significant differences were
observed for the relative percent of annexin V+ ($P = 0.51$) or
CX3CR1+ ($P = 0.23$) cells in this lymphocyte subset.
However, when the absolute change from baseline was evalu-
at (i.e., $\Delta$ baseline), a significant change in CD4+/annexin V+
cells was observed following exercise at 76% $V_{O2,\text{max}}$
($P = 0.0001$, \textit{Fig. 2}). At 87% $V_{O2,\text{max}}$, the elevation in CD4+/annexin V+
cells remained ($P = 0.0001$), and an absolute increase
was noted in CD4+/CX3CR1 cells compared to baseline
($P = 0.001$, \textit{Fig. 2}).

\[\text{CD8+}\]

Similar to what was observed for CD4+ cells, CD8+ cell count
changed minimally at 76% and 87% $V_{O2,\text{max}}$, but was signifi-
cantly greater at exhaustion compared to all other time points
($P = 0.01$, \textit{Fig. 1}). A significant decrease in the relative
percent of expressed migration receptor CX3CR1 was observed in the 1-h
post condition compared to immediately following exercise
($P = 0.04$, \textit{Fig. 3a}). No significant differences were noted for the
relative percentage of annexin V+ cells ($P = 0.89$).
However, when the absolute number of CD8+/annexin V+ cells
for each exercise intensity was calculated as the difference from
baseline, a disproportionately greater increase in this apoptotic
marker was observed at 76% $V_{O2,\text{max}}$ and was significantly
greater ($P = 0.0001$) compared to the change observed in CD8+
cell count (\textit{Fig. 4}). At 87% and 100% of $V_{O2,\text{max}}$, CD8+/CX3CR1
cellular expression displayed the largest rise from baseline and
was significantly different compared to the expected rise in
CD8+ cell count (87% $P = 0.001$, 100% $P = 0.0001$, \textit{Fig. 4}).

\[\text{CD19+}\]

No significant differences were noted for CD19+ cell count
($P = 0.36$) or relative CD19+/annexin V+ cell expression with any
exercise intensity ($P = 0.62$). A significant increase was noted in
the percent of expressed CX3CR1 migration receptor at 1-h post
exercise compared to resting values ($P = 0.02$, \textit{Fig. 3b}).
Expressed as the absolute change from baseline, CD19+/CX3CR1+ cells
displayed a significantly greater increase at each level of exercise
intensity when compared to the associated change in cell count
(76% $P = 0.001$, 87% $P = 0.0001$, 100% $P = 0.001$; \textit{Fig. 5}). On the
other hand, CD19+/annexin V+ cells were unaffected at the 76%
$V_{O2,\text{max}}$ intensity, significantly increased during 87% $V_{O2,\text{max}}$
effort ($P = 0.001$), and then significantly decreased by the mea-
urement taken following exhaustive exercise ($P = 0.0001$).

Discussion

The purpose of this investigation was to evaluate the exercise-
induced apoptotic response in lymphocyte subsets with increasing
levels of intensity. The intent was to replicate our previous
study in which the morphological technique was employed [16],
and utilize the common early-phase apoptotic marker, annexin
V, to extend the present knowledge of the effect of exercise on
specific lymphocyte subfractions. In addition, to more fully
explain the lymphocytopenia response observed in the post-
exercise period following exercise, we also employed the
lymphocyte cell migration marker, CX3CR1. We hypothesized that
there would be differences in the contribution of apoptosis and
cellular migration between the CD4+, CD8+, and CD19+ lympho-
cyte subfractions with progressively increasing levels of
exercise intensity. We found that lymphocyte subsets responded
differently with increasing levels of intensity with respect to cell
count, apoptosis, and cell migration. In relative terms, a significant
increase noted in CD4+ and CD8+ cell count immediately
following exhaustive exercise. No effect was found with regards

\[\text{Fig. 1} \quad \text{lymphocyte concentration in subjects (N = 12) at rest (Pre) and}
\text{following treadmill running at 76% } V_{O2,\text{max}}, \text{87% } V_{O2,\text{max}}\text{, exhaustion (i.e.,}
\text{100% } V_{O2,\text{max}}, \text{Post), and 1-h after the bout (1 h Post). Data are presented as}
\text{mean } \pm \text{SEM. } ^{*}\text{signifies significantly greater than all other conditions for}
\text{specific lymphocyte subset.}

