Comparison of the lymphocyte response to interval exercise versus continuous exercise in recreationally trained men

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A B S T R A C T

The purpose of this investigation was to compare changes in circulating lymphocyte subset cell counts between high-intensity interval exercise (HIIE), sprint interval exercise (SIE), and moderate-intensity continuous exercise (MICE). Recreationally active men (n = 11; age: 23 ± 4 yr; height: 179.9 ± 4.5 cm; body mass: 79.8 ± 8.7 kg; body fat %:12.6 ± 3.8%; VO_2max: 46.6 ± 3.9 ml·kg^{-1}·min^{-1}) completed a maximal graded exercise test to determine maximal oxygen uptake (VO_2max) and three duration-matched cycling trials (HIIE, SIE, and MICE) in a randomized, counterbalanced fashion. HIIE consisted of fifteen 90-s bouts at 85% VO_2max interspersed with 90-s active recovery periods. SIE consisted of fifteen 20-s bouts at 130% maximal power and 160-s active recovery periods. MICE was a continuous bout at 65% VO_2max. Total exercise duration was 53 min in all three trials, including warm-up and cool-down. Blood was collected before, immediately post, 30 min, 2 h, 6 h, and 24 h post-exercise. Changes in lymphocyte subset counts, and surface expression of various markers were analyzed via flow cytometry. Changes were assessed using mixed model regression analysis with an autoregressive first order repeated measures correction. Significant decreases were observed in absolute counts of CD56^{dim} NK cells, CD19$^+$ B cells, CD4$^+$ T cells, and CD8$^+$ T cells 30 min and 24-h post-exercise in all three trials. Despite resulting in greater total work and oxygen consumption, MICE elicited similar changes in lymphocyte subset counts and receptor expression compared to both SIE and HIIE. Similarly, while the two interval trials resulted in differing oxygen consumption and total work, no differences in the lymphocyte response were observed. Though both forms of exercise resulted in declines in circulating lymphocyte cell counts, neither exercise type provides an immune-related advantage when matched for duration.

1. Introduction

Interval exercise is a time-efficient style of training involving repeated bouts of relatively intense exercise interspersed by short periods of active or passive recovery. Studies have shown that high-intensity interval exercise (HIIE) can improve cycling performance (Hazell et al., 2010), cardiorespiratory fitness (Hazell et al., 2010; Astorino et al., 2011), and body composition (Hazell et al., 2014). Current terminology defines HIIE as 'near maximal' repeated efforts performed at intensities between 85 and 95% of maximal heart rate and sprint interval exercise (SIE) as efforts performed at maximal or supramaximal intensities (Hazell et al., 2010; Gillen et al., 2014; MacInnis and Gibala, 2017; Wood et al., 2016). In comparison, moderate intensity continuous exercise (MICE) is defined as

exercise performed in a continuous fashion at lower intensities than those used during interval training (MacInnis and Gibala, 2017). The benefits of interval training for improving aerobic performance have been well-documented in both recreational and elite athletes ranging across a wide variety of sports (MacInnis and Gibala, 2017; Gibala et al., 2006; Helgerud et al., 2007; Ní Chéilleachair et al., 2017; Rønnestad et al., 2016). However, the effects of interval exercise on immune health are not clearly understood.

The rapid release of lymphocytes into circulation (lymphocytosis) during and immediately following exercise is one of the most documented exercise-induced effects on the immune system (Kakanis et al., 2010; Lancaster et al., 2004; Nieman et al., 1989, 1994, 1995; Ronsen et al., 2001). Exercise-induced lymphocytosis is proportional to the intensity

and duration of exercise and is driven by the actions of catecholamines and increased shear stress associated with enhanced blood flow during exercise (Dhabhar, 2018; Graff et al., 2018; Walsh et al., 2011). Lymphocytosis is quickly followed by a return to pre-exercise cell numbers or below. It has previously been proposed that natural killer (NK) cells and $CD8^+$ T cells that egress the blood are destined for peripheral tissues (Bigley et al., 2014; Turner et al., 2010), though it is plausible that circulating cells may return to the marginated pool and secondary lymphoid organs from which they were mobilized (Dhabhar et al., 2012).

In addition to changes in lymphocyte mobilization, markers of early activation (Timmons et al., 2006) and migration (Friedman et al., 2012) have previously been used to evaluate the impact of exercise on immune function. CD69 is a membrane-bound receptor that serves as an early marker of lymphocyte activation due to its rapid upregulation upon activation in most leukocytes, primarily lymphocytes (Cibrián and Sánchez-Madrid, 2017). Importantly, CD69 expression, determined by either the number of cells expressing this marker or as the density (median fluorescence intensity) of the receptor on the cell subset, has been linked to NK cell cytotoxicity (Lanier et al., 1986). CD69 expression on NK cells may therefore be a plausible marker of NK cell function. CX3C chemokine receptor 1 (CX3CR1) is expressed by T cell and NK cell subsets, dendritic cells, and monocytes, and is the only known receptor for the chemotactic cytokine CX_3CL1, commonly known as fractalkine (Imai et al., 1997; Jung et al., 2000). Membrane-bound fractalkine is upregulated in endothelial cells by pro-inflammatory cytokines, which promotes the capture and eventual egress of expressing monocytes and lymphocytes from circulation (Imai et al., 1997). Changes in CX3CR1 expression on lymphocyte subsets has previously been used to determine if exercise-induced declines in circulating lymphocyte cell counts are due to migration from the vascular compartment (Friedman et al., 2012). To this end, CX3CR1 expression has been shown to be elevated in T cell subsets following both continuous and interval exercise (Friedman et al., 2012; Navalta et al., 2012, 2014, 2016).

Despite the well-documented performance benefits of interval exercise, studies evaluating and comparing the immunoregulatory effects of interval exercise versus continuous exercise are lacking. Though the clinical significance of transient exercise-induced changes in circulating lymphocyte counts remain under debate (Campbell and Turner, 2018, 2019; Simpson et al., 2020), comparing the effects of interval exercise and traditional endurance exercise on lymphocyte subset cell counts may provide further rationale for choosing one exercise type over the other. Moreover, previous research has reported dissimilar physiological and perceptual responses between SIE and HIIE (Wood et al., 2016); therefore, HIIE and SIE may also result in dissimilar immune responses. Additionally, though interval training is widely regarded as a time-efficient strategy to enhance aerobic performance, the present investigation matched the duration of the protocols to ensure that any differences observed in the immune responses would result from the disparities in training intensity and/or workload rather than exercise duration. Therefore, the purpose of this study was to compare changes in circulating lymphocyte subset counts between duration-matched bouts of MICE, SIE, and HIIE.

