High-intensity training reduces CD8$^+$ T cell redistribution in response to exercise

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Abstract

Purpose: We examined whether exercise-induced lymphocytosis and lymphocytopenia are impaired with high-intensity training. **Methods:** Eight trained cyclists ($\dot{V}O_2$ max: 64.2 ± 6.5 $mL \cdot kg^{-1} \cdot min^{-1}$) undertook one week of normal-, and a second week of high-intensity training. On day seven of each week, participants performed a cycling task, consisting of 120 min sub-maximal exercise followed by a 45 min time trial. Blood was collected before, during and after exercise. $CD8^+$ T lymphocytes ($CD8^+$TLs) were identified, as well as $CD8^+$TL sub-populations on the basis of CD45RA and CD27 expression. **Results:** High-intensity training (18,577 ± 10,984 cells·μL× ~165 min) was associated with a smaller exercise-induced mobilization of $CD8^+$TLs compared with normal-intensity training (28,473 ± 16,163 cells·μL× ~165 min, $p = 0.09$). The response of highly cytotoxic $CD8^+$TLs ($CD45RA^+CD27^-$) to exercise was smaller following one week of high- (3,144 ± 924 cells·μL× ~165 min) compared with normal-intensity training (6,417 ± 2,143 cells·μL× ~165 min; $p < 0.05$). High-intensity training reduced post-exercise $CD8^+$TL lymphocytopenia (−436 ± 234 cells·μL) compared to normal-intensity training (−630 ± 320 cells·μL; $p < 0.05$). This was driven by a reduced egress of naïve $CD8^+$TLs ($CD27^+CD45RA^+$). High-intensity training was associated with reduced plasma epinephrine (−37%) and cortisol (−15%) responses ($p < 0.05$). **Conclusions:** High-intensity training impaired $CD8^+$TL mobilization and egress in response to exercise. Highly cytotoxic $CD8^+$TLs were primarily responsible for the reduced mobilization of $CD8^+$TLs, which occurred in parallel with smaller neuro-endocrine responses. The reduced capacity for $CD8^+$TLs to leave blood post-exercise with high-intensity training was accounted for primarily by naïve, and also, highly cytotoxic $CD8^+$TLs. This impaired $CD8^+$TL redistribution in athletes undertaking intensified training may imply reduced immune-surveillance.

Key words: Exercise training, $CD8^+$ T Lymphocytes, immune surveillance, epinephrine, cortisol, humans.

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Introduction

Paragraph 1. Epidemiological evidence shows that athletes undertaking prolonged periods of high-intensity exercise training are more susceptible to infectious disease (e.g., upper respiratory tract infection (URTI) (12). Longitudinal studies demonstrate a 40% increase in the incidence of URTI in athletes undertaking a very high training load (11). To better understand the mechanisms that may underlie these observations, a variety of immune parameters have been investigated in response to, and following bouts of exercise (23). For example, intense exercise can alter several aspects of cell mediated immunity including; impaired neutrophil function, natural killer (NK) cell cytotoxicity, and lymphocyte cytokine production (12,21,34). These immune parameters, however, appear not to be predictive of viral infection incidence in athletes (23).

Paragraph 2. One process that has not been examined in the context of high-intensity exercise training is lymphocyte redeployment. Lymphocytes continuously traffic into and out of the blood and this process is essential to immune surveillance and elimination of virally infected self cells (20). The trafficking pattern of lymphocytes is very sensitive to exercise and is largely driven by $CD8^+$ T cells ($CD8^+$TL) and Natural Killer (NK) cells (39). Indeed, the exercise-induced mobilization and egress of lymphocytes into and out of the blood, respectively, referred to as lymphocytosis and lymphocytopenia, is one of the most established effects of exercise on the immune system (6). It has been argued that this exercise-induced mobilization of cells evolved to reflect a 'primed' immune system under conditions where tissue damage and infection is more likely (6).

Paragraph 3. Likewise, the post-exercise lymphocytopenia, whereby cell counts fall below baseline levels, is also thought to be important for immune surveillance and antigen detection (6,20,39).

Recently, it has become clear that lymphocytopenia is driven by a preferential egress of highly cytotoxic $CD8^+$TLs from peripheral blood (39). Moreover, animal studies have shown that lymphocytes migrate to 'front-line' locations such as the skin and lungs in response to exercise (20). Thus, changes in the cellular composition of peripheral blood during and following exercise appear to be adaptive immunological processes. If high-intensity exercise training is associated with decreased lymphocyte trafficking, and potentially impaired immune surveillance, then this might provide one explanation why athletes are at greater risk of infection following high-intensity exercise training (4,6).

Paragraph 4. The mobilization and egress of $CD8^+$TLs and NK cells in response to exercise is primarily driven by specific highly cytotoxic sub-populations (2,39). Thus, the trafficking of $CD8^+$TLs is determined by the kinetics of four $CD8^+$ sub-populations: a subset of antigen inexperienced cells; the naïve population (NA; $CD45RA^+CD27^+$) and three types of antigen-experienced memory cells; central memory (CM; $CD45RA^-CD27^+$); effector memory (EM; $CD45RA^-CD27^-$); and terminally differentiated $CD8^+$TL which have re-expressed the 'naïve' cell marker CD45RA (EMRA; $CD45RA^+CD27^-$) (15,35,39). NA and CM $CD8^+$TL re-circulate between the blood and secondary lymphoid organs, whereas EM and EMRA $CD8^+$TL preferentially migrate to the peripheral tissues, such as the skin and lungs (15,35). EM and EMRA subsets are able to employ immediate effector functions, such as the killing of virally infected cells (15,35).

Paragraph 5. NK cells also can be divided into sub-populations with distinct functional properties, identified as cells that express high levels of CD56 ($CD56^{hi}$) and cells which express low levels of CD56 ($CD56^{lo}$) (5). The $CD56^{lo}$ subset is comparable to $CD8^+$ EM and EMRA cells; preferentially migrating into peripheral tissues and capable of rapidly killing target cells (22).

Paragraph 6. Considering the association between exercise training load and infection risk (12), it is unknown whether the redeployment of $CD8^+$TL and NK cells is maintained or impaired during a period of high-intensity exercise training. Therefore, this study compared the exercise-induced mobilization and subsequent egress of $CD8^+$TL and NK cells, and their sub-populations, following one week of either normal- or high-intensity exercise training. To improve the relevance and applicability of results, lymphocyte responses were assessed following controlled sub-maximal exercise (simulating aspects of training), in addition to a maximal effort time trial (which simulates competition). On the basis of prior research, it was hypothesised that lymphocytosis (i.e., cell mobilization) and lymphocytopenia (i.e., cell egress) of $CD8^+$TL and NK cells would be reduced following a period of high-intensity training. Further, we speculated that any effects of high-intensity training would be most prominent in the most cytotoxic $CD8^+$TL and NK cell sub-populations.

Methods

Participants

Paragraph 7. Eight well-trained male cyclists (Mean ± SD; age 27 ± 8 yr; body mass index 22.8 ± 2.2 $kg \cdot m^{-2}$ maximal oxygen uptake ($\dot{V}O_2$ max) 64.2 ± 6.5 $mL \cdot kg^{-1} \cdot min^{-1}$) took part in this study. Participants were non-smokers, not taking any medication and had remained free from respiratory infection for four weeks. All participants provided informed written consent and the study was approved by the Research Ethics Committee of the School of Sport and Exercise Sciences, University of Birmingham, UK.

