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

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1 **Abstract**

2 **Purpose:** We examined whether exercise-induced lymphocytosis and lymphocytopenia are
3 impaired with high-intensity training. **Methods:** Eight trained cyclists ($\dot{V} O_2$ max: 64.2 ± 6.5
4 $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) undertook one week of normal-, and a second week of high-intensity training. On
5 day seven of each week, participants performed a cycling task, consisting of 120 min sub-maximal
6 exercise followed by a 45 min time trial. Blood was collected before, during and after exercise.
7 CD8^+ T lymphocytes ($\text{CD8}^+\text{TLs}$) were identified, as well as $\text{CD8}^+\text{TL}$ sub-populations on the basis
8 of CD45RA and CD27 expression. **Results:** High-intensity training ($18,577 \pm 10,984 \text{ cells}\cdot\mu\text{L}\times$
9 $\sim 165 \text{ min}$) was associated with a smaller exercise-induced mobilization of $\text{CD8}^+\text{TLs}$ compared with
10 normal-intensity training ($28,473 \pm 16,163 \text{ cells}\cdot\mu\text{L}\times \sim 165 \text{ min}$, $p = 0.09$). The response of highly
11 cytotoxic $\text{CD8}^+\text{TLs}$ ($\text{CD45RA}^+\text{CD27}^-$) to exercise was smaller following one week of high- ($3,144$
12 $\pm 924 \text{ cells}\cdot\mu\text{L}\times \sim 165 \text{ min}$) compared with normal-intensity training ($6,417 \pm 2,143 \text{ cells}\cdot\mu\text{L}\times \sim 165$
13 min ; $p < 0.05$). High-intensity training reduced post-exercise $\text{CD8}^+\text{TL}$ lymphocytopenia ($-436 \pm$
14 $234 \text{ cells}\cdot\mu\text{L}$) compared to normal-intensity training ($-630 \pm 320 \text{ cells}\cdot\mu\text{L}$; $p < 0.05$). This was
15 driven by a reduced egress of naïve $\text{CD8}^+\text{TLs}$ ($\text{CD27}^+\text{CD45RA}^+$). High-intensity training was
16 associated with reduced plasma epinephrine (-37%) and cortisol (-15%) responses ($p < 0.05$).
17 **Conclusions:** High-intensity training impaired $\text{CD8}^+\text{TL}$ mobilization and egress in response to
18 exercise. Highly cytotoxic $\text{CD8}^+\text{TLs}$ were primarily responsible for the reduced mobilization of
19 $\text{CD8}^+\text{TLs}$, which occurred in parallel with smaller neuro-endocrine responses. The reduced capacity
20 for $\text{CD8}^+\text{TLs}$ to leave blood post-exercise with high-intensity training was accounted for primarily
21 by naïve, and also, highly cytotoxic $\text{CD8}^+\text{TLs}$. This impaired $\text{CD8}^+\text{TL}$ redistribution in athletes
22 undertaking intensified training may imply reduced immune-surveillance.

23
24 **Key words:** Exercise training, CD8^+ T Lymphocytes, immune surveillance, epinephrine, cortisol,
25 humans.

26

27

28

29 **Introduction**

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

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

50
51 **Paragraph 3.** Likewise, the post-exercise lymphocytopenia, whereby cell counts fall below baseline
52 levels, is also thought to be important for immune surveillance and antigen detection (6,20,39).

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

61

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

72

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

77

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

90

91 **Methods**

92 **Participants**

93 **Paragraph 7.** Eight well-trained male cyclists (Mean \pm SD; age 27 ± 8 yr; body mass index $22.8 \pm$
94 $2.2 \text{ kg}\cdot\text{m}^{-2}$ maximal oxygen uptake ($\dot{V}\text{O}_2 \text{ max}$) $64.2 \pm 6.5 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) took part in this study.
95 Participants were non-smokers, not taking any medication and had remained free from respiratory
96 infection for four weeks. All participants provided informed written consent and the study was
97 approved by the Research Ethics Committee of the School of Sport and Exercise Sciences,
98 University of Birmingham, UK.

99

100 **Experimental design**

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

107

108 **Preliminary exercise testing and familiarization**

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

119 **Exercise training conditions**

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

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

131

132 **Exercise trials**

133 **Paragraph 11.** Exercise trials took place on the seventh day of each exercise training condition.