2. Materials and methods

A convenience sample of 16 recreationally trained men were recruited to participate in this experimental study. Women were excluded due to sex-related differences in the immune response to exercise (Aomatsu et al., 2013; Asai et al., 2001; Klein and Flanagan, 2016). Five participants were removed from the investigation prior to analysis due to noncompliance with study protocols (n=1), discomfort with the seat of the cycle ergometer (n=1), scheduling conflicts (n=2), and difficulty with venipunctures (n=1). Therefore, a total of 11 participants (age: 23 ± 4 yr; height: 179.9 ± 4.5 cm; body mass: 79.8 ± 8.7 kg; body fat %:12.6 ± 3.8%; VO2max: 46.6 ± 3.9 ml·kg^{-1}·min^{-1}) were included in the analysis. Inclusion criteria consisted of age 18–35 yr, free of any physical limitations that may affect performance, and a relative maximal

oxygen uptake (VO2max) ≥ 40 ml·kg^{-1}·min^{-1} as determined by a graded exercise test. Additionally, all participants were free of medications, performance enhancing drugs, and dietary supplements that may have antioxidant or recovery characteristics as determined by a health and activity questionnaire. Participants were recruited from the University and surrounding areas. Following an explanation of all procedures, risks, and benefits, each participant provided his written informed consent prior to participation in this study. The research protocol and the informed consent document were approved by the Institutional Review Board prior to participant enrollment. One adverse event was reported, whereby a participant reported a syncopal episode during the recovery period following an experimental trial. Further investigation into the event revealed noncompliance with the activity restrictions and sleep recommendations during the trial. The adverse event was reported to the Institutional Review Board and the participant was withdrawn.

Sample size was estimated a priori using G*Power and the procedures described by Beck (2013). Analyses based on the mean differences in T helper counts between the high- and moderate-intensity conditions at 2 h post-exercise reported by Nieman et al. (1994) indicated that a total sample size of 9 would produce a statistical power $(1 - \beta)$ of 0.93 at an alpha level of 0.05. Additional a priori power analyses were conducted to confirm sample size estimation. Based on the mean differences in total lymphocyte counts between the high- and moderate-intensity conditions at 2 h post-exercise reported by Nieman et al. (1993), a total sample size of 6 was required to a maintain power at 0.80. This data was used to estimate sample size a priori for the present study due to the similarities in study design and outcome variables.

2.1. Study design

Participants reported to the Exercise Performance and Recovery Laboratory (EPRL) on four occasions. On the first visit, participants completed baseline testing including anthropometrics and a maximal graded exercise test to establish workloads for the three subsequent visits. The remaining three visits included: a SIE trial, an HIIE trial, and a MICE trial. Participants completed each experimental trial in a randomized, counterbalanced fashion separated by a minimum of 72 h. During this time, participants were encouraged to abstain from strenuous exercise. For all trials, participants arrived to the EPRL in the morning (0600–0900) following a 10-h fast and provided pre-exercise (PRE) blood samples. Participants then consumed a standardized snack bar (Enjoy Life Foods; 140 Kcal, 4g fat, 26g CHO, 2g protein) and began exercise. Subsequent blood samples were collected immediately post (IP), as well as 30 min, 2 h, 6 h, and 24 h post-exercise. Participants consumed standardized meals immediately after the 2 h blood draw (Conagra Brands Marie Callender's; 510 Kcal, 21g fat, 56g CHO, 25g protein) and again after the 6 h blood draw (Conagra Brands Marie Callender's; 590 Kcal, 25g fat, 64g CHO, 27g protein). Participants were asked to refrain from any food or drink (except for water) until they reported back to the EPRL for the 24 h timepoint. An overview of the experimental design is presented in Fig. 1.

Participants reported to the laboratory on four occasions separated by a minimum of 72 h. The first visit consisted of baseline measurements including anthropometrics and a VO2max test. The remaining three visits included: a sprint interval exercise (SIE) trial, a high-intensity interval exercise (HIIE) trial, and a moderate intensity continuous exercise (MICE) trial. Participants completed each trial in a randomized, counterbalanced fashion. Blood samples were collected pre-exercise (PRE), immediately post (IP), and 30 min, 2 h, 6 h, and 24 h post-exercise. Participants consumed standardized meals immediately after PRE, 2 h, and 6 h.

2.2. Study procedures

Height (±0.1 cm) and body mass (±0.1 kg) were measured using a Health o meter® Professional scale (Model 500 KL, Pelstar® LLC, McCook,
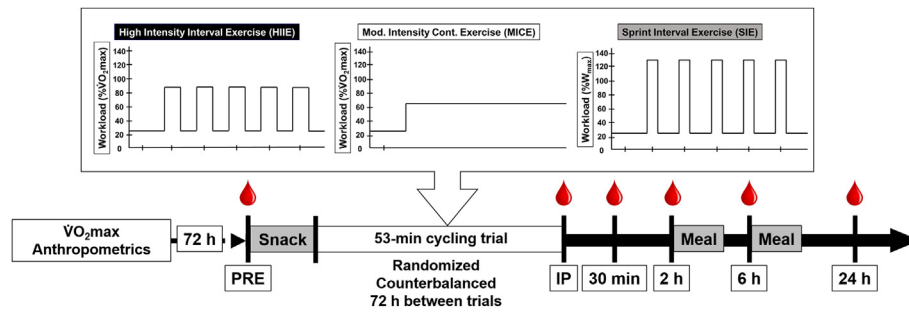
Fig. 1. Study design.

IL, USA). Body fat percentage was assessed via 7-site skinfold measurements according to the ACSM guidelines (American College of Sports Medicine et al., 2018). Body density was calculated using the Jackson-Pollock generalized skinfold equation (Jackson and Pollock, 1985) and body fat was estimated using the Siri equation (Siri, 1993). All skinfold measurements were performed by the same researcher using the same skinfold calipers (Lange Skinfold Caliper, Beta Technology, Santa Cruz, CA, USA).

Participants were fitted with a heart rate (HR) monitor (Polar Electro, Woodbury, NY, USA) and prepared for an incremental test to exhaustion on an electrically braked cycle ergometer (Velotron Dynafit Pro, QUARQ, Spearfish, SD, USA). For the first four stages, workload was set to 50 W and increased in a stepwise fashion by 50 W every 3 min. For the rest of the stages, workload increased by 30 W every 2 min until volitional fatigue. Throughout the test, pulmonary gas exchange data (Parvo Medics TrueOne, Sandy, UT, USA) and HR were monitored continuously, while ratings of perceived exertion (RPE; 6–20 scale) (Borg, 1998) were collected at the end of each stage. Participants were considered to have reached maximum capacity if their respiratory exchange ratio (RER) exceeded 1.1, RPE exceeded 17, or a plateau was observed in the participant's HR or oxygen consumption (VO_2), despite an increased workload. Test termination criteria followed the ACSM guidelines (American College of Sports Medicine et al., 2018). Once volitional fatigue was reached, maximal oxygen consumption and maximal workload [VO_2max and the maximum wattage attained (W_{max}), respectively] were recorded and used to determine the intensities for the experimental trials. Workloads for the experimental trials were determined by plotting average VO_2 over the final minute of each stage against intensity and calculating a regression equation.

2.2.1. Experimental trials

All experimental trials began with a 5-min warm-up at 25% VO_2max and ended with a 3-min recovery period at the same workload (Wood et al., 2016). HIIE consisted of fifteen 90-s bouts at 85% VO_2max separated by 90-s active recovery periods at 25% VO_2max. SIE consisted of fifteen 20-s bouts at 130% W_{max} and 160-s active recovery periods at 25% VO_2max. MICE consisted of a single 45-min bout at 65% VO_2max. Pulmonary gas exchange was collected intermittently during each session. For HIIE and SIE, gas exchange data was recorded before the warm-up, as well as during bouts 1, 5, 10, 15, and their respective recovery periods (for a total of 3 min), as well as the cool-down. For MICE, gas exchange data was recorded before the warm-up, during minutes 0–3, 12–15, 27–30, and 42–45, as well as during the cool-down (matching interval exercise). HR was continuously monitored and analyzed similar to gas exchange data. RPE was monitored starting at pre-exercise and after every other bout for SIE and HIIE, and every 6 min for MICE (matching interval exercise).