\[\text{Fig. 2} \quad \text{Absolute change from baseline in helper T lymphocytes (CD4+)}
\text{obtained from subjects (N = 12) following treadmill running at 76%}
\text{V}_{O2,\text{max}}, \text{87% } V_{O2,\text{max}}, \text{and to exhaustion (Post). Data is for cell count,}
\text{apoptosis (annexin V+), and cellular migration (CX3CR1+). } ^{*}\text{indicates}
\text{significantly greater than cell count at the given intensity.}

to relative apoptotic lymphocytes, but migration expression was significantly decreased at 1 h post-exercise in the CD8+ subfraction and increased 1 h post in CD19+ cells. However, when expressed as an absolute change from baseline and compared to the expected rest in cell volume, a different pattern emerged. We observed a significant increase in CD4+ and CD8+ apoptotic lymphocytes with moderate exercise intensity (76% VO_{2max}), followed by a significant increase in cellular migration markers in these cells, such that any effect was removed by the post-exercise time point. B lymphocytes displayed a different absolute response, such that cell migration was significantly initiated during moderate intensity exercise and remained elevated with each successive stepwise increase.

Typically immune changes have been reported in terms of a relative expression (i.e., the percent of apoptotic cells). In these terms, CD4+ and CD8+ cell count was observed, with a rise in CD19+ concentration that did not reach significance [9,23]. Regarding cell death in relative terms, no effect of exercise was noted in any of the lymphocyte subsets regardless of the exercise intensity. This finding is similar to what has been reported with a progressive treadmill protocol to exhaustion [9], as well as an endurance run on the treadmill for 2 h at 65% VO_{2max}[23]. In these investigations, a significant increase in CD4+ and CD8+ cell count was observed, with a rise in CD19+ concentration that did not reach significance [9,23]. Regarding the lymphocyte populations observed in the post-exercise period would be increased cell migration out of the vasculature, and this has been proposed previously rather than any contribution from apoptosis [3,24,25]. Our results utilizing the cell migration marker CX3CR1 would tend to confirm the results of these investigations, as relative changes in the expression of this receptor were noted in CD8+ and CD19+ cells at 1-h post exercise.

However, as we did note the presence of annexin V+ cells during each condition, this observation led us to consider whether lymphocytes are normally undergoing cell death in the lymphoid tissue at any given time. With exercise, the absolute increase in overall annexin V+ cells may simply reflect the mass movement of immune cells into the vasculature with activity, independent of cell death being induced by actual exercise. Given this, it would be logical that the change in the absolute number of apoptotic cells would match the absolute change in cell volume. Therefore, we decided to determine the percent change from baseline comparing cell count with cell death and migration, in specific lymphocyte subfractions. In this regard, our observations revealed a significant increase in annexin V+ helper lymphocytes (CD4+) at 76% VO_{2max} that is not matched with an
increase in CD4+ concentration (Fig. 2). The increase in the absolute change of apoptotic CD4+ cells remained significantly elevated at 87% VO2max, however this was accompanied by an even greater increase in CX3CR1+ helper T lymphocytes which suggests that apoptotic cells of this subset may migrate out of the vascular system and into the lymphoid pool at moderate to high intensities of aerobic exercise. Upon completion of exhaustive exercise, the CD4+ concentration stabilized with regard to the cell death and cell migration marker that were employed in the present investigation (Fig. 2). In a study design utilizing only a post-exercise blood draw, it would be reasonable to conclude that exercise has no effect on CD4+ apoptosis, however our results indicate that the cell death process in the helper T lymphocyte subset is initiated at moderate intensities of continuous aerobic exercise, similar to what has previously been reported for overall lymphocytes using moderate intensity exercise [11,16,18,28]. However, we demonstrate for the first time that with continuous exercise to exhaustion at greater intensities these cells tend to migrate out of the vascular compartment, providing a potential explanation for investigations reporting increases in lymphocyte apoptosis [16,19,28] and those that observe no differences [3,22,25,27]. While more research is necessary in this regard, it is possible that the increased cell death of CD4+ lymphocytes with prolonged moderate intensity exercise contributes to immunologic susceptibility, or the so-called “open window” [17,29], and that migration of these cells with high intensity exercise may represent a protective mechanism to minimize a functional decrease in the post-exercise period.