Experimental design

Paragraph 8. Participants were engaged in a seven day period of prescribed normal-intensity exercise training, followed by another seven day period of high-intensity exercise training (see *Exercise training conditions*). The order of these conditions was not randomised. On the final day of each condition, and at least 18 h after the last exercise session, participants undertook a standardized exercise trial (see *Exercise trials*), in which immune cell responses to exercise were quantified.

Preliminary exercise testing and familiarization

Paragraph 9. Participants visited the laboratory for two preliminary visits prior to the experimental period. During the first visit, participants undertook a maximal oxygen uptake test ($\dot{V}O_2$max test; workloads of 35 watts, 3 minute intervals) to exhaustion on a cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) as described previously (14). In the final min of each stage, heart rate was recorded (Polar Vantage NV, Kempele, Finland) and expired gas samples were collected using Douglas bags. The intensity of each acute exercise trial was determined using $\dot{V}O_2$ max values assessed during preliminary testing, whereas the intensity of the two experimental training conditions (normal- and high-intensity exercise training) was derived using percentages of maximal heart rate (HRmax) as previously described (40) (see *Exercise training conditions*). The second preliminary visit consisted of a familiarisation exercise trial conducted under fasted conditions.

Exercise training conditions

Paragraph 10. Five training zones, defined in accordance with British Cycling guidelines (14) were defined: zone 1: <70% HRmax, zone 2: 70-80% HRmax, zone 3: 80-90% HRmax, zone 4: 90-95% HRmax, and zone 5: >95% HRmax. During the normal-intensity training condition, participants engaged in their usual level of regular exercise (one session/day, 4-5 training days/wk). The high-intensity training condition required athletes to markedly increase training load: exercise volume,

(i.e., duration of each training session) and exercise intensity (i.e., difficulty of each training session) were increased by ~70% relative to normal-intensity training (1-2 sessions/day, 7 training days/wk). In the high-intensity training condition, cyclists typically undertook one sprint interval session per day and one continuous ride. To rule out immunological differences during exercise trials as a result of possible changes in $\dot{V}O_2$max following the normal- and high-intensity exercise training conditions, a $\dot{V}O_2$max test was conducted on day 6 of each training condition.

Exercise trials

Paragraph 11. Exercise trials took place on the seventh day of each exercise training condition. Trials started at ~06:30 after an overnight fast. Following a 15 min rest, a baseline blood sample was drawn from a forearm vein using an indwelling catheter. The catheter was flushed regularly with saline to maintain patency. Each exercise trial was identical, and consisted of two stages completed in short succession. First, participants cycled for 120 min at a fixed exercise intensity equivalent to ~60% $\dot{V}\,O_2$ max (sub-maximal effort cycling). Second, participants undertook a maximal-effort time trial lasting ~45 min (~85-100% $\dot{V}\,O_2$ max). The time trial required a target amount of work (mean ± SD, energy target; 670 ± 52 kJ) to be completed in as short a time as possible (18). The total amount of work to be performed was individualized, based on their Watt-max achieved during the $\dot{V}\,O_2$ max test, and was calculated using the following formula:

$$\text{Total work (J)} = 0.7 \times \text{Watt-max} \times 2700 \text{ s.}$$

A two-stage exercise trial was employed to assess endurance performance in a glycogen depleted state for a separate investigation (40).

Paragraph 12. Physiological and psychological data (e.g., HR, RPE, $\dot{V}\,O_2$ and RER) were collected at 20 min intervals during sub-maximal effort cycling. To minimize distraction to the cyclist during

149 the maximal-effort time trial, only HR and RPE were assessed at 25, 50, 75 and 100% completion
150 of this phase. Cyclists could monitor task progress (i.e., kJ of energy expended) however
151 performance time, power output or cadence (RPM) data were not made available.
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153 ***Paragraph 13.*** In addition to the resting sample before exercise, blood was collected in the final
154 min of each exercise stage and also 1 h post-exercise. Pilot data showed that 1 h post-exercise was
155 the nadir of lymphocytopenia.
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157 **Dietary control**
158 ***Paragraph 14.*** To rule out possible immunological changes during exercise which might be caused
159 by a differential energy expenditure between the normal- and high-intensity exercise training
160 conditions, diet was controlled to maintain energy balance (i.e., energy intake was equal to energy
161 expenditure in both training conditions) (40). Daily energy requirements were calculated by the
162 summation of basal metabolic rate (kcal•day^{-1}) (predicted using the Harris Benedict equation) and
163 estimated energy expenditure during cycling activity (26). All food and drink consumed by
164 participants was provided, and was matched to the energy expenditure of each training condition
165 using internet-based nutrition software (weightlossresources.co.uk). In both training conditions, the
166 macronutrient composition of the diet was equivalent to 1.5 g of protein•kg^{-1} body mass·day^{-1} and 6
167 g of CHO•kg^{-1} body mass·day^{-1}, with the remainder of energy derived from fat. Calorie intake was
168 higher during high-intensity training (4410 ± 437 kcal•day^{-1}) compared with normal-intensity
169 training (3711 ± 456 kcal•day^{-1}, $p < 0.05$). Participants consumed only food and drink provided, and
170 did not consume alcohol and caffeine. All athletes maintained a stable body weight throughout the
171 experimental period.

Flow Cytometry

Paragraph 15. Blood was collected into K_3EDTA Vacutainers and processed within 5 h. Whole blood was incubated with two separate antibody panels for 25 min at room temperature. Panel 1; CD45RA FITC, CD27 PE, CD3 PERCP and CD8 APC. Panel 2; CD16 FITC, CD56 PE, CD3 PERCP and CD14 APC (Antibodies from Pharmingen San Diego, USA, except CD3 PERCP; Becton Dickinson San Jose, USA). Erythrocytes were lysed using FACS lysing solution (Becton Dickinson, San Jose, USA). Cells were washed by centrifugation at 500 × *g* for 6 min at 4˚C, and re-suspended in 300 µL of 2% paraformaldehyde phosphate buffered saline. Cells were stored in the dark at 4˚C and read on a FACS calibur flow cytometer (Becton Dickinson, San Jose, USA), collecting 20,000 lymphocytes.

Paragraph 16. $CD8^+$TL subsets were identified by the expression of the cell-surface molecules CD45RA in and CD27, as previously described (15,39). NK cells were identified as being $CD3^-$ and $CD56^+$. The NK sub-populations; $CD56^{hi}$ and $CD56^{lo}$ were determined by CD16 expression as described by Cooper *et al.* (5). Data were analyzed using FlowJo v5.2 (Tree Star Inc., Aston, Oregon). Whole blood cell counts (i.e., total leukocytes, lymphocytes, granulocytes and monocytes) were determined based on the Coulter principle using an automated haematology analyzer (Coulter GEN-S, Beckman-Coulter, Miami, USA).

Neuro-endocrine measurements

Paragraph 17. Plasma cortisol and epinephrine concentrations were measured using commercially available kits (Cortisol, IDS, Tyne and Wear, UK; CAT-COMBO, IDS, Tyne and Wear, UK, respectively). Plasma was obtained by centrifugation at 1500 × *g* for 10 min at 4˚C. Samples were stored at −80˚C until analysis. Epinephrine was not measured 1 h post-exercise due to the short half-life in the blood following the exercise stimulus.

