Attenuation of T-lymphocyte demargination and adhesion molecule expression in response to moderate exercise in physically fit individuals

Suzi Hong,1 Todd A. Johnson,1 Noha H. Farag,1 Harold J. Guy,2 Scott C. Matthews,1 Michael G. Ziegler,2 and Paul J. Mills1

Departments of 1Psychiatry and 2Medicine, University of California, San Diego, California

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Suzi Hong,1 Todd A. Johnson,1 Noha H. Farag,1 Harold J. Guy,2 Scott C. Matthews,1 Michael G. Ziegler,2 and Paul J. Mills1

Address for reprint requests and other correspondence: S. Hong, Dept. of Psychiatry, Univ. of California, 9500 Gilman Dr., La Jolla, CA 92039-0804 (E-mail: shong@ucsd.edu).

LEUKOCYTOSIS, THE RECRUITMENT of leukocytes into the peripheral blood, occurs in response to acute exercise. Dramatic increases in mixed lymphocytes in circulation are seen shortly after the beginning of extended (e.g., 2.5 h) treadmill exercise and disappear after 30 min of rest (39). After 2 h of rest following exercise, lymphocyte numbers typically fall below the resting values (43) or remained elevated under an environmental challenge (i.e., high heat) (25). Natural killer (NK) cells (22) and neutrophils (38, 44) are major immune cell subsets that contribute to the rise in leukocyte numbers in response to exercise; marked increases (two- to threelfold above resting levels) in NK cell numbers are consistently seen after acute exercise challenges of various types (16, 31). The numbers of CD4+ T or CD19+ B cells do not change as significantly as the aforementioned cell types (30). In addition, a dose response is seen in exercise-induced leukocytosis; for example, a higher intensity of exercise task leads to greater circulating NK cell numbers for a given duration (40). These effects of exercise on immune cell demargination are mediated, in part, by sympathetic nervous system (SNS) activation (24).

In contrast to the large number of studies that report leukocytosis in response to acute exercise, the literature on the effects of chronic exercise (training) or physical fitness on peripheral blood leukocyte numbers and adhesion molecule expression is limited and inconclusive. Ten weeks of aerobic training increased circulating CD4+ T, naïve CD4+ T, CD8+ T, and B-lymphocyte numbers at rest (18, 19), whereas 7 wk of training increased the percentage of NK cells with no changes in CD4+ T, CD8+ T, or B cells (36). There were no differences, however, in circulating CD4+ T, CD8+ T, or NK cells at rest between elite rowers (34) or trained gymnasts (7) compared with age-matched untrained individuals. Studies examining how physical fitness affects leukocyte trafficking in response to stress, including acute exercise, are also inconclusive. No effects of regular physical activity on lymphocyte subpopulations were shown in response to submaximal exercise (27, 28) or in response to a speech stressor (29). However, physically active individuals show attenuated leukocytosis in selected lymphocyte subsets in response to a speech stressor (15). In addition, resistance exercise led to an increase in leukocyte numbers in untrained individuals but to no change in trained individuals (37). Exercise training or its converse, inactivity, can lead to changes in other end-organ responsiveness. For example, decreased heart rate (HR) at rest or during exercise after training is commonly known as “training-induced bradycardia” (41). On the other hand, low physical fitness or detraining can lead to augmented plasma norepinephrine (NE) responses to stress in a stressor-dependent manner (42).

Exercise training also leads to downregulation of β-adrenergic receptor density on lymphocytes at rest (35). However, it is unclear whether exercise training leads to adaptations in immune cell trafficking.