We observed a similar response with regard to the CD8+ subfraction of lymphocytes. The relative expression of annexin V on the surface of cytotoxic T lymphocytes revealed no significance at any of the exercise intensities utilized. However, similar to what we observed with the CD4+ subset, the absolute change from rest in CD8+ lymphocytes matched with markers of apoptosis and cellular migration at various levels of exercise intensity displayed an alternative interpretation. At 76% VO2max we noted a significant absolute increase in CD8+/annexin V+ that did not match the change in either cell count or cell migration (Fig. 4). Although not significant, this increase in the absolute change of apoptotic CD8+ cells remained elevated above cell count values at 87% VO2max, but accompanied by an even greater significant increase in CD8+/CX3CR1+ lymphocytes. In the blood sample obtained immediately following the exhaustive exercise bout, CD8+ apoptotic cells were reduced compared to cell count while CX3CR1 expression on cytotoxic lymphocytes was further significantly increased. It has been reported that with exercise CD8+ cells are more responsive with regard to the cellular adhesion molecules CD62L [5] and ICAM-1 [26] than helper T lymphocytes. Our findings provide further evidence in this regard, extending the time frame through 1-h post-exercise where we observed a significant decrease in the relative percentage of cytotoxic T cells displaying the fractalkine receptor CX3CR1. While not measured in the present study, previous investigations have surmised that exercise has a greater influence on the CD8+ subset compared with CD4+ due to epinephrine and the larger number of β2-adrenergic receptors observed on cytotoxic lymphocytes [6,21].

Regardless of intensity, treadmill exercise had a marked effect on the absolute change of CD19+ cells expressing CX3CR1. While the expression of this chemokine receptor has been reported to be minimal at rest [7], we report for the first time that moderate exercise is associated with an increase in CX3CR1 on B lymphocytes, and that further increases in exercise intensity provokes an even greater absolute rise (Fig. 5). Furthermore, the influence of exercise on this migration receptor appears to persist for at least 1 h following the exercise bout (Fig. 3b). Previous investigations have reported exercise to have no effect on the CD19+ cell count [9,23], and the present results tend to confirm these findings. It is reasonable to conclude that CD19+ B lymphocytes do not experience a significant elevation with exercise due to the migration of these cells out of the vasculature and into the lymphoid pools. While continued investigation into this phenomenon is warranted, it is possible that this response represents a protective mechanism in these cells that carry out humoral immunity.

One limitation with regards to the present investigation is that the subject pool consisted of males aged between 28–40 years. As a group, a BMI of 28 would classify the individuals who participated in the current study as overweight, and this may have some bearing on the results of the investigation. Future studies may wish to determine these immunological responses in relative terms to account for variations in lean body mass/fat mass between participants. In addition, it should be noted that due to the nature of treadmill exercise and the necessity of a blood draw, the protocol was discontinuous with 5-min rest periods before successive exercise bouts. Given the findings of the present study, it is possible that the rest period between bouts of increasing exercise intensity could have had an impact on the expression of apoptotic and migratory markers obtained in each subfraction. Future investigations utilizing either a continuous mode of exercise with increasing intensity or a continuous mode of blood sampling are warranted.

In conclusion, the primary aim of this investigation was to assess both the apoptotic and migratory response of CD4+, CD8+, and CD19+ lymphocyte subsets to increasing intensities of treadmill running. We found that lymphocyte subfractions displayed a differential response when expressed as the absolute change from baseline. Helper T lymphocytes are more prone to apoptosis at moderate levels of exercise intensity, and tend to migrate from the vasculature at greater intensities. Cytotoxic T lymphocytes are also influenced by the cell death process to a greater extent at moderate exercise, but apoptosis is diminished and superceded at high levels of exercise intensity by the migratory response. Lastly, exercise appears to greatly influence B lymphocytes toward cellular migration from the vasculature into the lymphoid pools at all intensities of exercise.

Acknowledgements

The authors wish to thank Elizabeth A. Fedor, Holly B. Kell and Greg Lee for their technical contribution to this investigation.

References


Patlar S. Effects of acute and 4-week submaximal exercise on leukocyte and leukocyte subgroups. Isokinetics Exerc Sci 2010; 18: 145–148


Simpson RJ, Florida-James GD, Whyte GP, Guy K. The effects of intensive, moderate and downhill treadmill running on human blood lymphocytes expressing the adhesion/activation molecules CD54 (ICAM-1), CD18 (β2 integrin) and CD55. Eur J Appl Physiol 2006; 97: 109–121