196 **Statistical analyses**

197 ***Paragraph 18.*** Leukocyte, neuro-endocrine, physiological and psychological responses to exercise
198 were analyzed within-subjects using two-way repeated measures analyses of variance (ANOVA).
199 Post-hoc least-significant difference (LSD) tests identified time-point differences. To examine
200 differences in immune cell counts between training conditions over the defined exercise period
201 (measurements collected at baseline, sub-maximal exercise and maximal exercise, only), data were
202 expressed as incremental area under the curve (iAUC) (33). iAUC is routinely used to detect
203 differences across multiple time-points (33). An advantage of using this technique is that both the
204 magnitude of response (increases in immune cell counts above baseline) and changes over time
205 (i.e., time intervals between consecutive measurements of immune cell counts) are accurately
206 captured, irrespective of non-uniform time intervals between measurements (10,33). Post-exercise
207 lymphocytopenia was expressed as absolute change in cell number (i.e., cell count upon immediate
208 cessation of time trial minus cell count 1 h post-exercise). Differences in mobilization or egress of
209 cells between training conditions were examined with paired samples *t*-tests, or ANOVA, including
210 key variables entered as individual covariates (ANCOVA). This strategy allows investigation of
211 variables mediating observed relationships. Key variables included change in epinephrine, cortisol
212 (expressed as iAUC), HR, RPE, performance time and power output between normal and high-
213 intensity exercise training. This approach is routinely used in analyses of this sort (39) and provides
214 a robust statistical test for mediation (17).

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216 ***Paragraph 19.*** All data were expressed as means ± SEM, unless otherwise stated. iAUC was
217 calculated using PRISM software (v4 Graph pad INC., San Diego, CA) with baseline set as the
218 basal immune cell sub-population/subset count measured in the corresponding training condition.
219 Data were analysed using SPSS 18.0 for Windows (SPSS Inc., USA). Significance was set at the p
220 < 0.05 level.

Results

Exercise training conditions

Paragraph 20. Training volume (duration of exercise per week) during high-intensity training was increased compared to normal-intensity training (Mean ± SD; 1084 ± 203 min *vs.* 650 ± 173 min, respectively; Paired samples *t*-test; $t_{(7)} = -14.9$, $p < 0.001$). As a measure of exercise intensity (i.e., difficulty of exercise training each week), average heart rate during training was increased during high- compared to normal-intensity training (Mean ± SD; 140 ± 7 bpm *vs.* 131 ± 5 bpm, respectively; Paired samples *t*-test; $t_{(7)} = -6.2$, $p < 0.05$). The high-intensity training regimen did not improve cardio-respiratory fitness, but instead caused a slight reduction (Normal-intensity training: 63.3 ± 4.9 $mL \cdot kg^{-1} \cdot min^{-1}$; High-intensity training: 56.9 ± 7.2 $mL \cdot kg^{-1} \cdot min^{-1}$, Paired samples *t*-test; $t_{(7)} = 1.9$, $p < 0.05$).

Physiological and psychological responses to exercise

Paragraph 21. Physiological responses to sub-maximal cycling were not different between trials, ($\dot{V}O_2$ and RER data not shown) although RPE was greater following high-intensity training (see supplementary table which shows exercise duration, power output, RPE and HR responses to exercise following normal- and high-intensity training). Time to completion (min) and power output (watts) during maximal-effort cycling were slightly reduced following high-intensity training (Paired samples *t*-test; $t_{(7)} < 1.9$, $p < 0.05$), however, HR and RPE were not significantly different (Paired samples *t*-test; $t_{(7)} > 4.4$, $p > 0.05$; see supplementary table). Physiological, and psychological variables were not significant covariates mediating any of the training-induced immunological responses to acute exercise (all interaction effects; $F_{(1,6)} < 3.2$, $p > 0.05$, $\eta_2 < 0.3$). In other words, changes in these variables between training conditions did not confound any of the results reported below.

Leukocyte responses to exercise

Paragraph 22. Table 1 presents total leukocyte and leukocyte sub-population responses to exercise following the normal- and high-intensity training conditions, respectively. In both training conditions, total leukocytes, lymphocytes, granulocytes and monocytes showed the largest increase upon immediate completion of the maximal-effort time trial. Sixty minutes post-exercise, total leukocytes and granulocytes remained elevated, monocytes returned to baseline, and lymphocytes fell below pre-exercise levels (see Table 1, main effects of time all; $F_{(1,7)} > 8.5$, $p < 0.05$). High-intensity training was associated with a smaller exercise-induced mobilization of leukocytes (Mean ± SD iAUC; 366 ± 50 *vs.* 467 ± 69 cells/μL; high- and normal-intensity training, respectively) and granulocytes (Mean ± SD iAUC; 255 ± 51 *vs.* 377 ± 76 cells/μL; high- and normal-intensity training, respectively) (Paired samples *t*-tests both; $t_{(7)} > 3.2$, $p < 0.05$). There were no significant differences in total lymphocyte and monocyte responses to exercise between training conditions (see Table 1).

Total $CD8^+$ T Lymphocyte responses to exercise

Paragraph 23. Figure 1 shows $CD8^+$TL responses to exercise following the normal- and high-intensity training conditions. $CD8^+$TL increased during exercise (~300 %) and fell below baseline levels 1 h post-exercise (~45 %) (see Figure 1 A, Main effects of time $F_{(1,7)} = 29.8$; $p < .05$). There was a trend for a smaller exercise-induced mobilization of $CD8^+$TL in the high-intensity training condition (see Figure 1 B, Paired samples *t*-tests iAUC between training conditions; $t_{(7)} = 2.1$; $p = 0.09$). The egress of $CD8^+$TL 1 h post-exercise (Paired samples *t*-tests between training conditions; $t_{(7)} = 3.047$; $p < 0.05$) was attenuated in the high-intensity training condition (see Figure 1 C).

$CD8^+$ T Lymphocyte subset responses to exercise

Paragraph 24. Figure 2 shows EMRA $CD8^+$TL responses to exercise following the normal- and high-intensity training conditions. The mobilization of EMRA $CD8^+$TL was smaller in the high-intensity condition (see Figure 2 B, Paired samples *t*-test iAUC between conditions; $t_{(1,7)} = 2.5$; $p < 0.05$). 1 h post exercise, the number of circulating EMRA $CD8^+$TL was reduced by ~65% compared to pre-exercise with normal-intensity training. In the high-intensity condition, there was a trend for a smaller post-exercise egress; EMRA CD8+TL fell by ~55% relative to baseline (see Figure 2 C, Paired samples *t*-tests between conditions; $t_{(1,7)} = 2.2$; $p = 0.06$).

Paragraph 25. There were no differences in the mobilization of NA, CM and EM $CD8^+$TL to exercise between the normal and high-intensity training conditions (data only shown for NA, see Figure 3 A-B). The post-exercise egress of NA $CD8^+$TL was smaller in the high-intensity training condition (see Figure 3 C, Paired samples *t*-tests between training conditions; $t_{(7)} = 3.6$; $p < 0.05$), however the egress of CM and EM was unaffected (data not shown),

NK cell responses to exercise

Paragraph 26. NK cells increased during exercise (~420%) and fell below baseline 1 h post-exercise (~36%). NK $CD56^{lo}$ cells showed a larger mobilization into peripheral blood (~430%) compared to $CD56^{hi}$ cells (~240%) without any differences between the normal- and high-intensity training conditions (data not shown).