Cellular adhesion molecules (CAM) (i.e., selectins, integrins, etc.) play a pivotal role in leukocyte circulation and homing. L-selectin (CD62L) is expressed by leukocytes and is critical in the initial capture and rolling of leukocytes on the endothelial surface by binding to endothelial glycolipids (i.e., GlyCAM-1, CD34, etc.). Integrins, including CD11a, play an important role in firm attachment of leukocytes on the endo-
thelial surface. SNS activation during exercise may lead to selective recruitment of immune cells into the circulation by affecting surface adhesion molecule expression. CD62L expressing T lymphocytes and CD11a density on mixed lymphocytes increase significantly in response to mental and exercise stress (13, 15, 23).

The purpose of the present study was to determine the effects of physical fitness on leukocyte subpopulations and CAM expression in response to a moderate exercise challenge. We used a standardized exercise challenge based on each individual’s physical fitness level as assessed by a peak oxygen consumption (\(VO_2\); \(VO_2\) peak) level. It was hypothesized that the two groups of different cardiovascular fitness levels will exhibit different responses to exercise; a possible attenuation in exercise-induced immune cell demargination may occur in fit individuals via diminished cellular responsiveness and sympathetic activation. Catecholamine levels were assessed in plasma as a guide to SNS activation.

MATERIALS AND METHODS

Subjects. Thirty-two healthy volunteers with normal or high blood pressure (BP) (age 20–55 yr) from the community gave written, informed consent. The protocol was approved by the University of California, San Diego (UCSD) Institutional Review Board and was conducted at the UCSD General Clinical Research Center and UCSD Clinical Trials Center. All subjects underwent a history and physical examination by a physician, and a normal resting electrocardiogram (ECG) was confirmed. Individuals who had a history of heart disease, liver or renal disease, diabetes, psychosis, severe asthma, pregnancy, morbid obesity, or current use of prescription medication, except for liver or renal disease, diabetes, psychosis, severe asthma, pregnancy, and metabolic equivalent were monitored. Oxygen saturation levels, RPE, and BP were recorded every 3 min. Subjects were informed to notify the investigator immediately of warning signs of excess exertion (e.g., faintness, chest pain, shortness of breath, dizziness, muscle cramps). Following the 20-min treadmill test, subjects continued to walk slowly for 2 min as a cool-down period, and blood samples were acquired after 10 min after the cool-down in a supine position.

Depending on the individual’s body weight-adjusted \(VO_2\) peak (ml·kg\(^{-1}\)·min\(^{-1}\)), subjects were categorized into the “fit” or “nonfit” group, according to previously published age- and gender-dependent criteria (2). For example, for a 45-yr-old woman, \(VO_2\) peak (ml·kg\(^{-1}\)·min\(^{-1}\)) higher than 29 ml·kg\(^{-1}\)·min\(^{-1}\) was considered fit compared with a 40 ml·kg\(^{-1}\)·min\(^{-1}\) cutoff value for a 25-yr-old man to be considered fit.

Flow cytometric analyses. Blood was preserved with EDTA and maintained at room temperature (23°C) until analyses. Complete blood count (CBC) analysis was performed using a Cell-Dyn 4000 CBC counter (Abbott Diagnostics, Santa Clara, CA). Within 3 h of collection, whole blood (100 µl) was stained with various monoclonal antibodies (20 µl) for phenotypic markers, followed by 15-min incubation in the dark. Monoclonal antibodies against phenotype (CD3, CD4, CD8, CD56/CD16, and CD45RA and RO), adhesion (CD62L, CD11a), and activation [human leukocyte antigen (HLA)-DR] markers were conjugated to four-color fluorochromes, FITC, phycoerythrin (PE), peridinin chlorophyll protein, or allophycocyanin (BD-PharMingen, San Diego, CA). After staining with antibodies, erythrocytes were removed using 2 ml of FACScans Brand Lysing Solution (Becton-Dickinson, San Jose, CA). Following 10-min incubation and centrifugation (5 min at 300 g), the supernatant was aspirated, and cells were washed and resuspended in PBS with 5% formaldehyde.