2.2.2. Dietary recall

Participants provided a 24-h dietary recall prior to each exercise trial and were asked to maintain their regular diet for the duration of the investigation. Each participant was asked to record as accurately as possible all the food and drink they consumed on the previous day. The United States Department of Agriculture FoodData Central (USDA Agricultural Research Service, Beltsville, MD, USA) was used to analyze the self-reported dietary recalls for total kilocalorie intake and macronutrient proportions (carbohydrate, protein, and fat).

2.2.3. Blood measurements

Venous blood samples were collected at PRE, IP, 30 min, 2 h, 6 h, and 24 h post-exercise. The PRE, IP, 30 min, and 2 h blood samples were obtained using a Teflon cannula placed in a superficial forearm vein. The cannula was maintained patent using a non-heparinized isotonic saline solution (Becton Dickinson, Franklin Lakes, NJ, USA). The 6 h and 24 h blood samples were obtained using a Vacutainer® blood collection needle (Becton Dickinson). With the exception of IP, participants lain supine for 15 min prior to each blood draw. Approximately 10 mL of blood was collected at each timepoint in a 10 mL Vacutainer® tube containing K_2EDTA.

Total lymphocyte (LY) counts were analyzed via an automated hematology analyzer (Coulter® AC·T diff 2™, Beckman Coulter, Brea, CA).

2.2.4. Cell staining

A 100 μL sample of K_2EDTA-treated peripheral whole blood was used to identify lymphocyte subpopulations and surface markers by direct immunofluorescent labeling of cell surface antigens with mouse and rat anti-human monoclonal antibodies. Prior to data collection, every antibody was titrated to determine optimal antibody concentrations. At each timepoint, a sample was heat-treated to induce cell death and determine viability dye expression for the exclusion of non-viable cells. Briefly, 200 μL of whole blood were transferred to a 12 × 75 mm polystyrene test tube and placed in 65 °C water for 1 min. The sample was then cooled in 4 °C armor beads (Lab Armor, LLC, Cornelius, OR, USA) for 1 min. Finally, another 200 μL of fresh whole blood was mixed into the test tube and used for analysis.

Cells were first stained with an amine-reactive fluorescent dye (Zombie Green™, BioLegend, San Diego, CA, USA) and incubated while protected from light for 15 min to determine cell viability. Following incubation, samples were centrifuged (Centrifuge 5810 R, Eppendorf) at 300*g* for 8 min and washed with 2 mL of 1 × wash buffer containing 1% fetal bovine serum (Avantor® Seradigm Premium Grade FBS, Radnor, PA, USA) and 0.1% sodium azide (NaN_3; VWR Chemicals BDH®, Radnor, PA, USA) in a 1 × phosphate buffered saline (Corning® 10X PBS, Corning, NY, USA) solution. Samples were then centrifuged at 300*g* for 8 min, and the supernatant was discarded. Nonspecific binding was blocked with Fc receptor blocking solution (Human TruStain FcX™, BioLegend). Antibodies were pre-conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), or peridinin chlorophyll protein complex (PerCP; BioLegend, San Diego, CA) and are shown in Table 1. Surface staining was performed by adding 25 μL of the appropriate antibody cocktail (Table 1) diluted in PBS to the cell suspension and incubating in the dark for 15 min at room temperature. After staining, samples were lysed with 2 mL of RBC Lysis/Fixation solution (BioLegend) and subsequently mixed and incubated in the dark for 12

Table 1
Flow cytometry antibody panel.

	FITC	FITC	PE	PerCP	APC
Blank	–	–	–	–	–
Viability (heat-treated)	Zombie	–	–	–	–
Cocktail 1	Zombie	CD3 (HIT3a)	CD56 (5.1H11)		–
Cocktail 2	Zombie	CD3 (HIT3a)		CD16 (3G8)	–
Cocktail 3	Zombie	CD3 (HIT3a)	CD56 (5.1H11)	CD16 (3G8)	–
Cocktail 4	Zombie	CD3 (HIT3a)	CD56 (5.1H11)	CD16 (3G8)	CD69 (FN50)
Cocktail 5	Zombie	CD3 (HIT3a)	CD19 (HIB19)	–	
Cocktail 6	Zombie	CD3 (HIT3a)	CD19 (HIB19)	–	CD69 (FN50)
Cocktail 7	Zombie	–	–	CD3 (HIT3a)	
Cocktail 8	Zombie	–	CD4 (RPA-T4)	CD3 (HIT3a)	–
Cocktail 9	Zombie	–	CD8a (RPA-T8)	CD3 (HIT3a)	–
Cocktail 10	Zombie	–	CD4 (RPA-T4)	CD3 (HIT3a)	CX3CR1 (2A9-1)
Cocktail 11	Zombie	–	CD8a (RPA-T8)	CD3 (HIT3a)	CX3CR1 (2A9-1)
Cocktail 12	Zombie	–	CD4 (RPA-T4)	CD3 (HIT3a)	CD69 (FN50)
Cocktail 13	Zombie	–	CD8a (RPA-T8)	CD3 (HIT3a)	CD69 (FN50)

Antibody (clone). FITC = Fluorescein isothiocyanate. PE = Phycoerythrin. PerCP = peridinin chlorophyll protein complex. APC = allophycocyanin.

min. Following incubation, samples were centrifuged and washed once more. Finally, samples were fixed in a 300 μL solution of 2% para-formaldehyde (Santa Cruz Biotechnology, Dallas, TX, USA) in PBS.

2.2.5. Flow cytometry

Flow cytometry analysis of stained samples was performed on an ACEA NovoCyte™ Flow Cytometer (ACEA Biosciences, Inc., San Diego, CA, USA), equipped with two lasers providing excitation at 488 and 640 nm, four band pass filters, and ACEA's NovoExpress™ software. Events were gated based on forward scatter (FSC), side-scatter (SSC), and median fluorescence intensity (MFI) as depicted in Fig. 2. Compensation for fluorescence spillover was performed bi-weekly prior to and during data collection by staining of anti-mouse and anti-rat Ig, κ/negative control compensation particles (BD CompBeads, BD Biosciences, San Jose, CA, USA). Unstained blood samples and fluorescence-minus-one (FMO) controls were used to determine optimal gate positions (Roederer, 2002). A 100 μL sample was used to analyze changes in peripheral blood lymphocyte subset counts and surface markers. Lymphocyte subsets were identified as B cells (CD3⁻CD19⁺), cytotoxic NK cells (CD3⁻CD56^{dim}CD16⁺), regulatory NK cells (CD3⁻CD56^{bright}CD16⁻), T helper cells (CD3⁺CD4⁺), and T cytotoxic cells (CD3⁺CD8⁺). CD69 expression was assessed on each lymphocyte subset and reported as MFI and %positive ($\frac{CD69^{+}\,count}{Total\,count} \times 100$). CX3CR1 expression was assessed on T cell subsets and reported similarly. The gating strategy employed for lymphocyte subsets is depicted in Fig. 2. Changes in lymphocyte counts were expressed as % of total lymphocytes and absolute number of cells per μL of peripheral blood. Absolute number of lymphocyte subsets was determined by multiplying the percentage of gated cells expressing the surface marker combinations of interest by the total blood lymphocyte counts measured by the automated hematology analyzer (Rooney et al., 2018).