Neuro-endocrine responses to exercise

Paragraph 27. Figure 4 shows the plasma epinephrine and cortisol responses to exercise following the normal- and high-intensity training conditions. Epinephrine concentrations showed the expected

increase in response to exercise (see Figure 4 A, main effects of time; $F_{(1,7)} = 4.9$; $p < 0.05$). High-intensity training was associated with a smaller epinephrine response to exercise (Mean ± SD iAUC between conditions; 24,133 ± 31,792 pg/mL× ~165min *vs.* 33,120 ± 36,078 pg/mL× ~165min; high- and normal- intensity training, respectively; Paired samples *t*-test; $t_{(7)} = 3.3$, $p < 0.05$).

Paragraph 28. Cortisol concentrations increased in response to the maximal-effort time trial and remained elevated at 1 h post-exercise (see Figure 4 B, main effects of time; $F_{(1,7)} = 20.0$; $p < 0.05$). Cortisol responses to exercise were reduced after high-intensity training (Mean ± SD iAUC; 10,344 ± 3382 ng/mL× ~165min; 8754 ± 1258 ng/mL× ~165min during normal- and high-intensity training, respectively; Paired samples *t*-test; $t_{(7)} = 2.4$, $p < 0.05$).

Paragraph 29. Epinephrine and cortisol concentration were not significant covariates mediating any of the training-induced immunological responses to acute exercise (all interaction effects; $F_{(1,6)} < 3.5$, $p > 0.05$, $\eta_2 < 0.4$). In other words, the reduced neuro-endocrine response to exercise with high-intensity training, was not responsible for the training-induced reduced immune responses to exercise.

Discussion

Paragraph 30. This study investigated whether $CD8^+$TL mobilization and subsequent egress from blood in response to a standardized bout of exercise, is affected by a period of high-intensity exercise training. Using a validated model of high-intensity training (14), our data suggest that both the mobilization and egress of $CD8^+$TL to an acute bout of intense exercise is reduced after a week of high-intensity training in well trained cyclists. Further analyses showed that these effects observed in total $CD8^+$TL were driven by a differential response of $CD8^+$TL sub-populations.

EMRA $CD8^+$TL cells exhibited a smaller mobilization during exercise with high-intensity exercise training. Following exercise, the reduced egress of $CD8^+$TL was largely driven by a smaller egress of NA and to a lesser extent, a smaller egress of EMRA cells.

Paragraph 31. The smaller mobilization of $CD8^+$TL with high-intensity exercise training occurred in parallel with a reduced epinephrine response to exercise. $CD8^+$TL, and in particular the EMRA sub-population, express the ß2 adrenergic receptor very densely at the cell surface (19). Exercise is associated with increased adrenergic activity, and these cells become selectively mobilized via an adrenergic-dependent mechanism (8,19). Moreover, epinephrine infusion alone causes a similar mobilization of lymphocytes (8,19) which confirms previous assumptions that exercise-induced lymphocytosis is largely dependent on epinephrine release (28). The observed blunting of epinephrine and $CD8^+$TL responses to acute exercise following high-intensity training is consistent with previous reports. For example, physically fit individuals, who engage regularly in exercise training, exhibit a smaller exercise-induced lymphocytosis compared to unfit individuals, which is independent of absolute exercise intensity (16,29). It has previously been reasoned that ß2 adrenergic receptors are desensitized due to repeated exercise-induced adrenergic activity (16,29). Also consistent with our findings is the observation that relative to unfit individuals, physically fit individuals tended to show more modest epinephrine responses to stress and exercise tasks (16). Thus, our findings and those of others, suggest that periods of high-intensity exercise training are associated with a reduced $CD8^+$TL mobilization, which might be in part mediated by ß2 adrenergic receptor down-regulation, a decline in adrenergic output, or a combination of the two.

Paragraph 32: Adrenergic stimulation is not the only mechanism behind exercise-induced lymphocytosis. For example, lymphocytes are mobilized non-specifically due to increased cardiac output and associated shear forces (36). Indeed, our results suggest that the smaller mobilization of

lymphocytes following high-intensity training is likely to be mediated by several mechanisms. For example, our analyses showed that the smaller epinephrine response to exercise following high-intensity training was not a significant covariate in the smaller training-induced immune responses to exercise. This indicates that the reduced epinephrine response to exercise is not entirely responsible for our observation. Thus, other known processes (e.g., cardiac output and associated shear stresses), as well as unknown factors (e.g., possible changes in lymphocyte adhesion molecule, or tissue ligand expression with high-intensity training) also might have mediated the reduced mobilization of cells in response to exercise.

Paragraph 33. Although the mobilization of lymphocytes into blood during exercise is well studied, investigation of post-exercise lymphocytopenia and the mechanism behind this process remains unclear. Cortisol is proposed as a neuro-endocrine mediator of lymphocyte extravasation from blood (9,32). Indeed, Dimitrov et al. (7) showed that cortisol infusion causes a selective egress of NA $CD8^+$TL from peripheral blood. Consistent with these findings, we observed a smaller NA $CD8^+$TL egress occurring in parallel with a reduced cortisol release with high-intensity exercise training. Together, these findings imply that an impaired ability of NA $CD8^+$TL to leave blood post-exercise might, at least in part, be mediated by a blunted cortisol response. However, our mediation analyses suggest that a smaller cortisol response to exercise following high-intensity training was not responsible for the reduced NA $CD8^+$TL (or any other $CD8^+$TL population) lymphocytopenia. Thus, if cortisol does indeed play a role in lymphocytopenia, it might be related to down-stream mechanistic processes. For example, as with lymphocyte ß2 adrenergic receptor down regulation with chronic adrenergic stimulation (16), glucocorticoid receptor density might similarly be affected with chronic cortisol stimulation. Likewise, high-intensity exercise training and/or chronic cortisol release might influence other aspects of lymphocytopenia not investigated in this study. For example, lymphocyte migration to bone marrow, as observed with exercise in rodents (20), is

dependent on lymphocyte expression of the adhesion molecule CXCR4 (25). In turn, the actions of CXCR4 are dependent on bone-marrow derived ligands (e.g., CXCL12), and the expression of CXCR4 and CXCL12 is partly governed by cortisol levels (27,31). Thus, it is not surprising that measuring cortisol levels alone does not explain the reduced lymphocytopenia following high-intensity exercise training in this study.

Paragraph 34. It has also been proposed that lymphocytopenia is a result of exercise-induced apoptosis in blood (24). However, apoptosis cannot fully explain the often observed −50% fall in lymphocytes following exercise, as <10% of cells in blood become apoptotic (24). In addition, blood lymphocyte numbers recover within several hours of exercise completion. Replacement of 'deleted' lymphocytes so quickly is therefore unlikely. A more accepted view is that $CD8^+$TLs, important for the detection and elimination of antigen, extravasate from peripheral blood post-exercise, as part of immune surveillance (6,20). As periods of intense exercise training are associated with an increased incidence of infection in athletes, we predicted that high-intensity exercise training would result in a reduced egress of total $CD8^+$TLs post-exercise. Analysis of $CD8^+$TL numbers following exercise confirmed our hypothesis. Further analysis of $CD8^+$TL sub-populations showed, although not statistically significant, that there was a strong trend fewer EMRA $CD8^+$TL to leave the blood post-exercise. Together, these findings imply that high-intensity exercise training might be associated with a reduced trafficking of lymphocytes to peripheral tissue, which in turn might be associated with compromised immune surveillance.

