Original Research

Leukocyte Subset Changes in Response to a 164-km Road Cycle Ride in a Hot Environment

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ABSTRACT

International Journal of Exercise Science 9(1): 34-46, 2016. The purpose of this observational study was to determine the circulating leukocyte subset response to completing the 2013 Hotter'N Hell Hundred recreational 164-km road cycle event in a hot and humid environmental condition. Twenty-eight men and four women were included in this study. Whole blood samples were obtained 1-2 hours before (PRE) and immediately after (POST) the event. Electronic sizing/sorting and cytometry were used to determine complete blood counts (CBC) including neutrophil, monocyte, and lymphocyte subsets. The concentration of circulating total leukocytes ($10^3 \cdot \mu L^{-1}$) increased 134% from PRE to POST with the greatest increase in neutrophils (319%, $p<0.0001$). Circulating monocytes (including macrophages) increased 24% ($p=0.004$) and circulating lymphocytes including B and T cells increased 53% ($p<0.0001$). No association was observed between rolling time or relative intensity and leukocyte subset. Completing the Hotter n' Hell Hundred (HHH), a 100 mile recreational cycling race in extreme (hot and humid) environmental conditions, induces a substantial increase in total leukocytes in circulation. The contribution of increases in specific immune cell subsets is not equal, with neutrophils increasing to greater than 4-fold starting values from PRE to POST race. It is likely that exercise in stressful environmental conditions affects the complement of circulating immune cells, although activational state and characterization of specific leukocyte subsets remains unclear. The observed increase in circulating cell sub-populations suggests that the circulating immune surveillance system may be acutely affected by exercise in hot and humid conditions.

KEY WORDS: White blood cells, heat, ultra-endurance, neutrophils, lymphocyte, monocyte, exercise

INTRODUCTION

In recent decades participation in long-distance endurance exercise events (*e.g.*, Ironman triathlons, ultra-marathons, and ultra-distance cycling events), has increased substantially among recreational exercisers and competitive athletes (7, 17, 34). Many of these events occur in extreme environmental conditions including high altitude or low or high ambient temperature. Such extreme environmental conditions provide substantial additional physiological stress beyond that induced by

the long-distance exercise alone and thus can provide additional perturbations to homeostasis. High intensity endurance exercise and environmental stress independently can affect immune system function, but the combination of multiple stressors likely has a more drastic effect on the immune system (23).

Long-distance exercise alone affects populations of circulating immune cells that function in non-specific immunity. Marathon (42.195 km) runners exhibit increases in total leukocytes (~4 fold), monocytes (~2.5 fold), neutrophils (~5.5 fold) (8, 14), with only a minor increase (~1.25 fold) (8) in lymphocytes from before to immediately after the event. Ultra-marathon (≥160km) runners display a similar increase in total leukocytes and monocyte/neutrophil subsets, but exhibit a less dramatic increase in circulating lymphocytes compared to traditional marathon runners (27, 37). Similar results of a 1-2 fold increase in leukocytes, monocytes, neutrophils, has also been observed for 75-km cycling events (19, 27, 46). Such long-distance exercise events impose a substantial mechanical and systemic physiological demand on the body, even in thermoneutral environments (46). It is likely that micro-trauma to musculature and vascular system, leakage of endotoxins from the gut to the circulation, and glycogen depletion (13, 20, 39, 40, 43) occur during ultra-endurance exercise and can instigate activation, differentiation, and demargination of leukocytes and greater systemic inflammation (13).

Hot and humid environmental conditions magnify the effect of exercise that occurs in thermoneutral environments. Studies of shorter duration running and cycling during hot and cold water immersion (40 min) (6, 33) and in temperature controlled laboratory settings (40 min to 4 h) (3, 16, 21, 23, 38) demonstrate that exercise in a hot environment (35-39°C) elicits greater increases in total leukocytes, neutrophils, and lymphocytes than exercising in a cold to moderate temperature environment (18-23°C) (6, 21, 33). One study emphasized the inefficacy of a thermal clamp (16) in reducing the exercise-induced changes in circulating leukocyte populations. However, it is interesting to note that the exercise protocol in this study did not elicit core body temperature increases above and beyond the passive heating control in which participants did not exercise. It is likely that the core temperature increase associated with high intensity exercise alone has a role in instigating immune responses, in addition to the damage associated with long duration, eccentric exercise.