A flow cytometer (FACSCalibur, Becton-Dickinson), equipped with 15 mW argon (488 m) and red diode (635 nm) lasers and with CellQuest software (version 3.2, 1998), was used to quantify leukocyte subpopulations and adhesion molecule expression. Isotypic controls were used to determine nonspecific antibody binding, and events of 8,000–15,000 were analyzed per tube. Leukocytes and adhesion molecule expression were obtained in percentage of total or gated cells and transferred into absolute numbers using the CBC. For surface CAM density, estimation of antibodies bound per cell was performed using Quantibrite PE beads (Becton-Dickinson). Antibodies bound per cell, being the number of antibodies that bind to the specific cell or microbead population, provides a good approximation of antigen density expressed on the cell. The Quantibrite PE beads were run at the same instrument settings as the assay, and the FL2 (PE) axis was converted into the number of PE molecules bound per cell. Analyses of the flow cytometry data were performed using FlowJo analysis software (Tree Star, Ashland, OR).

Catecholamines. Blood for catecholamines was preserved in EDTA on ice before and after the task and after a 10-min quiet rest. After centrifugation in a refrigerated centrifuge, plasma was stored at −80°C until the assays were done. Epinephrine (Epi) and NE were determined by radioenzymatic assay, as described previously (17). The intra- and interassay coefficients of variation for the assay were 6.5 and 11%, respectively. Catecholamine data were log-transformed for normalization before statistical analyses.

Statistical analysis. To examine leukocyte demargination responses to and recovery from exercise in fit vs. nonfit individuals, time-by-group interactions were analyzed using two-way, time (pre, post, and 10-min recovery) by group (low and high fitness) repeated-measures analysis of covariance. Age and gender were used as covariates to control for the effects of age and gender on the immune cell responses and physical fitness levels (SPSS Statistical Software 11.0). Statistical significance was determined at an alpha level of 0.05. Bonferroni post hoc tests were done where appropriate, and simple treadmill exercise was performed at 65–70% of estimated \(VO_2\) peak. Throughout the exercise ECG, HR, respiratory exchange ratio (R), and metabolic equivalent were monitored. Oxygen saturation levels, RPE, and BP were recorded every 3 min. Subjects were informed to notify the investigator immediately of warning signs of excess exertion (e.g., faintness, chest pain, shortness of breath, dizziness, muscle cramps). Following the 20-min treadmill test, subjects continued to walk slowly for 2 min as a cool-down period, and blood samples were acquired after 10 min after the cool-down in a supine position.
RESULTS

Demographics. The demographic data for subjects in the fit and nonfit groups are presented in Table 1. There were no significant differences in age, body mass index, and BP or HR at rest between the two groups, although there was a trend for these to be slightly lower in the fit group. There was no difference in gender distribution between the groups. By definition, \( \bar{V}O_2 \text{peak} \) (\( \text{ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \)) was significantly higher in the fit group than in the nonfit group \( (P < 0.001) \). Although there were six individuals with high BP, the distribution of individuals with normal vs. high BP was not significantly different in fitness groups. In addition, there was no effect of hypertension status on leukocyte demargination responses to acute moderate exercise.

Metabolic responses during exercise in the fit vs. nonfit groups. As shown in Table 2, average BP and HR during the 20-min treadmill exercise at 65–70% \( \bar{V}O_2 \text{peak} \) were not significantly different between the two groups. In addition, average R and RPE were not different between the two groups. Average R values of 0.99 were shown, and average RPE values of 13 (“somewhat hard”) were indicative of a moderate exertion in both groups. Average \( \text{V}O_2 \) (\( \text{ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \)) values during 20-min steady-state exercise were significantly higher for the fit group as expected \( (P < 0.001) \).