Paragraph 35. An alternative possible implication of the current findings is that beneficial effects of regular exercise on immunity might be reduced if an excessive volume of exercise training is undertaken. A hypothetical but attractive framework put forward by Simpson (37) suggests exercise as a mechanism for the 'deletion' of clonally expanded virus-specific T cells, which deleteriously

accumulate with ageing. The T cell compartment is assumed to be relatively stable or 'fixed' because thymic output of naive T cells becomes almost negligible around the time of adolescence (3). Thus, the 'immune space' available for the expansion of memory T cells upon infection with a novel pathogen is limited, and the relative 'size' of the naive T cell pool declines with ageing (1,3). This narrowing of the T cell repertoire (also known as immune senescence) is associated with lower antibody responses to vaccination, exacerbated inflammation and an increased susceptibility to infection (1,3). Simpson (37) suggests that regular exercise might promote apoptotic removal of some virus-specific T cells by extravasation from blood post-exercise and subsequent exposure to pro-apoptotic signals (e.g., reactive oxygen species). In support, a cross-sectional study by Spielmann *et al.* (38) showed that individuals with a high aerobic fitness (compared to less fit individuals) had lower proportions of senescent T cells in blood (e.g., $CD8^+$TL EMRA cells). In the present study, athletes undertook a very high training load, which, as with immune senescence, is also associated with increased susceptibility to infection (12). Subsequently, these athletes showed a smaller egress of senescent EMRA $CD8^+$TLs. Although speculative, our results, interpreted in the context of the theory proposed by Simpson (37), suggest that fewer senescent cells may be 'deleted' from the T cell repertoire during a period of high-intensity exercise training. Thus, the possibility of delayed immune senescence with regular exercise as suggested by Simpson (37), might not be gained from excessive volumes of exercise training. Although this is an attractive implication of the current findings and relevant to infection risk and possible immune senescence in athletes, we emphasise that our interpretations are speculation at present.

Paragraph 36. It is important to note that the high-intensity exercise training manipulation in the present study was not designed to improve fitness. Such improvements in fitness might have made the exercise trial following the high-intensity training condition less demanding. Interestingly, high-intensity training caused a small reduction in $\dot{V}O_2$ max which is common following very intense

training regimens (14). Thus, it could be argued that the exercise trial following high-intensity training was in fact more demanding, providing further support that the smaller immune-response observed was not caused by an 'easier' exercise trial. In support, HR and RPE during maximal exercise were not different between trials. Further, our mediation analyses showed that performance (i.e., exercise duration and power output), physiological (i.e., HR) and psychological (i.e., RPE) variables were not significant covariates in the training-induced reductions in immune cell mobilization with acute exercise. In other words, training-induced alterations in these variables did not confound the observed immunological responses to exercise.

Paragraph 37. As well as alterations in the adaptive immune compartment, the present study showed that high-intensity exercise training influenced the response of granulocytes, but not NK cells (cell populations of the innate immune system). Given that similar to the $CD8^+$TL EMRA population, NK cells exhibit high adrenergic sensitivity (4), the failure of high-intensity exercise to influence NK cells might seem surprising. However, as NK cells mobilize more than $CD8^+$TLs (2), suggesting a greater adrenergic sensitivity or receptor density, it is possible that the relatively small reduction in sympathetic activity with high-intensity training was not enough to affect these highly exercise-sensitive cells. Moreover, although adrenergic activity is just one of the mechanisms behind lymphocytosis (6), as mentioned earlier, other well established processes (e.g., cardiac output and increased shear forces) as well as less investigated processes (e.g., tissue homing and adhesion molecule expression) may play a significant roles in the context of high-intensity training. Unlike NK cells, the mobilization of granulocytes into peripheral blood during exercise was reduced with high-intensity training. These effects observed in the total granulocyte pool were likely driven by neutrophils, an exercise-sensitive sub-population (50-60%) of granulocytes, which exhibit both α- and β-adrenergic receptors (13). These cells are important for the elimination of microbial pathogens (30). Although direct measurements of granulocyte or neutrophil function were

not conducted, our data provide some support for the possibility that innate immune responses might be impaired following high-intensity exercise training (41).

Paragraph 38. When interpreting the results of this study, it should be considered that in humans, the exact destination of cells leaving peripheral blood post-exercise is unknown. Animal studies provide strong evidence for exercise-induced leukocyte migration to peripheral tissues such as the skin, and lungs (6,20). In these models, it is thought that leukocyte tissue migration facilitates the detection and elimination of antigen. Another consideration is that clinical diagnosis of URTI episodes were not made in this study. It therefore remains unclear whether altered redistribution patterns of $CD8^{+}$TLs following high-intensity exercise training translates into increased susceptibility to infection. Future studies are needed to investigate whether altered leukocyte responses to bouts of intense exercise are predictive of clinically diagnosed infectious disease episodes in athletes. Functional measures of lymphocytes (e.g., activation, proliferation and cytokine production) and granulocytes or neutrophils (e.g., chemotaxis, phagocytosis and superoxide production) might provide useful adjunct measurements for follow-up investigations. Further, in light of the present results, mechanistic studies are now warranted to better understand how high-intensity exercise training influences lymphocytosis and lymphocytopenia. For example, relevant to lymphocytosis, cyclic-adenosine monophosphate (cAMP) assays on isoproterenol stimulated T-cell subsets could be conducted *in vitro* to examine whether ß2 adrenergic receptors are indeed desensitized. Relevant to lymphocytopenia, adhesion and tissue homing molecule expression could be investigated *ex vivo* with athletes undertaking very large volumes of exercise. Similar analyses could be made in *vitro*, examining lymphocyte cell-surface molecule expression in response to cortisol exposure.

Paragraph 39. We recently showed that $CD8^+$TL mobilization and egress in response to exercise was amplified in CMV+ individuals (39). The within-subjects study design employed in the present study negates the possibility that CMV status confounded our observation of a blunted mobilization and egress of $CD8^+$TL sub-populations in response to high-intensity exercise training. However, investigations into possible interactions between lymphocyte responses to acute exercise, high-intensity exercise training, and CMV serostatus is justified. For example, it might be hypothesized that compared to CMV− individuals, CMV+ individuals would exhibit a smaller 'blunting' effect of high-intensity training because they exhibit exaggerated lymphocyte responses to exercise.

Paragraph 40. In summary, we show that the redistribution of $CD8^+$TLs in response to a standardized bout of exercise was reduced by a period of high-intensity training. Although the mechanism(s) behind this observation remains unclear, it is possible that aspects of immune-surveillance might be impaired with very large volumes of exercise training.

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The results of the present study do not constitute endorsement by ACSM.

Conflict of Interest Statement: All authors declare that there are no conflicts of interest.

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495 Reference List

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612 **Table captions**

613

614 **Table 1:** Total leukocyte and leukocyte sub-population responses to exercise following normal- and

615 high-intensity training.

616

617 **Supplementary table:** Physiological and psychological responses to exercise following normal-

618 and high-intensity training.