Despite the growing popularity of recreational long-distance exercise events no field or controlled laboratory study appears to have investigated the non-specific immune response to such prolonged endurance events (>4 h) in a thermally stressful hot environment. Long-distance exercise in moderate temperature conditions induces an increase in leukocytes and in laboratory settings a hot environment augments the leukocyte response to shorter-distance exercise. Therefore, the purpose of this observational study was to determine the leukocyte subset response to completing a recreational 164-km road cycle event in an extremely hot and humid environment.

Exercise duration and relative exercise intensity (42) likely have significant effects on the immune response, independent of the extreme environmental conditions. Thus, a secondary purpose of this study was to examine the associations among the time to complete the event, relative intensity (%VO_{2max}) and changes in leukocyte subsets.

METHODS

Participants

In order to determine the leukocyte subsets response to completing a recreational 164-km road cycle event in a hot ambient temperature we recruited adults registered for participating in the 2013 Hotter'N Hell Hundred cycling event (HHH). The procedures of the study were reviewed and approved by the University Institutional Review Board and undertaken in compliance with the Helsinki Declaration. On the day of the HHH, participants provided a blood sample in the morning 1-2 hours prior to the start of the event (PRE) and immediately upon completion of the event (POST). Blood samples were analyzed for total leukocyte as well as leukocyte subsets: neutrophils, monocytes, and lymphocytes. In order to describe the immune response that recreational riders experience when completing the HHH, we aimed to minimize any effect of study involvement on participant behavior before and during the event. Therefore, no instructions or restrictions were provided with regard to ride strategies (*e.g.*, hydration/diet intake, number rest stops, pace of the ride, or length of the rest stops).

Twenty-eight men and four women volunteered to participate and were enrolled in this study. Participants' demographic information and ride characteristics (*e.g.*, pace) are presented in Table 1 and 2, respectively.

Table 1. Participant's demographic information (Mean±SD).

	Men (n=28)	Women (n=4)
Age	49 ± 8	42 ± 12
Body mass (kg)	88.2 ± 12.5	61.7 ± 7.7
Height (cm)	179.0 ± 4.9	166.3 ± 6.6
Body fat (%)	18.5 ± 5.7	26.3 ± 6.4

Table 2. Participants' ride characteristics.

	Min	Max	Mean ± SD
Total Rolling Time (hr)	4.7	8.2	6.4 ± 1.0
Number of Stops	0	8	4 ± 2
Total Stop Time (min)	0.0	41.7	12.4 ± 8.0
Heart Rate $_{avg}$	125	158	142 ± 9
Heart Rate $_{max}$	148	181	167 ± 10
Relative Intensity (%VO2max)	61	85	76 ± 6
PRE_{Usg}	1.003	1.032	1.018 ± 0.001
$POST_{Usg}$	1.013	1.035	1.026 ± 0.005
Body Mass Loss (%)	-0.9	4.3	2.5 ± 1.2
Carbohydrate Intake (g)	42.2	769.1	290.8 ± 157.2

Core temperature was not measured in the present study but based on findings from a prior year's HHH event (same course and similar populations, environmental conditions) it was expected to rise approximately 1.6 °C from before to after the ride (1). Participants were recruited through the HHH official event registration website, through invitation emails sent to all HHH entrants, and at the official HHH onsite event registration center (Wichita Falls, TX). Potential participants attended a

Table 3. Environmental Conditions at the start/finish line and at the 97-km aid station.