Physical fitness and leukocyte redistribution after acute exercise. Significant time effects were shown for all of the leukocyte subsets assessed. Significant increases in the number of leukocyte subsets after the exercise were shown, and they returned closely to the resting values after a 10-min recovery period \( (P \text{ range } 0.00 \text{ to } 0.04) \). There were significant interactions between fitness level (group) and leukocyte responses to exercise (time). Interactions were found for CD3\(^+\) \( [F(1,22) = 6.29, P = 0.02] \), CD4\(^+\) T \( [F(1,22) = 6.81, P = 0.016] \), and CD8\(^+\) T \( [F(1,22) = 4.88, P = 0.038] \) cells. Among T-cell subsets, naive (CD45RA\(^+\)CD62L\(^+\)) \( [F(1,22) = 4.72, P = 0.041] \) and memory (CD45RO\(^+\)) \( [F(1,22) = 6.52, P = 0.018] \) CD4\(^+\) cells and naive \( [F(1,22) = 12.0, P = 0.002] \) CD8\(^+\) cells were significant for group-by-time interaction (Figs. 1 and 2). Memory CD8\(^+\) cells did not exhibit significant group-by-time interaction \( (P = 0.075) \). In addition, HLA-DR nonexpressing memory CD4\(^+\) \( [F(1,22) = 4.57, P = 0.044] \) cells were significant for group-by-time interaction, whereas numbers of HLA-DR\(^-\) memory CD8\(^+\) cells did not show group-by-time interaction \( (P = 0.131) \). Interestingly, HLA-DR-expressing memory CD8\(^+\) cells did not even increase significantly after exercise, regardless of the fitness groups. Physically fit subjects in our sample showed an attenuated response in selected immune cell numbers compared with nonfit subjects after moderate exercise. There were no differences between the two fitness groups on the response of neutrophils, monocytes, NK cells, or B cells in response to exercise. We also examined group-by-time interactions for the hematocrit values to examine possible effects of fitness on plasma volume changes during exercise; however, the interactions were not significant. Simple effect for group revealed that there were no statistical differences in leukocyte subset numbers between the groups before exercise, although slightly lower immune cell numbers were shown at rest among fit compared with nonfit individuals.

CAM expression in response to exercise in the fit vs. nonfit groups. CD62L (L-selectin)-expressing CD4\(^+\) \( [F(1,22) = 6.37, P = 0.019] \) and CD8\(^+\) cells \( [F(1,22) = 8.53, P = 0.008] \) were significant for group-by-time interaction, with physically fit subjects showing reduced responses. However, CD4\(^+\) and CD8\(^+\) cells that did not express CD62L (CD62L\(^-\)) did not show significant interactions (Fig. 3). We also examined lymphocytes that expressed high, medium, and low levels of CD11a based on the mean florescent intensity. Significant group-by-time interactions were found for CD11a\(^\text{med} \) \( [F(1,22) = 6.43, P = 0.019] \) and CD11a\(^\text{low} \) \( [F(1,22) = 6.90, P = 0.015] \)-expressing lymphocytes, with physically fit subjects showing reduced responses (Fig. 4). CD11a\(^\text{high} \)-expressing lymphocytes significantly increased after exercise in both groups, but did not show significant group-by-time interaction \( (P = 0.068) \). None of the densities of CD54 (ICAM-1) on leukocyte subsets was significant for group-by-time interaction.

Catecholamines. Exercise induced significant SNS activation, as indicated by elevated circulating catecholamine levels. Exercise led to a threefold increase in plasma NE \( [F(1,24) = 72.46, P < 0.001] \) and a fourfold increase in Epi \( [F(1,24) = 21.8, P < 0.001] \) levels across the groups. However, there were no significant time-by-group interactions between the changes in NE or Epi levels before and after exercise by fitness. The fit group showed slightly lower NE levels after exercise compared