A. Gating was performed midflow to exclude debris and **(a)** multiplets using forward scatter properties. **(b)** Lymphocytes were identified based on forward and side scatter. **B.** Non-viable cells positive for Zombie

Green were excluded. **(c)** T cytotoxic (CD8⁺) cells and **(d)** T helper (CD4⁺) cells were identified based on CD3, CD4, and CD8 expression. **C.** Non-viable and CD3⁺ cells were excluded. **(e)** CD19⁺ B cells and **(f)** natural killer cell subsets (CD56^{bright} and CD56^{dim}) were identified based on CD19, CD56, and CD16 expression. **D.** CX3CR1 surface expression [Median Fluorescence Intensity (MFI) and %positive] were measured on T cell subsets. **(E)** CD69 expression was measured on all lymphocyte subsets.

2.3. Statistical analysis

Prior to statistical procedures, all data were assessed to ensure normal distribution, homogeneity of variance, and sphericity. If the assumption of sphericity was violated, a Greenhouse-Geisser correction was applied. The Shapiro-Wilk test was used to determine normality. Changes in VO₂, total lymphocyte, and lymphocyte subset (CD19⁺ B cells, CD56^{bright} and CD56^{dim} NK cells, CD4⁺ and CD8⁺ T cells) counts, as well as expression of CD69 and CX3CR1 were analyzed using mixed model regression analysis with an autoregressive first order repeated measures correction. Mixed-model regression analysis, with trial and timepoint as fixed factors, was used to account for interindividual differences in resting lymphocyte subset counts and receptor expression and missing timepoints for VO₂. Dietary intake during the 24 h prior to each experimental trial and total work performed during each trial were analyzed using one-way, repeated measures ANOVA. In the event of a significant F ratio, the least significant difference (LSD) post-hoc analysis was used for pairwise comparisons. If a significant interaction was observed, follow-up one-way, repeated measures ANOVA and LSD pairwise comparisons were used to determine time effects within each trial and trial effects at each timepoint. Time effects were further analyzed using partial eta squared (η^2_p). Interpretations of η^2_p were evaluated according to Cohen (1988) at the following levels: small effect (0.01–0.058), medium effect (0.059–0.137), and large effect (>0.138). Significance was accepted at α ≤ 0.05. Data were analyzed using IBM SPSS Statistics for Windows (version 26.0; IBM Corp., Armonk, NY). All data are presented as mean (SD).

3. Results

Dietary intake during the 24 h prior to the experimental trials is presented in Table 2. No main effect for trial (F = 0.008, p = 0.992, η^2_p = 0.001) was noted for total caloric, carbohydrate, fat, or protein intake during the 24 h prior to each experimental trial.

3.1. Oxygen consumption during exercise

A trial × time interaction (F = 12.369, p < 0.001, η^2_p = 0.706) was observed for VO₂ (Fig. 3A). No significant differences in VO₂ were observed between trials before exercise. During bout 1 (3 min for MICE), VO₂ was higher in both MICE (p = 0.005) and HIIE (p = 0.001) compared to SIE (p = 0.005). During bout 5 (15 min for MICE), VO₂ was higher in MICE compared to both SIE (p = 0.010) and HIIE (p = 0.019), and higher in HIIE compared to SIE (p = 0.002). During bout 10 (30 min for MICE), VO₂ was higher in MICE compared to both SIE and HIIE, and higher in HIIE compared to SIE ($p's$ < 0.001). During bout 15 (45 min for MICE), VO₂ was higher in MICE and HIIE compared to SIE ($p's$ < 0.001). During the cool-down, VO₂ was higher in MICE compared to SIE and HIIE ($p's$ ≤ 0.001).

3.2. Total work during exercise

A main effect of trial (F = 289.806, p < 0.001, η^2_p = 0.967) was observed for total work performed during exercise (Fig. 3B). Total work was greater in the MICE trial compared to both HIIE and SIE and higher in HIIE compared to SIE ($p's$ < 0.001).
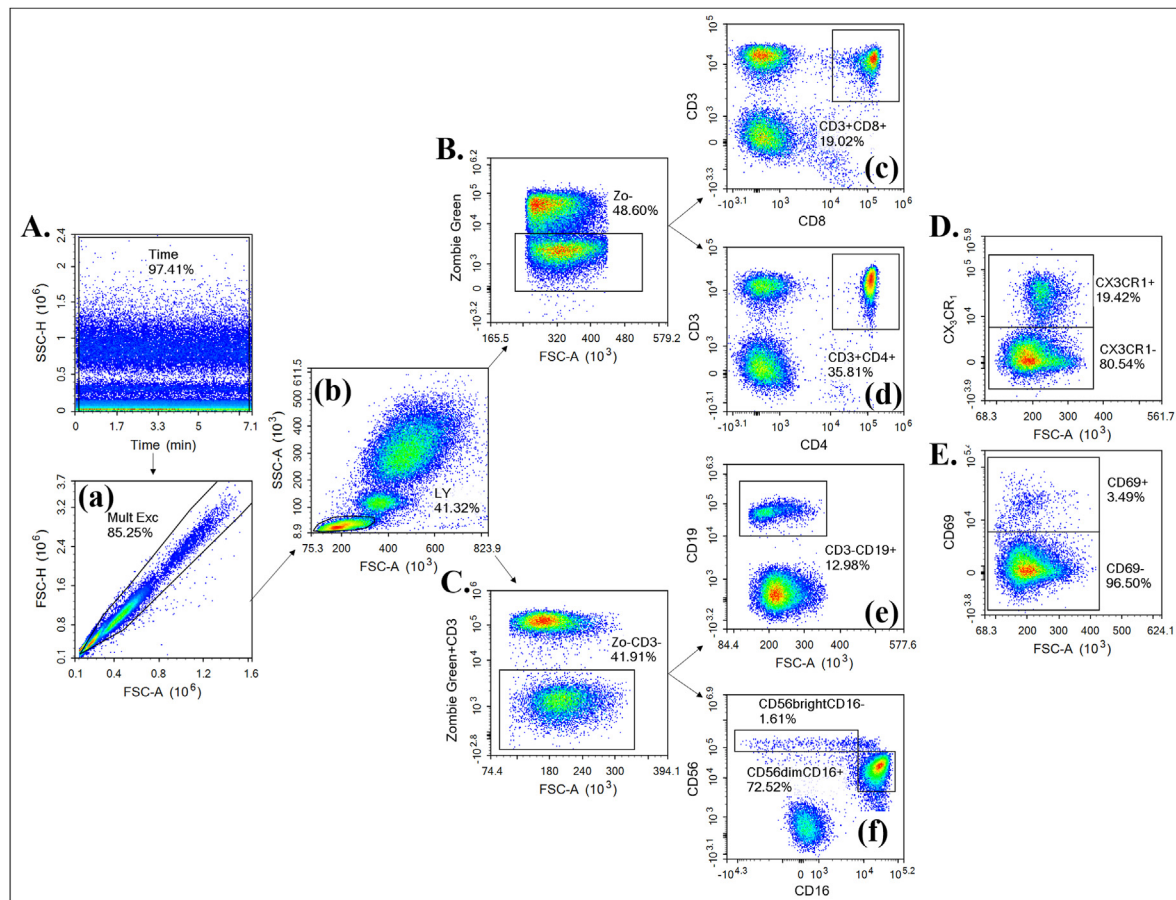
Fig. 2. Flow cytometry gating procedure.