	Start/Finish Line					97km Aid Station				
	Min	Max	Mean	±	SD	Min	Max	Mean	±	SD
Dry Bulb (°C)	25.1	43.7	35.3	±	5.0	27.2	41.4	32.7	±	5.0
Humidity (%)	32.6	78.0	47.2	±	14.5	23.7	65.3	41.1	±	17.5
WBGT Out (°C)	23.9	36.2	31.5	±	3.8	25.5	34.1	30.0	±	2.7

mandatory informational session prior to providing written informed consent. Participants completed medical history and training questionnaires. Upon review of the medical history, and with follow-up questions as needed, all participants were approved for participation by the on-site medical doctor. To be included in the study, volunteers were required to 1) be apparently healthy, 2) be 18-62 years of age, 3) have previously completed a 160-km cycle ride, and 4) intent to complete the HHH ride within 9 hours. Participants were excluded if they met any of the following criteria: 1) tobacco product user; 2) taking cholesterol-lowering, blood pressure, or anticoagulant medications; 3) healing from a musculoskeletal injury; 4) liver, kidney, cardiovacular, gastrointestinal or blood disease or severe metabolic or endocrine disorders (*e.g.,* diabetes); 5) past history of exertional heatstroke or exercise-heat inotlerance; 6) use of anabolic hormonal substances. No participant reported being pregnant.

Protocol

A detailed description of the anthropometrics measurements used in this study has previously been published (15). Briefly, anthropometric measurements were obtained on one of the two days preceding the event. Body weight and height was measured using a floor scale and a tape measure attached to the wall, respectively. Percent body fat was estimated using three-site (men: chest, abdomen, thigh; women: triceps, suprailiac, thigh) skinfold thickness equations (18) using calibrated skinfold calibers (Bodycare Harpenden Caliper, England).

The HHH is among the largest recreational road cycle events (>10,000 participant annually) in the USA and held every late-August in Wichita Falls, TX. The 164-km course covers largely flat terrain (15) and is usually held under hot and humid conditions (average Wichita Falls environmental conditions for late-August is 35°C and humidity is 59%, with little to no cloud cover). On event day, dry bulb temperature, relative humidity, and wet bulb globe temperature were recorded at the start/finish line (7:31am to 3:24pm) and at the 97-km aid station (8:17am to 12:32pm) using the Kestrel Meter 4400 Heat Meter (Nielson-Kellerman Co., Boothwyn, PA, USA). The details of the environmental conditions are presented in Table 3.

The ride began at 0700h and depending on starting position a particular rider might not pass the start line until as late as 0800h. Participants without a computer-downloadable heart rate monitoring device were provided one to wear during the ride (Polar Electro Inc., Lake Success, NY);

participants having their own computer-downloadable heart rate monitoring device were requested to provide heart rate data files via electronic mail within 7d after the event. Participant total ride time was determined using either the participant's own timer or a timer provided by the research team and was confirmed using official HHH ride electronic chip monitoring system. Participants were instructed to record the number of stops and the duration of each stop. The rolling time was calculated as the difference between the total event time and the resting time. Heart rate was collected by standard telemetry using either participants' own device or one provided by the investigators.

Whole blood samples and urine sample were obtained 1-2 hours prior to the start of the ride (PRE) and immediately after (POST) completion of the event without fasting. Upon arrival, participants were weighted and then sat for a few minutes after which 30ml of blood were collected from an antecubital vein using venipuncture by a 21 gauge needle with an evacuated tube containing EDTA, while participants remained in a seated position. After blood collection, participants provided a small urine sample for determination of urine specific gravity. Participants were instructed to be arrive for the PRE time point before performing their event warm-up. The EDTA-treated whole blood samples were refrigerated and stored at 4°C until biochemical analyses on the following morning.

Participants were instructed to remember the food consumption during the ride and if possible, kept the food labels. Upon arrival at the medical tent after the ride, participants recorded the quantity and the type of food that were consumed or participants provided the food labels to investigators for them to record.

Prior to analysis, blood samples were diluted with an equal volume of phosphate buffered saline and results were adjusted to account for this dilution. Then complete blood count was measured in duplicate using a 20-µl sample of diluted EDTA-whole blood loaded into an Automatic Hematology Analyzer (BC-3200; Mindray, Mahwah, NJ). In this automated procedure, total (total leukocytes) WBC and three-part differentiation (lymphocytes, monocytes, and neutrophils) of WBC are measured using electrical impedance. The analyzer has a precision of ±2.5% and a linear range from 0.3~99.9 10^9cells·L^{-1} for WBC. The complete blood count analysis also included determination of hemoglobin (Hb) concentration (using a cyanide free method) and hematocrit (Hct). Plasma volume change (ΔPV) was determined by the change in Hct and Hb from PRE to POST using established methods (9) (ΔPV: 7.1 ± 12.6%). The POST immune cell (total leukocytes, lymphocytes, monocytes, and neutrophils) concentration was adjusted according to individual ΔPV. Urine samples were analyzed for urine specific gravity by a refractometer. The percent of body mass loss (%BML) was the fluid loss as percent of pre-body mass, and it was corrected for the weight of food consumed during the ride.