619

620

621 **Figure legends**

622

623 **Figure 1:** Total $CD8^+$TL responses to exercise following normal- and high-intensity exercise
624 training. A) changes in cell number over time; B) mobilization of cells expressed as incremental
625 area curve (iAUC) during exercise; C) egress of cells 1 hour post-exercise. SM: sub-maximal
626 exercise, MAX: maximal exercise, Post 1 h: 1 hour following maximal exercise. Values are means
627 ± SEM (n = 8). †significantly different from baseline in corresponding training period ($p < 0.05$).
628 *significantly different from normal-intensity training ($p < 0.05$).

629

630 **Figure 2:** $CD45RA^+$ effector memory $CD8^+$TL (EMRA) responses to exercise following normal-
631 and high-intensity exercise training. A) changes in cell number over time; B) mobilization of cells
632 expressed as incremental area curve (iAUC) during exercise; C) egress of cells 1 hour post-exercise.
633 SM: sub-maximal exercise, MAX: maximal exercise, Post 1 h: 1 h following maximal exercise.
634 Values are means ± SEM (n = 8). †significantly different from baseline in corresponding training
635 period ($p < .05$). *significantly different from normal-intensity training ($p < 0.05$).

636

637 **Figure 3:** Naïve (NA) $CD8^+$TL ($CD45RA^+CD27^+$) responses to exercise following normal- and
638 high-intensity exercise training. A) changes in cell number over time; B) mobilization of cells
639 expressed as incremental area curve (iAUC) during exercise; C) egress of cells 1 hour post-exercise.
640 SM: sub-maximal exercise, MAX: maximal exercise, Post 1 h: 1 hour following maximal exercise.
641 Values are means ± SEM (n = 8). †significantly different from baseline in corresponding training
642 period ($p < .05$). *significantly different from normal-intensity training ($p < 0.05$).

643

644 **Figure 4**: Neuroendocrine responses to exercise following normal- and high-intensity exercise
645 training. A) Plasma epinephrine; B) plasma cortisol. Concentrations expressed as pg/ml and ng/ml

646 respectively. SM: sub-maximal exercise, MAX: maximal exercise, Post 1 h: 1 hour following
647 maximal exercise. Values are means ± SEM (n=8). †significantly different from baseline in
648 corresponding training week ($p < .05$). *significantly different from normal-intensity training at
649 corresponding time-point ($p < 0.05$).
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Table 1

Total leukocyte and leukocyte sub-population responses to exercise following normal- and high-intensity training.

Cells × 10^9/L	Normal-intensity training				High-intensity training				Main effect of time (both normal- and high-intensity training)
	Baseline	Submaximal-cycling (120 min)	Maximal-cycling (~45 min)	Post 1 h	Baseline	Submaximal-cycling (120 min)	Maximal-cycling (~45 min)	Post 1 h	
Leukocytes	4.8±1.0	8.2±1.9†	12.8±3.6†	10.2±4.1†	4.7±0.8	7.6±1.6†	10.3±3.0$^{\dagger *}$	8.4±1.7†	$F_{(1,7)} = 11.5$; $p < 0.05$
Lymphocytes	2.0±0.4	2.8±0.6†	4.0±0.5†	1.5±0.3†	1.9±0.3	2.7±0.5†	3.6±0.7†	1.6±0.4†	$F_{(1,7)} = 62.3$; $p < 0.05$
Granulocytes	2.5±2.4	4.8±1.3	8.5±3.1†	8.4±0.3	2.5±0.7	4.5±1.2†	6.3±2.5$^{\dagger *}$	6.6±0.4	$F_{(1,7)} = 8.5$; $p < 0.05$
Monocytes	0.4±0.1	0.6±0.2†	0.7±0.2†	0.5±0.2	0.3±0.1	0.5±0.1†	0.6±0.2†	0.3±0.1	$F_{(1,7)} = 13.2$; $p < 0.05$

Values are means ± SD. Main effect of time combines both normal- and high-intensity training values. †significantly different from baseline in corresponding training week ($p < .05$). *significantly different from Normal-intensity training in corresponding time-point ($p < .05$).

Supplementary table: Physiological and psychological responses to exercise following normal- and high-intensity training.

Power output and performance time during maximal-cycling was reduced following high-intensity training despite no significant differences in heart rate and perceived effort. None of these variables mediated or confounded the reduced immunological response to exercise.

Parameter	Normal-intensity training			High-intensity training			Main effect of time (both normal- and high-intensity training)
	Baseline	Submaximal-Cycling	Maximal-cycling	Baseline	Submaximal-cycling	Maximal-cycling	
Exercise duration (min:sec)	-	120:0±00:00	42:35±02:45	-	120:00±00:00	48:13±04:59*	*n/a*
Power output (watts)	-	177±14	267±47	-	177±14	237±46*	$F_{(1,7)} = 41.6$; $p < 0.05$
RPE	-	11±1	18±1	-	13±1*	18±1	$F_{(1,7)} = 56.7$; $p < 0.05$
HR (beats·min^{-1})	50±6	128±8†	159±4†	53±3*	125±10†	153±5†	$F_{(1,7)} = 575.4$; $p < 0.05$

Values are means ± SD. Data were not collected 1h post-exercise. Main effect of time combines data from normal- and high-intensity training and shows increased physiological demand between baseline (HR only), submaximal and maximal-cycling. HR is an average of measurements made continuously during submaximal and maximal cycling. RPE during submaximal-cycling is an average from 20, 40, 60, 80, 100 and 120 min. RPE during maximal cycling is an average from 25, 50, 75 and 100% of task completion. VO2 and RER were measured at the same periods as RPE during sub-maximal cycling: no differences were observed between trials (data not shown for clarity). †significantly different from baseline in corresponding training week ($p < .05$). *significantly different from Normal-intensity training at corresponding time-point ($p < .05$).