Table 2
Dietary intake.

	MICE	SIE	HIIE
Caloric Intake (kcal·day^{-1})	2879.8 (977.8)	2890.2 (1116.4)	2843.8 (721)
Carbohydrate (g·day^{-1})	313.3 (167.3)	318.5 (131.6)	317.2 (95.3)
Fat (g·day^{-1})	116.5 (39.1)	128.7 (48)	122.2 (33.9)
Protein (g·day^{-1})	122.4 (41.8)	116.9 (55.7)	125.7 (53.9)

Mean (SD).

3.3. Changes in total lymphocyte counts

A trial × time interaction (F = 2.070, $p = 0.046$, $\eta^2_p = 0.301$) was observed for changes in LY (Fig. 4A). At IP, LY was higher in HIIE ($p = 0.008$) and MICE ($p = 0.006$) compared to SIE. For all three trials, LY increased from PRE to IP (p's ≤ 0.005), decreased to below PRE numbers at 30 min (p's ≤ 0.004), returned to PRE numbers at 2H (p's > 0.05), and were decreased at 24H relative to PRE ($p \leq 0.043$). A significant increase in LY was observed at 6H relative to PRE for HIIE only ($p = 0.015$).

3.4. Changes in lymphocyte subset counts and percentages

A main effect of time (F = 15.006, $p < 0.001$, $\eta^2_p = 0.602$) was observed for changes in CD19$^+$ counts (Fig. 4B). CD19$^+$ counts decreased from PRE to 30 min ($p = 0.001$), returned to PRE numbers at 2H ($p = 0.323$), increased at 6H ($p = 0.007$), and decreased to below PRE numbers at 24H ($p = 0.001$).

A main effect of time (F = 20.563, $p < 0.001$, $\eta^2_p = 0.606$) was observed for changes in CD4$^+$ counts (Fig. 4C). CD4$^+$ counts increased from PRE to IP ($p < 0.001$), decreased to below PRE numbers at 30 min

($p < 0.001$), returned to PRE at 2H ($p = 0.097$), rose again at 6H relative to PRE ($p = 0.020$) and decreased below PRE numbers at 24H ($p < 0.001$).

A main effect of time (F = 28.520, $p < 0.001$, $\eta^2_p = 0.657$) was observed for changes in CD8$^+$ counts (Fig. 4D). CD8$^+$ counts increased from PRE to IP ($p < 0.001$), decreased to below PRE numbers at 30 min ($p < 0.001$), returned to PRE numbers at 2H ($p = 0.086$), increased again at 6H relative to PRE ($p = 0.017$) and decreased below PRE numbers at 24H ($p < 0.001$).

A main effect of time (F = 37.337, $p < 0.001$, $\eta^2_p = 0.846$) was observed for changes in CD56^{dim} counts (Fig. 4E). CD56^{dim} counts increased from PRE to IP ($p < 0.001$), decreased below PRE numbers at 30 min ($p < 0.001$), returned to PRE numbers at 2H ($p = 0.102$), and decreased below PRE numbers at 24H ($p = 0.032$).

A main effect of time (F = 12.344, $p < 0.001$, $\eta^2_p = 0.602$) was observed for changes in CD56^{bright} counts (Fig. 4F). CD56^{bright} counts increased from PRE to IP ($p < 0.001$) and returned to PRE numbers at 30 min ($p = 0.116$).

Changes in lymphocyte subset proportions are shown on Table 3. No significant trial × time interactions nor main effects of trial were observed for changes in the proportions of any lymphocyte subset. However, main effects of time were observed for all lymphocyte subset proportions.

3.5. Changes in CD69 expression

A main effect of time (F = 4.145, $p = 0.003$, $\eta^2_p = 0.293$) was observed for changes in CD69 MFI on CD19$^+$ cells (Supplemental Fig. 1). CD69 MFI on CD19$^+$ cells increased from PRE to IP ($p = 0.002$), remained elevated at 30 min ($p = 0.010$), 2H ($p = 0.034$), and 6H ($p = 0.034$), and
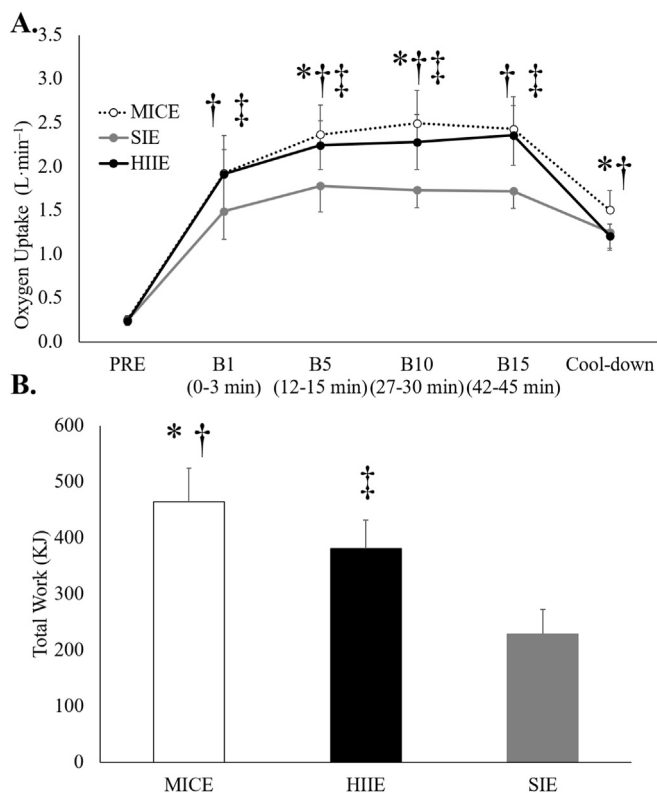
Fig. 3. Oxygen Consumption and Total Work During Exercise.
Trials: MICE, moderate-intensity continuous exercise; SIE, sprint interval exercise; HIIE, high-intensity interval exercise. Timepoints: PRE, before onset of exercise; B1, first interval and 3 min of exercise for MICE; B5, fifth interval and 15 min of exercise for MICE; B10, tenth interval and 30 min of exercise for MICE; B15, fifteenth interval and 45 min of exercise for MICE; Cool-down, 3-min recovery following trials. *Significant difference between MICE and HIIE; † Significant difference between MICE and SIE; ‡ Significant difference between HIIE and SIE. Data are presented as mean (SD).

returned to PRE MFI at 24H ($p = 0.492$). A main effect of time ($F = 5.076$, $p = 0.002$, $\eta^2_p = 0.441$) was observed for changes in CD69 %positive of $CD19^+$ cells (Supplemental Table 1). The % of $CD19^+$ cells expressing CD69 increased from PRE to 2H ($p = 0.027$), remained elevated at 6H ($p = 0.001$), and returned to PRE proportions at 24H ($p = 0.970$).