134 Trials started at ~06:30 after an overnight fast. Following a 15 min rest, a baseline blood sample
135 was drawn from a forearm vein using an indwelling catheter. The catheter was flushed regularly
136 with saline to maintain patency. Each exercise trial was identical, and consisted of two stages
137 completed in short succession. First, participants cycled for 120 min at a fixed exercise intensity
138 equivalent to ~60% $\dot{V}O_{2\max}$ (sub-maximal effort cycling). Second, participants undertook a
139 maximal-effort time trial lasting ~45 min (~85-100% $\dot{V}O_{2\max}$). The time trial required a target
140 amount of work (mean \pm SD, energy target; 670 ± 52 kJ) to be completed in as short a time as
141 possible (18). The total amount of work to be performed was individualized, based on their Watt-
142 max achieved during the $\dot{V}O_{2\max}$ test, and was calculated using the following formula:

$$143 \quad \text{Total work (J)} = 0.7 \times \text{Watt-max} \times 2700 \text{ s.}$$

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

146

147 **Paragraph 12.** Physiological and psychological data (e.g., HR, RPE, $\dot{V}O_2$ and RER) were collected
148 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.

152

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.

156

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 ($\text{kcal}\cdot\text{day}^{-1}$) (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 $1.5 \text{ g of protein}\cdot\text{kg}^{-1} \text{ body mass}\cdot\text{day}^{-1}$ and 6
167 $\text{g of CHO}\cdot\text{kg}^{-1} \text{ body mass}\cdot\text{day}^{-1}$, with the remainder of energy derived from fat. Calorie intake was
168 higher during high-intensity training ($4410 \pm 437 \text{ kcal}\cdot\text{day}^{-1}$) compared with normal-intensity
169 training ($3711 \pm 456 \text{ kcal}\cdot\text{day}^{-1}$, $p < 0.05$). 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.

172 **Flow Cytometry**

173 **Paragraph 15.** Blood was collected into K₃EDTA Vacutainers and processed within 5 h. Whole
174 blood was incubated with two separate antibody panels for 25 min at room temperature. Panel 1;
175 CD45RA FITC, CD27 PE, CD3 PERCP and CD8 APC. Panel 2; CD16 FITC, CD56 PE, CD3
176 PERCP and CD14 APC (Antibodies from Pharmingen San Diego, USA, except CD3 PERCP;
177 Becton Dickinson San Jose, USA). Erythrocytes were lysed using FACS lysing solution (Becton
178 Dickinson, San Jose, USA). Cells were washed by centrifugation at 500 × g for 6 min at 4°C, and
179 re-suspended in 300 µL of 2% paraformaldehyde phosphate buffered saline. Cells were stored in the
180 dark at 4°C and read on a FACS calibur flow cytometer (Becton Dickinson, San Jose, USA),
181 collecting 20,000 lymphocytes.

182

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

190 **Neuro-endocrine measurements**

191 **Paragraph 17.** Plasma cortisol and epinephrine concentrations were measured using commercially
192 available kits (Cortisol, IDS, Tyne and Wear, UK; CAT-COMBO, IDS, Tyne and Wear, UK,
193 respectively). Plasma was obtained by centrifugation at 1500 × g for 10 min at 4°C. Samples were
194 stored at -80°C until analysis. Epinephrine was not measured 1 h post-exercise due to the short
195 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).

215

216 **Paragraph 19.** All data were expressed as means \pm 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.

221 **Results**

222 **Exercise training conditions**

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

232

233 **Physiological and psychological responses to exercise**

234 **Paragraph 21.** Physiological responses to sub-maximal cycling were not different between trials,
235 ($\dot{V}O_2$ and RER data not shown) although RPE was greater following high-intensity training (see
236 supplementary table which shows exercise duration, power output, RPE and HR responses to
237 exercise following normal- and high-intensity training). Time to completion (min) and power output
238 (watts) during maximal-effort cycling were slightly reduced following high-intensity training
239 (Paired samples *t*-test; $t_{(7)} < 1.9$, $p < 0.05$), however, HR and RPE were not significantly different
240 (Paired samples *t*-test; $t_{(7)} > 4.4$, $p > 0.05$; see supplementary table). Physiological, and
241 psychological variables were not significant covariates mediating any of the training-induced
242 immunological responses to acute exercise (all interaction effects; $F_{(1,6)} < 3.2$, $p > 0.05$, $\eta^2 < 0.3$). In
243 other words, changes in these variables between training conditions did not confound any of the
244 results reported below.