Figure 1

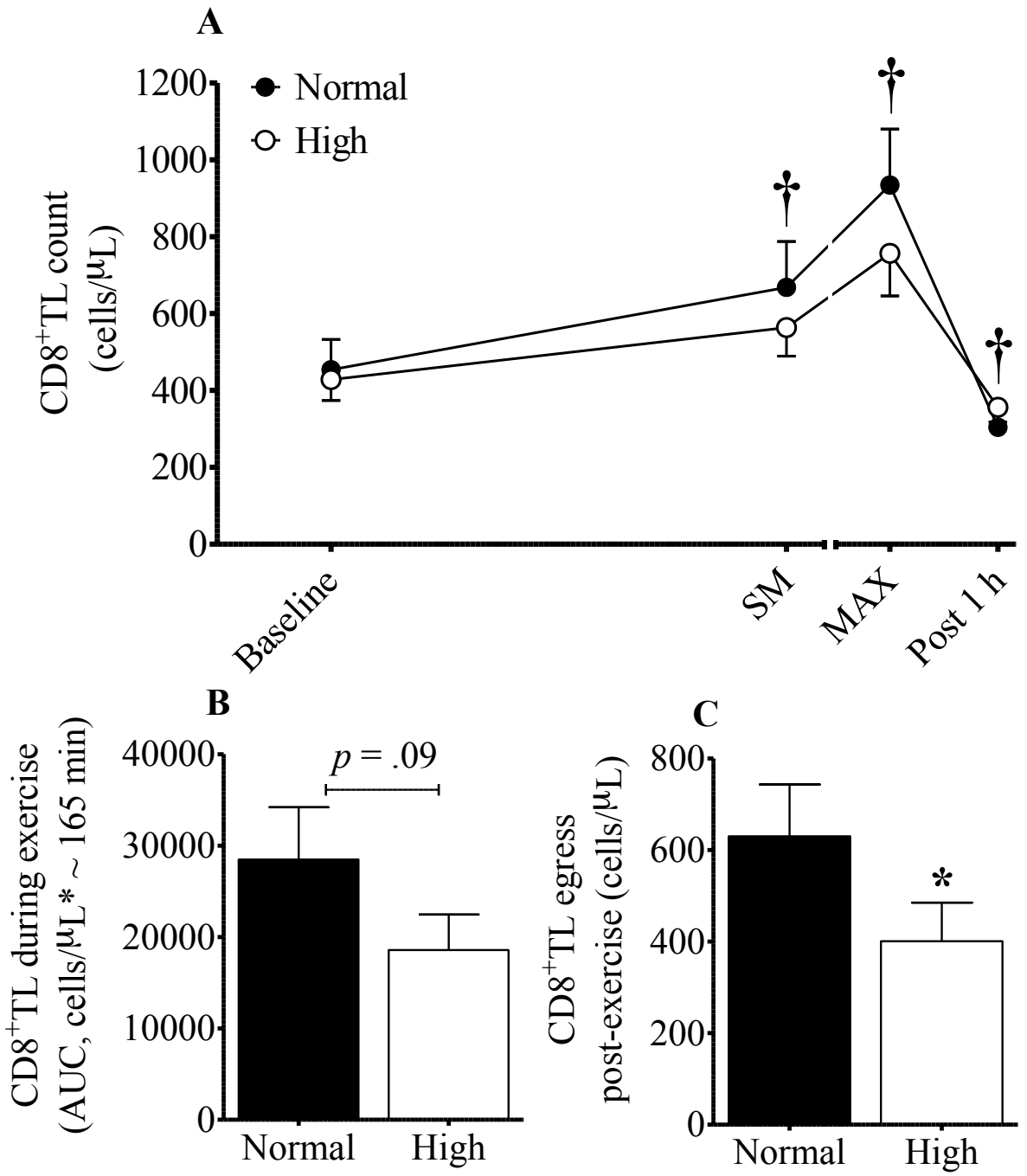

A
Normal
High
CD8+TL count (cells/μL)
1200
1000
800
600
400
200
0
Baseline
SM
MAX
Post 1 h
B
CD8+TL during exercise (AUC, cells/μL* ~ 165 min)
p = .09
40000
30000
20000
10000
0
Normal
High
C
CD8+TL egress post-exercise (cells/μL)
800
600
400
200
0
*
Normal
High

Figure 2

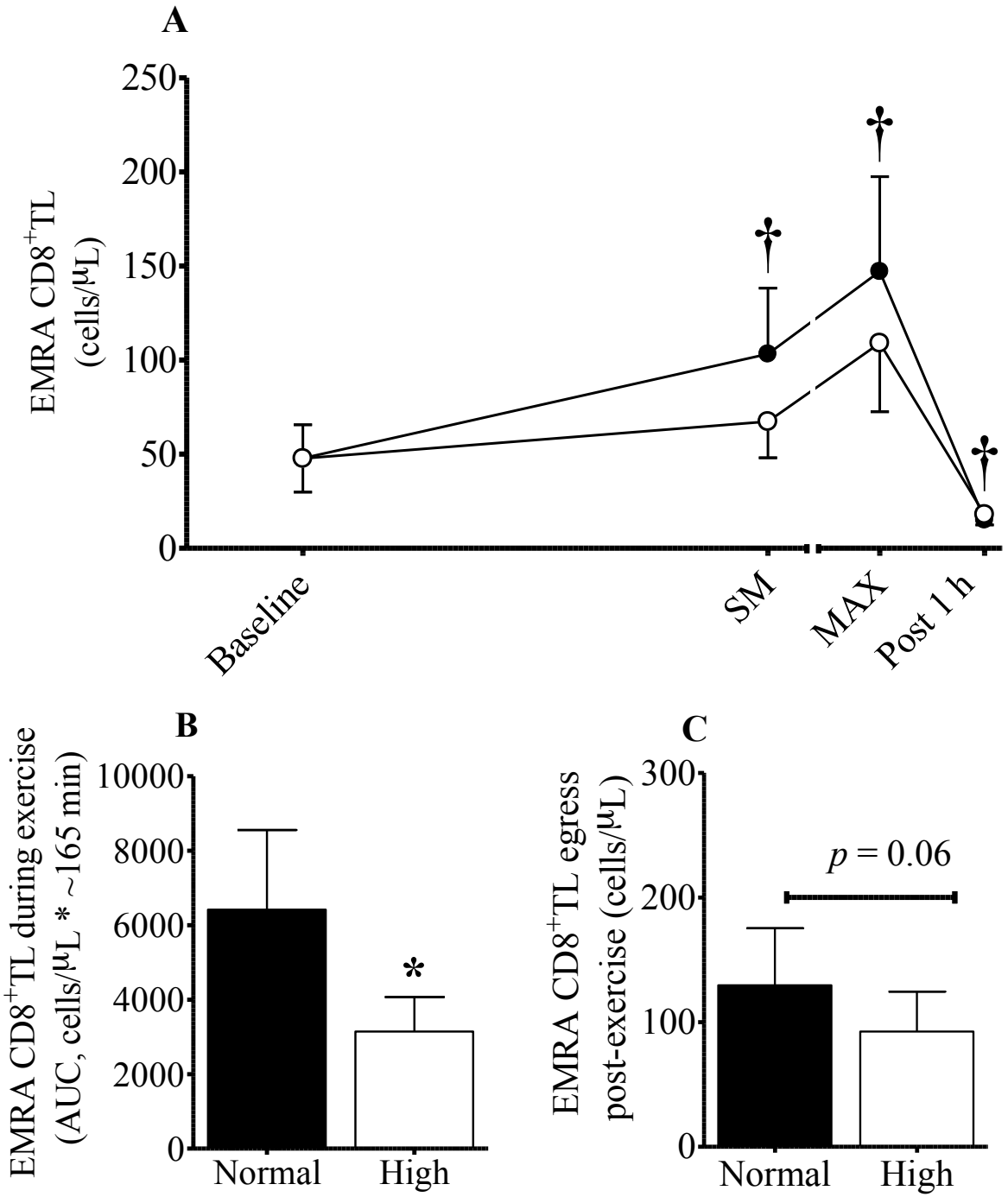

A
250
200
150
100
50
0
EMRA CD8+TL (cells/μL)
Baseline
SM
MAX
Post 1 h
B
EMRA CD8+TL during exercise (AUC, cells/μL * ~165 min)
10000
8000
6000
4000
2000
0
*
Normal
High
C
EMRA CD8+TL egress post-exercise (cells/μL)
300
200
100
0
p = 0.06
Normal
High