A main effect of time ($F = 6.092$, $p < 0.001$, $\eta^2_p = 0.301$) was observed for changes in CD69 MFI on $CD4^+$ cells (Supplemental Fig. 2A). CD69 MFI on $CD4^+$ cells decreased from PRE to 2H ($p = 0.028$), remained decreased at 6H ($p = 0.002$) and returned to PRE MFI at 24H ($p = 0.104$). A main effect of time ($F = 4.825$, $p = 0.002$, $\eta^2_p = 0.436$) was observed for changes in CD69 %positive of $CD4^+$ cells (Supplemental Table 1). The % of $CD4^+$ cells expressing CD69 decreased from PRE to 6H ($p = 0.007$) and remained decreased at 24H ($p = 0.013$).

A main effect of time ($F = 10.860$, $p < 0.001$, $\eta^2_p = 0.605$) was observed for changes in CD69 MFI on $CD8^+$ cells (Supplemental Fig. 2B). No changes in CD69 MFI on $CD8^+$ cells were observed relative to PRE. However, CD69 MFI on $CD8^+$ cells was higher at IP relative to 2H ($p < 0.001$), 6H ($p < 0.001$), and 24H ($p = 0.007$). A main effect of time ($F = 7.655$, $p < 0.001$, $\eta^2_p = 0.400$) was observed for changes in CD69 % positive of $CD8^+$ cells (Supplemental Table 1). The % of $CD8^+$ cells expressing CD69 increased from PRE to IP ($p < 0.001$), returned to PRE proportions at 30 min ($p = 0.071$), decreased below PRE proportions at 6H ($p < 0.001$), and returned to PRE proportions once again at 24H ($p = 0.166$).

A main effect of time ($F = 5.270$, $p = 0.001$, $\eta^2_p = 0.454$) was observed for changes in CD69 MFI on $CD56^{dim}$ cells (Supplemental Fig. 3A). CD69 MFI on $CD56^{dim}$ cells decreased from PRE to 2H ($p = 0.02$) and remained decreased at 6H ($p = 0.04$) and 24H ($p = 0.044$). A main effect of time ($F = 5.704$, $p < 0.001$, $\eta^2_p = 0.394$) was observed for changes in CD69 %positive of $CD56^{dim}$ cells (Supplemental Table 1). The % of $CD56^{dim}$ cells expressing CD69 decreased from PRE to 2H ($p = 0.032$), remained decreased at 6H ($p = 0.003$) and returned to PRE proportions at 24H ($p = 0.067$).

A main effect of time ($F = 4.054$, $p = 0.003$, $\eta^2_p = 0.260$) was observed for changes in CD69 MFI on $CD56^{bright}$ cells (Supplemental Fig. 3B). CD69 MFI on $CD56^{bright}$ cells decreased from PRE to 2H ($p = 0.042$), remained decreased at 6H ($p = 0.005$), and returned to PRE MFI
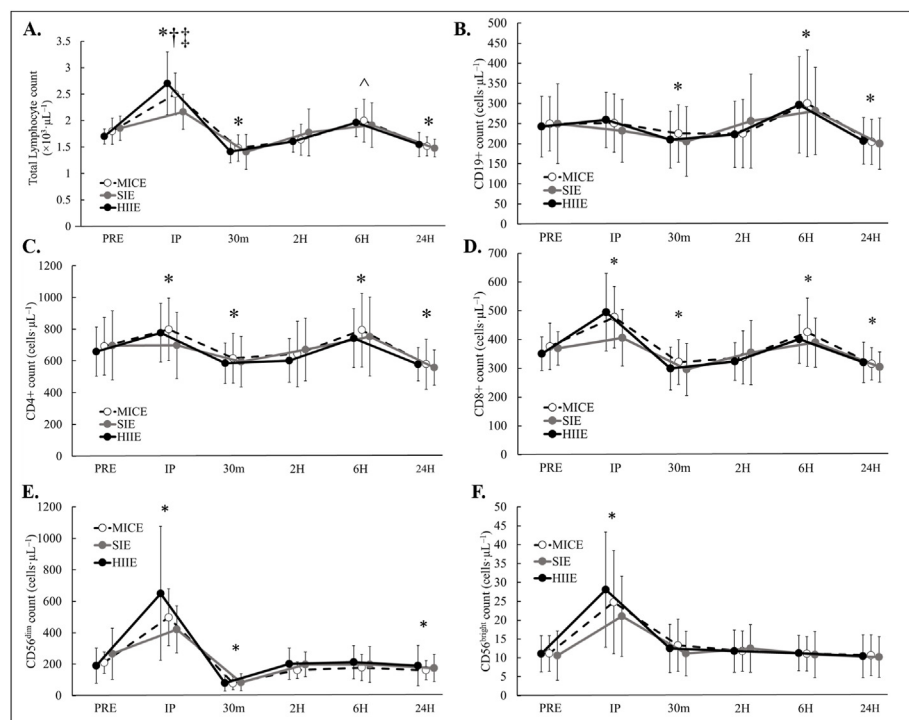

Fig. 4. Changes in Total Lymphocyte and Lymphocyte Subset counts.
(A) Total lymphocyte counts. (B) $CD19^+$ B cell counts. (C) $CD4^+$ T cell counts. (D) $CD8^+$ T cell counts. (E) $CD56^{dim}$ NK cell counts. (F) $CD56^{bright}$ NK cell counts *Significantly different from PRE, main effect of time. Data are presented as mean (SD). Significantly differently from PRE in HIIE only; †Significant difference between MICE and SIE; ‡ Significant difference between HIIE and SIE. Data are presented as mean (SD).

Table 3
Changes in lymphocyte subset proportions.