Estimated relative exercise intensity during the ride was calculated through the estimation of $\%VO_{2max}$ from exercise heart rate using an equation which was validated by Franklin et al. (11) (i.e. $\%VO_{2max} = 1.33 \times (\%HR_{max}) - 37.3$). The percent of maximum

heart rate (%HR_{max}) was calculated using the maximum heart rate (HR_{max}) and the average heart rate (HR_{avg}) recorded during the cycling event. Although it is not possible to determine if the recorded HR_{max} represents the true HR_{max}, the recorded HR_{max} (167 ± 10 bpm) was similar to but slightly less than the age predicted HR_{max} (i.e. 208 – (0.7 X age); 174 ± 6 bpm) (44).

Statistical Analysis

Data were analyzed using SPSS version 20.0 (IBM, Chicago, IL). Tests for normality of distribution and homogeneity of variance were performed for all data sets. Data sets that did not meet assumptions of linear statistics were log10 transformed and retested; after transformation all data sets met these assumptions. Paired T-tests were used to evaluate the mean PRE and POST differences in total leukocytes, and each leucocyte subset (neutrophils, monocytes, and lymphocytes) concentrations. To examine the effect intensity and duration on total leukocytes and each leucocyte subset, two different analyses were utilized. First, Pearson's product-moment correlation coefficients between PRE to POST changes and ride characteristics (estimated relative exercise intensity and total rolling time) were calculated. Second, participants were grouped into quartiles based on rolling time. Then data for the fastest and slowest quartiles were examined using 2 (Time point: PRE vs POST) × 2 (Rolling time: fastest vs slowest quartile) ANOVAs with repeated measures on Time point. The level of significance for all analyses was set at $\alpha = 0.05$. Data are presented as mean ± standard deviation unless otherwise noted.

RESULTS

The concentrations of total leukocytes and of each leukocyte subset increased significantly from PRE to POST. The percent increase was 134% for total leukocytes ($p<0.0001$), 319% for neutrophils ($p<0.0001$), 24% for monocytes ($p=0.004$), and 53% for lymphocytes ($p<0.0001$). The data for leukocyte subset is presented in Table 4 and individual responses are presented in Figures 1-4.

Table 4. Immune marker concentrations before (PRE) and after (POST, ΔPV adjusted) 164-km road cycling (Mean±SD).

Variables	PRE	POST
Leukocytes ($10^3 \bullet \mu l^{-1}$)	5.4 ± 1.1	12.9 ± 3.4*
Lymphocytes ($10^3 \bullet \mu l^{-1}$)	2.5 ± 0.6	3.7 ± 1.5*
Monocytes ($10^3 \bullet \mu l^{-1}$)	1.2 ± 0.4	1.5 ± 0.6*
Neutrophils ($10^3 \bullet \mu l^{-1}$)	1.8 ± 0.6	7.6 ± 2.8*

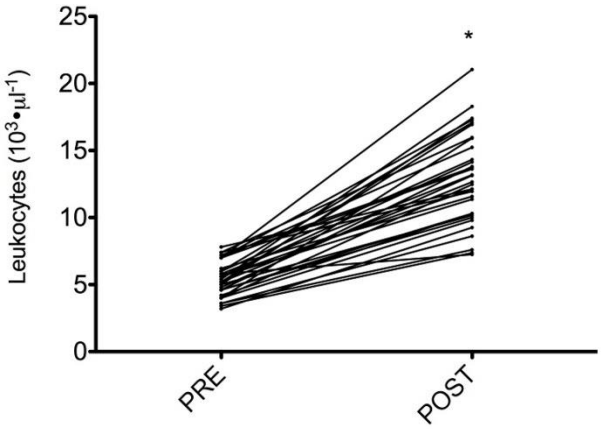

Figure 1. Individual leukocyte concentrations before (PRE) and immediately post event (POST). *POST significantly ($p < 0.05$) different from PRE.