Figure 3

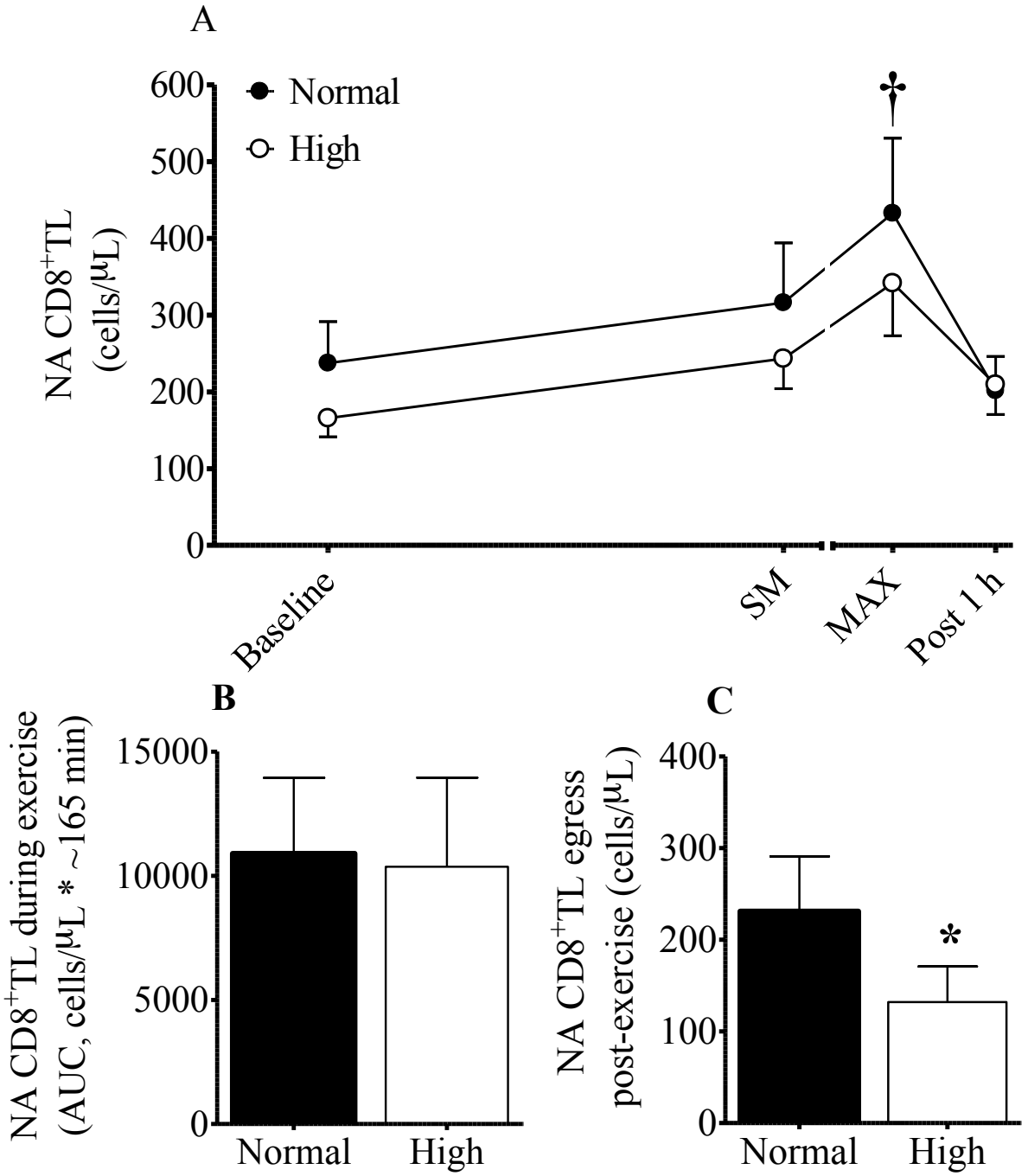
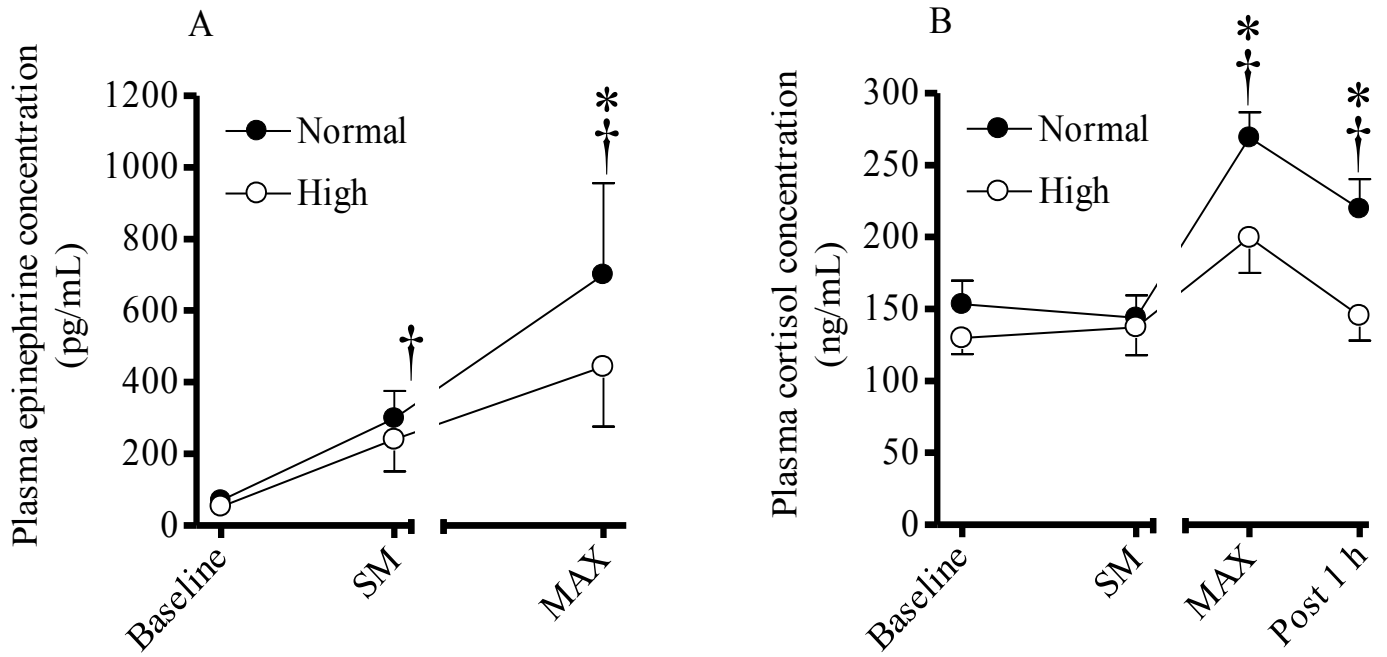

A
Normal
High
NA CD8+TL (cells/μL)
600
500
400
300
200
100
0
†
Baseline
SM
MAX
Post 1 h
B
NA CD8+TL during exercise (AUC, cells/μL * ~165 min)
15000
10000
5000
0
Normal
High
C
NA CD8+TL egress post-exercise (cells/μL)
400
300
200
100
0
*
Normal
High

Figure 4


A
Plasma epinephrine concentration (pg/mL)
1200
1000
800
600
400
200
0
Normal
High
†
*
†
Baseline
SM
MAX
B
Plasma cortisol concentration (ng/mL)
300
250
200
150
100
50
0
Normal
High
*
†
*
†
Baseline
SM
MAX
Post 1 h