%LY		PRE	IP	30 min	2H	6H	24H	F[a]	p	η^2_p
CD19^{+}	MICE	14.1 (4.5)	10.1 (2.7)*	15.2 (4.1)	13.7 (4.5)	14.8 (5.2)	13.6 (3.8)			
	SIE	13.4 (5.2)	10.7 (3.4)*	14.3 (4.2)	14.0 (3.6)	14.5 (4.3)	13.5 (4.3)	50.232	<0.001	0.844
	HIIE	14.2 (4.1)	10.0 (3.6)*	14.7 (3.8)	13.6 (3.9)	14.8 (5.1)	13.6 (4.4)			
CD4^{+}	MICE	38.3 (7.3)	32.1 (6.9)*	41.2 (5.7)*	38.7 (7.1)	39.4 (6.5)	37.9 (7.3)			
	SIE	37.0 (7.4)	31.9 (6.6)*	42.6 (7.7)*	37.9 (6.8)	38.9 (5.7)	37.6 (5.4)	73.588	<0.001	0.844
	HIIE	38.7 (7.8)	29.4 (7.4)*	41.3 (5.9)*	37.3 (6.1)	37.7 (6.9)	37.7 (6.4)			
CD8^{+}	MICE	20.9 (4.0)	19.1 (3.4)*	21.8 (4.5)	20.6 (4.8)	21.2 (4.0)	20.9 (3.5)			
	SIE	20.0 (3.1)	18.7 (3.1)*	21.0 (4.0)	19.9 (3.9)	20.6 (3.9)	20.7 (4.2)	16.064	<0.001	0.530
	HIIE	20.7 (4.1)	18.3 (3.1)*	21.1 (4.2)	20.2 (3.9)	20.4 (3.4)	20.8 (3.9)			
CD56^{dim}	MICE	11.7 (3.5)	19.8 (6.5)*	5.4 (2.8)*	10.3 (4.1)	9.3 (4.8)	10.9 (4.5)			
	SIE	14.5 (9.5)	19.9 (7.6)*	6.1 (3.8)*	11.8 (5.7)	10.7 (6.6)	11.9 (5.7)	39.012	<0.001	0.796
	HIIE	11.3 (6.6)	22.8 (10.7)*	5.6 (3.6)*	12.8 (6.8)	11.3 (6.4)	11.9 (6.5)			
CD56^{bright}	MICE	0.6 (0.3)	1.0 (0.5)*	0.9 (0.5)*	0.7 (0.4)*	0.6 (0.3)	0.7 (0.4)			
	SIE	0.6 (0.4)	1.0 (0.5)*	0.8 (0.4)*	0.7 (0.4)*	0.6 (0.3)	0.7 (0.4)	6.829	<0.001	0.462
	HIIE	0.7 (0.3)	1.1 (0.6)*	0.9 (0.6)*	0.7 (0.4)*	0.6 (0.3)	0.7 (0.4)			

LY = Total lymphocytes. η^2_p = partial eta squared. *Significant difference from PRE. [a]Main effect of time. Mean (SD).

at 24H ($p = 0.792$). No significant trial × time interaction, main effect of time, or main effect of trial were observed for CD69 %positive of CD56^{bright} cells.

3.6. Changes in CX3CR1 expression on T cell subsets

A main effect of time (F = 7.682, $p < 0.001$, $\eta^2_p = 0.477$) was observed for changes in CX3CR1 MFI on CD4^{+} cells (Supplemental Fig. 4). CX3CR1 MFI on CD4^{+} cells increased from PRE to IP ($p = 0.025$) and returned to PRE MFI at 30 min ($p = 0.544$). No significant trial × time interaction, main effect of time, or main effect of trial were observed for CX3CR1 %positive of CD4^{+} cells.

A main effect of time (F = 3.704, $p = 0.011$, $\eta^2_p = 0.407$) was observed for changes in CX3CR1 MFI on CD8^{+} cells (Supplemental Fig. 4). No significant changes were observed relative to PRE. However, CX3CR1 MFI on CD8^{+} cells was lower at 30 min compared to 2H ($p = 0.020$) and 24H ($p = 0.038$). A main effect of time (F = 9.180, $p < 0.001$, $\eta^2_p = 0.580$) was observed for changes in CX3CR1 %positive of CD8^{+} cells (Supplemental Table 2). The % of CD8^{+} cells expressing CX3CR1 increased from PRE to IP ($p < 0.001$), decreased below PRE proportions at 30 min ($p = 0.002$), and returned to PRE proportions at 2H ($p = 0.108$).

4. Discussion

We present a direct comparison of the lymphocyte response following duration-matched acute bouts of SIE, HIIE, and MICE. Despite significant trial differences in total work and oxygen consumption, no significant differences in lymphocyte subset counts or expression of CD69 or CX3CR1 were found between SIE, HIIE, and MICE. In the present study, total work was highest in MICE (464.5 ± 60.0 KJ), followed by HIIE (381.4 ± 50.7 KJ) and SIE (229.2 ± 43.5 KJ). Accordingly, VO$_2$ was consistently higher in MICE compared to SIE and HIIE. However, total exercise duration was 53 min in all three trials, including warm-up and cool-down. Therefore, the current findings suggest that acute bouts of MICE, SIE, and HIIE elicit similar lymphocyte responses when matched for total duration.

The superior benefits of HIIE to improve aerobic capacity are well-documented (Helgerud et al., 2007; Ní Chéilleachair et al., 2017; Rønnestad et al., 2016); however, a common concern among athletes, coaches, and scientists is whether the high training loads associated with repeated intervals within a short period of time could compromise immune function and increase susceptibility to infections. These concerns are justified, given that high-intensity exercise has been shown to increase oxidative stress and increase lymphocyte apoptosis (Navalta et al.,

2007, 2009, 2012; Mars et al., 1998; Leal et al., 2021). It has been suggested that this exercise-induced increase in lymphocyte apoptosis may contribute to the declines in circulating lymphocyte cell counts (Mars et al., 1998). In the present study, declines in circulating lymphocyte cell counts were observed 30 min and 24-h post-exercise, which consisted of decreased absolute counts of CD56^{dim} NK cells, CD19^{+} B cells, CD4^{+} T cells, and CD8^{+} T cells. However, no differences were observed between MICE and interval exercise. Though no measurements of apoptosis or oxidative stress were included, the results of this study suggest that an individual HIIE or SIE training session, when matched for a duration of 53 min, does not promote greater declines in circulating lymphocytes compared to an individual MICE training session.

Our findings contrast with Turner and colleagues (Turner et al., 2016), who found that CD56^{+} NK cells were mobilized to a greater extent during continuous exercise compared with interval exercise, a response that was driven primarily by the cytotoxic subset (CD56^{dim}). Similar to the present study, Turner et al. (2016) compared duration-matched bouts of continuous and interval exercise: a 20-min continuous cycling bout at 80% VO$_2$max versus 10 × 1 min cycling intervals at 90% VO$_2$max and 1-min recovery periods. Total energy expenditure was significantly higher after continuous exercise compared with interval exercise, as was peak RPE. Notably, however, the intensity of the continuous trial was similar to that of the interval trial, and considerably higher than the intensity of the continuous bout in the present study (65% VO$_2$max). The differences in exercise duration and intensity are likely responsible for the discrepancy between our findings. Notwithstanding, similar to the present study, no trial differences were observed in LY, CD3^{+} T or CD8^{+} T cell counts (Turner et al., 2016). This indicates that CD56^{dim} NK cells may be more sensitive to differences in acute training variables than other lymphocyte subsets (Leal et al., 2021). A key limitation of this study was that post-exercise blood samples were limited to 30 min post-exercise. In the present study, significant changes in lymphocyte counts were observed at 6H and 24H post-exercise, though no trial differences were observed. Moreover, neither exercise trial resulted in declines in circulating lymphocyte cell counts, suggesting that 20 min of exercise is insufficient to measure this phenomenon (Turner et al., 2016).