Table 2. Metabolic and cardiorespiratory responses during a 20-min exercise at 65–70% \( \bar{V}O_2 \text{peak} \) in the low vs. high physical fitness groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>Low</th>
<th>High</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \bar{V}O_2 \text{peak}, \text{ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} )</td>
<td>13 (4.02)</td>
<td>26.54 (3.91)</td>
<td>5.77*</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>19.01 (4.33)</td>
<td>28.71 (4.34)</td>
<td>5.71*</td>
</tr>
<tr>
<td>Average diastolic blood pressure, mmHg</td>
<td>160.96 (24.15)</td>
<td>165.79 (20.98)</td>
<td>0.53</td>
</tr>
<tr>
<td>Average heart rate, beats/min</td>
<td>82.32 (13.74)</td>
<td>74.31 (9.27)</td>
<td>1.50</td>
</tr>
<tr>
<td>Average respiratory exchange ratio</td>
<td>143.59 (28.66)</td>
<td>149.71 (13.34)</td>
<td>0.72</td>
</tr>
<tr>
<td>Average ratings of perceived exertion</td>
<td>13.16 (2.35)</td>
<td>13.26 (1.86)</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Values are means (standard deviation). *P < 0.001.
with the nonfit group, but it was not significant. NE and Epi levels decreased after a 10-min supine rest following the exercise, but post hoc test revealed that they were significantly higher \( (P < 0.001) \) than the resting catecholamine levels (Fig. 5).

**DISCUSSION**

Compared with nonfit individuals, physically fit subjects showed attenuated demargination of selected lymphocyte subsets and CAM-expressing T cells. This was not derived from differences at rest, because the cell numbers were not significantly different between the groups before exercise. Also, the cell numbers were comparable to the values reported in other studies (18, 20) and to normal ranges for healthy adults. Attenuated increases in cell numbers after exercise in the fit group were observed among only selected T lymphocytes. There were no effects of fitness on demargination of neutrophils, monocytes, NK cells, or B cells in response to exercise, although their numbers significantly increased after exercise across the groups. In addition, a significant attenuation of demargination was found in CD4\(^+\) memory T cells that did not express the activation marker HLA-DR after exercise in the fit groups, but not among activated (HLA-DR expressing) memory T cells. Such findings imply that the effects of fitness on leukocytosis during exercise may be cell-type and activation-stage specific. It is unclear, though, why T lymphocytes, especially HLA-DR-nonexpressing CD4\(^+\) T memory cells, are largely influenced by cardiovascular fitness in their responses to exercise.

The leukocytosis induced by the 20-min moderate exercise was fairly transient and nearly disappeared within 10–15 min.
following cessation of exercise. Consistent R values of 0.99 during the exercise for both fitness groups indicated that all subjects exercised at a steady and moderate state. Also, RPE of 13 (“somewhat hard”) during the exercise confirms that the exercise intensity was moderately, not greatly, challenging. These observations agree with findings from previous studies (13, 39) and suggest that intensity and duration of exercise affect time to recovery (i.e., a longer or more intense exercise bout leads to more persistent leukocytosis).

Fit subjects underwent a higher absolute workload as indicated by VO₂ values (27–29 ml·kg⁻¹·min⁻¹) compared with that (18–19 ml·kg⁻¹·min⁻¹) of nonfit subjects during the exercise. However, both physiological and psychological responses to the workload, as mentioned above, indicated that both groups experienced a similar physiological stress of moderate exercise. Thus, in response to an exercise of the same intensity relative to their fitness level, fit individuals showed blunted lymphocytosis.

What are the possible mechanisms leading to the difference in lymphocyte responses to exercise between individuals with high vs. low cardiovascular fitness? Changes in plasma volume during exercise did not appear to be a factor because postexercise hemoconcentrations were not different between the two groups. Attenuation of increases in L-selectin (CD62L) and α-chain of LFA-1 (CD11a)-expressing cell numbers suggests that CAMs play a role. Our findings of increased CD62L and CD11a expression after exercise are in general agreement with previous studies (13, 24). Attenuation of T-lymphocyte demargination did not appear to be mediated by peripheral catecholamine responses in our study, because plasma NE and Epi levels were not significantly different between the two groups in response to exercise. However, it should also be noted that Epi and NE responses to exercise appeared marginally attenuated in the fit group compared with the nonfit group.