Both leukocytes and neutrophils increased in concentration uniformly, while monocytes and lymphocytes did not show this pattern. Thirteen of 32 participants had

a reduction from PRE to POST in monocyte concentration (range: -0.05 to -0.88 $10^3 \bullet \mu l^{-1}$) and eight of 32 showed a reduction from PRE to POST in lymphocyte concentration (range: -0.11 to -0.83 $10^3 \bullet \mu l^{-1}$). The cause of these findings is unclear but could represent naturally occurring individual differences or be due to differences in training state, heat acclimation, and/or immune function.

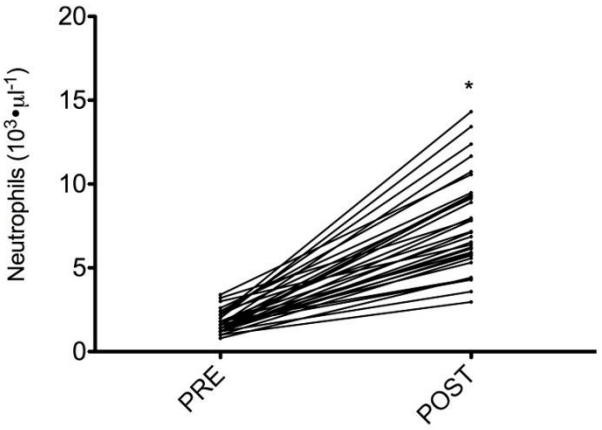

Figure 2. Individual neutrophil concentrations before (PRE) and immediately post event (POST). *POST significantly ($p < 0.05$) different from PRE.

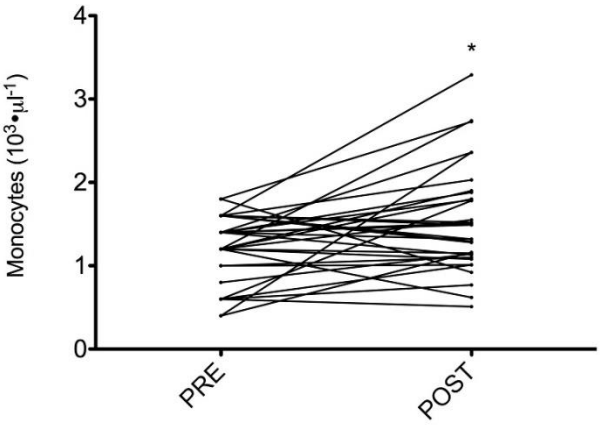

Figure 3. Individual monocyte concentrations before (PRE) and immediately post event (POST). *POST significantly ($p < 0.05$) different from PRE.

No significant relationship was observed between changes in total leukocytes or leukocyte subset concentrations from PRE to POST and ride characteristics (estimated relative exercise intensity and rolling time). The bivariate correlations ranged from -0.24 to 0.22. Furthermore, circulating total leukocytes and leukocyte subset concentrations were not different between the fastest and slowest quartile of riders.

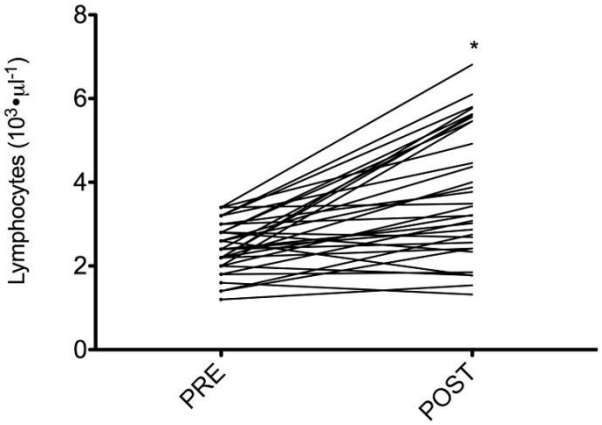

Figure 4. Individual lymphocyte concentrations before (PRE) and immediately post event (POST). *POST significantly ($p < 0.05$) different from PRE.