Our findings are also in contrast with O'Carroll and colleagues (O'Carroll et al., 2019), who compared a 45-min bout of continuous exercise at 70% peak oxygen uptake with 6 × 20 s maximal sprints on a cycle ergometer on circulating lymphocyte counts. LY, CD8^{+} T, and CD4^{+} T cell counts were elevated following both trials. These increases were significantly higher following interval exercise compared to continuous exercise. This may be due to the higher exercise intensities implemented by O'Carroll et al., (O'Carroll et al., 2019), whereby sprint intervals were

'maximal effort' and resulted in mean power outputs of $223 \pm 6\%$ of the power output at VO_2peak. In the present study, power outputs corresponded to 130% peak power and 85% VO_2max for SIE and MICE, respectively. Notably, lymphocyte measurements were limited to pre-exercise, immediately post-exercise, 2 h post-exercise, and 24 h post-exercise. In the current study, total lymphocytes, and lymphocyte subsets, except for CD56^{bright} cells, were depleted at 30 min post-exercise compared to pre-exercise. Though O'Carroll and colleagues (O'Carroll et al., 2019) reported greater lymphocyte mobilization immediately following interval exercise compared to continuous exercise, whether this greater mobilization is linked to greater declines in circulating lymphocyte cell counts following interval exercise remains unknown. Moreover, the transient declines in circulating lymphocyte cell counts observed in the present study were accompanied by considerable decreases in the proportions of CD56^{dim} cells, which have been shown to be most responsive to exercise (Bigley et al., 2014; Turner et al., 2016; Campbell et al., 2009), and were not measured by O'Carroll et al. (O'Carroll et al., 2019).

The present study provides further evidence for exercise-induced declines in circulating lymphocyte cell counts. Though exercise type had no effect, total lymphocyte counts were depleted 30 min post-exercise and remained depleted at 24H post-exercise. The proportion of CD56^{dim} NK cells significantly decreased from PRE to 30 min, while CD4^{+} T cell percentage was significantly elevated at this timepoint and the proportions of the other lymphocyte subsets remained unchanged. Furthermore, total lymphocyte counts were elevated at IP, and this rise was primarily driven by increases in CD56^{dim} NK cells, as the proportion of these cells was significantly elevated at this timepoint, while the proportion of other lymphocyte subsets were decreased. Researchers have proposed that decreased lymphocyte counts, particularly CD8^{+} T cells and NK cells, reflect the redistribution of these cells to peripheral tissues (Simpson et al., 2006). That is, exercise-induced increases in catecholamines promote leukocyte mobilization from secondary lymphoid organs and the marginated pool into the bloodstream, which subsequently exit circulation toward target organs in preparation for possible immune challenges (Dhabhar, 2018; Dhabhar et al., 2012). To this end, the percentage of CD8^{+} T cells expressing CX3CR1, which represents a marker of lymphocyte migration, was elevated at IP, and decreased significantly at 30 min, providing evidence for CD8^{+} T cell extravasation as a potential cause for their decrease in circulating numbers.

A key point in the debate surrounding the 'Open Window Theory' is whether exercise-induced declines in circulating lymphocyte cell counts are also accompanied by impaired immune cell function (Campbell and Turner, 2018, 2019; Simpson et al., 2020). To investigate this, we measured changes in CD69 expression on lymphocyte subsets following exercise. Lymphocyte expression of CD69 has been linked to early cell activation, proliferation, apoptosis, and cytotoxicity (Borrego et al., 1999; Tugores et al., 1992; Ziegler et al., 1994). CD69 is expressed by T cells within 30 min of T-cell receptor binding, by NK cells activated by interleukin-2 or interleukin-12, as well as by all monocytes and by polymorphonuclear leukocytes (De Maria et al., 1994; Gerosa et al., 1993; Simms and Ellis, 1996). Moreover, an in vitro study by McFarlin and colleagues (McFarlin et al., 2004) showed that the number of NK cells expressing CD69 increased immediately following exercise and then decreased to below pre-exercise numbers at 2 h and 4 h post-exercise. These changes closely mirrored the changes observed in the chromium-release (^{51}Cr) cytotoxicity assay, which is considered the gold standard for measuring T cytotoxic or NK cell-mediated cytotoxicity (Karimi et al., 2014). Therefore, CD69 expression may serve as an indirect measure of NK cell cytotoxicity (Lanier et al., 1986). In the present study, CD56^{dim} cells expressed the highest density (MFI) of CD69 and the greatest proportion of cells positive for CD69. Moreover, CD69 MFI on CD56^{dim} NK cells was significantly decreased 2H, 6H, and 24H post-exercise relative to PRE. These findings suggest that mobilized CD56^{dim} NK cells following exercise may exhibit decreased cytotoxicity. CD69 expression was also downregulated in CD8^{+} T, CD4^{+} T, and

CD56^{bright} NK cells but elevated in CD19^{+} B cells during recovery from exercise. Since the physiological function of CD69 is not clearly understood, the clinical significance of our findings related to CD69 expression is uncertain.

One of the limitations of this study was that we did not include an inactive control condition to establish the influence of exercise alone on the lymphocyte response. Though all trials were performed around the same time of day (0600–0900) to minimize the effect of diurnal variations, a detailed temporal analysis of lymphocyte trafficking measuring blood and salivary cortisol samples every 15 min over an 8-h period found that changes in CD8^{+} T, CD4^{+} T, and CD56^{+} NK cell numbers were strongly linked to circadian variations of salivary cortisol (Trifonova et al., 2013). To this end, the present study found significant increases in various lymphocyte subsets (CD19^{+} B cells, CD4^{+} T cells, and CD8^{+} T cells) at 6H. This secondary increase in circulating lymphocyte numbers during the 6–8 h recovery period has been reported previously (Kakanis et al., 2010), though the mechanisms are currently unknown. Though it is possible that this secondary increase in lymphocyte numbers represents a return to circulation following the egress during the early stages of recovery, it may also represent a response to diurnal variations, as no comparison was made to inactive controls. Another limitation of this study is the exclusion of women. Given that previous studies have demonstrated sex-related differences in the immune response to exercise (Turner et al., 2016; O'Carroll et al., 2019; Campbell et al., 2009), our results may not be applicable to women. Therefore, future studies should compare the immune responses to continuous exercise versus interval exercise in women. The small sample size is another limitation of our study. The high variability in lymphocyte subset counts among subjects may require a larger sample size to detect statistically significant differences in the immune response to interval exercise versus continuous exercise.

One of the strengths of this study was that dietary intake was standardized for each trial. Therefore, any trial-related differences in the immune response could not be attributed to differences in dietary intake. However, dietary intake was not normalized to body mass or activity levels, and caloric intake during exercise trials was 1,240 kilocalories for every participant. Given that our participants varied widely in body mass and activity levels, it is likely that, while all participants were calorie restricted, some participants had greater caloric deficits during exercise trials than others. Since calorie restriction can have an impact on circulating lymphocyte numbers (Meydani et al., 2016), inter-individual differences in caloric needs may have played a role in the immune response to exercise. Future studies should aim to normalize dietary intake during trials to individual caloric needs.

5. Conclusions

In summary, the present study is the first, to our knowledge, to compare the effects of duration-matched acute bouts HIIE, SIE, and MICE on lymphocyte numbers and expression of CD69 and CX3CR1. Despite resulting in greater total work and oxygen consumption, MICE elicited similar changes in lymphocyte subset counts and receptor expression compared to both SIE and HIIE. Similarly, while SIE and HIIE resulted in differing oxygen consumption and total work, no differences in the lymphocyte response were observed. These findings have practical applications to athletes and coaches seeking to improve aerobic performance via interval training or continuous training while minimizing immune perturbations. Specifically, though circulating lymphocyte concentrations and NK cytotoxicity may decline following both continuous exercise and interval exercise, neither exercise type provides an immune-related advantage when matched for duration.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2022.100415.

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