Sympathetic responses to exercise in individuals with varying fitness levels remain to be further investigated. Exercise
increases plasma catecholamine levels acutely (4, 21), and it was observed in our subjects. Downregulation of β-adrenergic receptors occurs in response to high circulating catecholamine levels or as a result of exercise training in humans (3, 35) and may explain the blunted cellular responses that we found among fit individuals. Thus whether the findings of different leukocyte responses between fit vs. nonfit individuals is the result of a possible adaptation of leukocytes among fit individuals. Thus whether the findings of different leukocyte responses between fit vs. nonfit individuals is the result of a possible adaptation of β-adrenergic receptors on leukocytes merits further examination. In addition, a possibility of attenuated sympathetic innervation of the secondary lymphoid organs in fit individuals remains to be examined. Given that noradrenergic projections and NE-containing nerve terminals are found in rat spleens (8, 9) and that leukocytosis during exercise is believed to be largely due to a mobilization of cells from the secondary lymphoid organs, sympathetic mediation of leukocyte demargination responses needs further investigation.

Attenuation of exercise-induced demargination appeared more pronounced among memory CD4 T cells compared with naive CD4 cells. Previously, our laboratory reported that demargination in response to a speech stressor was greater in memory T cells compared with that of naive CD4 or CD8 T cells (15). These findings may indicate that memory cells are mobilized more readily into the circulation in response to moderate stress, as also reported by Gannon and colleagues (12). Exercise led to a decrease in percentage of naive CD8+ T cells, and mobilization of T cells into the circulation was primarily a result of a redistribution of activated cells expressing CD45RO (CD62L−), as reported by Nielsen and colleagues (32). Therefore, recruitment of cells to the circulation during exercise appears to be from the secondary lymphoid organs, such as the spleen and lymph nodes, not the primary lymphoid organs, including the thymus and bone marrow (33). Therefore, mobilization of cells from the secondary lymphoid organs, not proliferation or differentiation, appears to be responsible for the lymphocytosis in response to stress, and such a phenomenon appears to be reduced with greater fitness.

There are a few shortcomings in the study. Although we have categorized individuals into the fit or nonfit groups based on age- and gender-dependent reference values, our study is cross sectional in nature. A future study may benefit from employing an exercise training study and examining the effect of observed improvements in fitness on immune cell responses to an acute exercise bout. Second, we included six individuals with high BP in the study. Although there were no statistical differences in the distribution of normotensive and hypertensive subjects between the fit and nonfit groups, it would be premature to state that there was no effect of high BP on fitness or immune cell trafficking responses to exercise. Given that there were only a few subjects with hypertension in our sample, accurately documenting the effects of hypertension is beyond the scope of our study. A larger scale clinical trial is desirable as a future direction to investigate immune cell adhesion molecule expression in response to exercise among hypertensive compared with normotensive individuals. Last, we did not control for the menstrual cycle in our 12 women subjects, although the gender effect was controlled in the statistical analyses. Future studies should document and control for a possible effect of menstrual cycle on immune cell demargination in response to exercise.

Our findings suggest that individuals of high cardiorespiratory fitness have attenuated immune cell responses to acute exercise. Because physically fit subjects had a smaller immune response to the same levels of exercise relative to their cardiovascular fitness, they would exhibit even smaller immune responses to the same absolute level of exercise performed by the less fit individuals. A “protective” role of exercise training against stress-induced immunosuppression was found in animal studies (5, 6, 10, 26). Fu and colleagues (11) found that exercise training dampened decreases in CD4+ T-cell numbers normally seen after exhaustive exercise in mice. Although immunoprotection against various stressors in physically fit individuals remains inconclusive, attenuated lymphocyte demargination in response to physical and psychological (15) stress clearly demonstrates that physical fitness alters immune responses in humans.

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