DISCUSSION

The primary purpose of this study was to determine the circulating leukocyte response to completing a recreational 164-km road cycle event in a hot ambient temperature. Our data indicated that leukocytes (134%) and the each leukocyte subset (neutrophils: 319%; monocytes: 24%; lymphocytes: 53%) increased from PRE to POST, but these increases were not affected by estimated relative exercise intensity. Considering the growing popularity of long-distance cycling, this is important new knowledge since this substantial immune response might affect the acute and long-term health of recreational cyclist who regularly perform long-distance cycling in the heat. Better understanding of the acute physiological effects of such exercise is

critical to defining proper recovery and monitoring health following rides and over the course of training. To our knowledge these results are the first to describe the non-specific immune response to a completion of a self-paced recreational long-distance road cycling in a hot (>30 °C) environment. Overall, this study provides unique physiological insight by showing that completing a 164-km cycling event (≥5-8h) without instructions or restrictions with regard to pace or food and fluid intake during the exercise induces substantial leukocytosis. These findings adds to the previous findings of increases in leukocyte subsets concentrations with prolonged endurance exercise in more moderate temperature conditions: including standard marathon running (8, 14, 46); 160-km running (27); 2-4h cycling (28, 35); and 3h treadmill running (26).

Long-distance endurance exercise induces leukocytosis (8, 14, 19, 26-28, 35, 37). Although all circulating leukocyte subsets increase with exercise, the largest increase is observed in neutrophils. Neutrophils are involved in phagocytosis of damaged tissue and thus are important to recovery from exercise. Despite of its importance to the host defense, neutrophils release reactive oxygen species, which further the process of muscle damage following exercise and contribute to inflammation (29). The exercise-induced increase in neutrophils has been suggested to be the result of demargination of cells from endothelial tissues and bone marrow to the circulation, a response which is mediated by catecholamine and cortisol, respectively (30). The absolute resting (PRE) circulating of total leukocytes and each leukocyte subset observed in the present study were all within the "normal" reference range for the general population (2) and for elite, albeit younger, cycle athletes (12); however, the absolute concentration of neutrophil was near the low end of the "normal" range. This low-end PRE neutrophil concentration likely explain the low relative proportion of neutrophils (33%) and the high relative proportion of lymphocytes (60%) among total circulating leukocytes. It is uncertain why the neutrophil proportion was low in the study population; however, it has been suggested that that plasma volume expansion, which is commonly observed among trained endurance athletes (5), might cause a selective reduction in neutrophil concentration but not in lymphocytes (12). Following 164-km of cycling in the heat, POST neutrophil concentrations were greater than 4-fold those observed for PRE. The magnitude of the increase in neutrophils is similar to the increase found for 100-km (31) and 160-km (27) running in a moderate (~23°C) ambient temperature although these events were likely performed at a lower relative intensity (10) Since running induces greater muscle damage than cycling at the same relative intensity (41), the similar neutrophil response suggests that a hot environment provides added stress to the immune system during exercise. This is supported by the previous finding that cycle exercise induced a greater increase in neutrophils during shorter-term heat exposure (40 min submersion in 39°C water) than cold exposure (18- 23°C water) (6, 33). A potential mechanism underlying this augmented neutrophil response when exercising in the heat could be changes in cortisol and catecholamine (epinephrine, and norepinephrine) concentrations. Exercise in the heat induces a greater

increase in the concentration of cortisol (2 fold), epinephrine (4 fold), and norepinephrine (2-3 fold) compared to exercise in the cold (6, 33, 38). Cortisol and catecholamine interact with glucocorticoid receptors (22) and β2-andrenergic receptors (24) of neutrophils, respectively, and influence the development, trafficking, and function of immunocytes (25, 36, 47).

Monocytes with inflammatory profiles migrate to damaged tissue and mature into macrophages after entering that tissue. Similar to neutrophils, macrophages are involved in phagocytosis of damaged tissue and thus facilitate muscle remodeling (45), but macrophages also serve an important role as a link between the non-specific and specific response by releasing cytokines into the circulation to attract other immune cells. The increase in monocytes (24%) in the present study is similar to that observed for 4 hours of ergometer cycling at room temperature (29%)(35) and for 40 min water immersed cycling at 39°C (15%) and 18°C (20%)(33). The similarity in these monocyte responses to exercise suggests that, unlike neutrophils and lymphocytes, the monocytes response is ambient temperature independent during cycle exercise. In contrast to the moderate increase in monocytes found with cycling, long distance running across a range of relative intensities, including intensities similar to that of the current study, induces a substantial increase in monocytes: marathon (150%) (8), 3 hour treadmill (67%) (26), 100 km (148%) (31), and 160 km (213%) (27) running in moderate temperature; no study appears to have investigated the monocyte response to long distance running in the heat. Running induces greater muscle damage than cycling at the same intensity (41) thus, it appears likely that the smaller monocyte increase observed in this and other cycling studies compared to that observed for running is due to the difference in muscle damage from the two exercise modes.

T and B lymphocytes function both in innate and adaptive immunity. The increase in lymphocytes in response to endurance exercise observed in this and previous studies is less pronounced than that of neutrophils (6, 8, 26, 32, 33, 38). The increase in lymphocytes (53%) in the present study following the completion of a 164-km cycling event in the heat (average temperature 35°C), is similar to the increase found in studies involving shorter-term exercise under heat exposure: 40 min cycling immersed in 39°C water (53-54%)(6, 33) and 90 min cycling at 70%VO_{2peak} at 35°C (38). In contrast, exercise in a cold environment (18- 23°C water) induced only a modest increase in lymphocyte concentration (21-25%) (6, 33). Using multiple regression, Cross et al. (6) found that cortisol concentration, growth hormone concentration, and rectal temperature accounted for 93% of the variance in lymphocyte concentrations following exercise in the heat and 87% of the variance following exercise in the cold(6). Thus as with neutrophils, the greater lymphocyte response to exercise in the heat than in the cold is likely, at least in part, the result of core temperature mediated stimulation of stress hormone release(6, 33).

The results of present study showed no significant relationship between the change in leukocyte subsets concentrations and time to complete the event (rolling time) or

intensity (%VO_{2max}). It was hypothesized that the slower riders would have a greater leukocyte response since they were exposed to the high temperature for longer. Although there was a large spread in the time to complete the event (4.7-8.2 hours) even the fastest rider spent more than 4 hours under heat exposure and thus the additional heat exposure for the slower riders appears to have not affected the leukocyte subsets response. It is possible that any potential effects of prolonged heat exposure on leukocyte subsets concentrations were counteracted by the lower absolute intensity of exercise performed by the slower riders. In contrast to the large range in time to complete the event, the range of the estimated relative intensity was rather small (61-85%) and thus not well suited for investigations of bivariate correlations. It appears that when completing a 164-km cycling event in the heat, the rolling time and self-determined relative intensity do not affect the leukocyte response.

It should be noted that the age range of participants in the current study was large (25-61 years) although most of the participants were between 40 and 58. Previous studies have found that age can significantly affect neutrophils (4), thus the findings might not apply to young adults. Gender can also affect immune cell volume, but since only 4 women volunteered for the current study, it is not possible to examine a potential gender effect on changes in immune cell volume during the 164 km ride in a hot ambient temperature.

Although in this observational study, we do not have clear information about the activational state and characterization within each immune cell subpopulation, the increases observed, most notably, in neutrophil and monocyte sub-populations vs. lymphocyte sub-populations, suggest that the exercise in combination with the environmental stress posed a significant stimulus for a circulating immune response. Furthermore, the lack of correlation to relative exercise intensity or duration suggests that in this event, the participants might have experienced a dramatic enough environmental stressor or overall exercise stress such that differences on the order of hours for duration time had no apparent effect on circulating concentrations of immune cells. This secondary finding suggests an intriguing hypothesis that there is a threshold of heat stress exposure beyond which any additional effects of exercise are either non-significant or negligible with regard to the leukocyte subset response. Long-distance endurance events, and the training preceding these events, which often occur in extreme environmental conditions, might affect the acute and long-term health of even in well-trained participants. Considering the growing popularity of these types of events, better understanding the acute effects of such events is critical to defining proper recovery and monitoring health over the course of training and post-ride.

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