245

246 **Leukocyte responses to exercise**

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

259

260 **Total CD8⁺ T Lymphocyte responses to exercise**

261 **Paragraph 23.** Figure 1 shows CD8⁺TL responses to exercise following the normal- and high-
262 intensity training conditions. CD8⁺TL increased during exercise (~300 %) and fell below baseline
263 levels 1 h post-exercise (~45 %) (see Figure 1 A, Main effects of time $F_{(1,7)} = 29.8$; $p < .05$). There
264 was a trend for a smaller exercise-induced mobilization of CD8⁺TL in the high-intensity training
265 condition (see Figure 1 B, Paired samples t -tests iAUC between training conditions; $t_{(7)} = 2.1$; $p =$
266 0.09). The egress of CD8⁺TL 1 h post-exercise (Paired samples t -tests between training conditions; t
267 $_{(7)} = 3.047$; $p < 0.05$) was attenuated in the high-intensity training condition (see Figure 1 C).

268

269 **CD8⁺ T Lymphocyte subset responses to exercise**

270 **Paragraph 24.** Figure 2 shows EMRA CD8⁺TL responses to exercise following the normal- and
271 high-intensity training conditions. The mobilization of EMRA CD8⁺TL was smaller in the high-
272 intensity condition (see Figure 2 B, Paired samples *t*-test iAUC between conditions; $t_{(1,7)} = 2.5$; $p <$
273 0.05). 1 h post exercise, the number of circulating EMRA CD8⁺TL was reduced by ~65% compared
274 to pre-exercise with normal-intensity training. In the high-intensity condition, there was a trend for
275 a smaller post-exercise egress; EMRA CD8⁺TL fell by ~55% relative to baseline (see Figure 2 C,
276 Paired samples *t*-tests between conditions; $t_{(1,7)} = 2.2$; $p = 0.06$).

277
278 **Paragraph 25.** There were no differences in the mobilization of NA, CM and EM CD8⁺TL to
279 exercise between the normal and high-intensity training conditions (data only shown for NA, see
280 Figure 3 A-B). The post-exercise egress of NA CD8⁺TL was smaller in the high-intensity training
281 condition (see Figure 3 C, Paired samples *t*-tests between training conditions; $t_{(7)} = 3.6$; $p < 0.05$),
282 however the egress of CM and EM was unaffected (data not shown),

283

284 **NK cell responses to exercise**

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

289

290 **Neuro-endocrine responses to exercise**

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

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

297

298 **Paragraph 28.** Cortisol concentrations increased in response to the maximal-effort time trial and
299 remained elevated at 1 h post-exercise (see Figure 4 B, main effects of time; $F_{(1,7)} = 20.0$; $p < 0.05$).
300 Cortisol responses to exercise were reduced after high-intensity training (Mean \pm SD iAUC; $10,344$
301 ± 3382 ng/mL \times ~165min; 8754 ± 1258 ng/mL \times ~165min during normal- and high-intensity
302 training, respectively; Paired samples t -test; $t_{(7)} = 2.4$, $p < 0.05$).

303

304 **Paragraph 29.** Epinephrine and cortisol concentration were not significant covariates mediating any
305 of the training-induced immunological responses to acute exercise (all interaction effects; $F_{(1,6)} <$
306 3.5 , $p > 0.05$, $\eta^2 < 0.4$). In other words, the reduced neuro-endocrine response to exercise with high-
307 intensity training, was not responsible for the training-induced reduced immune responses to
308 exercise.

309

310 **Discussion**

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

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

320

321 **Paragraph 31.** The smaller mobilization of CD8⁺TL with high-intensity exercise training occurred
322 in parallel with a reduced epinephrine response to exercise. CD8⁺TL, and in particular the EMRA
323 sub-population, express the β 2 adrenergic receptor very densely at the cell surface (19). Exercise is
324 associated with increased adrenergic activity, and these cells become selectively mobilized via an
325 adrenergic-dependent mechanism (8,19). Moreover, epinephrine infusion alone causes a similar
326 mobilization of lymphocytes (8,19) which confirms previous assumptions that exercise-induced
327 lymphocytosis is largely dependent on epinephrine release (28). The observed blunting of
328 epinephrine and CD8⁺TL responses to acute exercise following high-intensity training is consistent
329 with previous reports. For example, physically fit individuals, who engage regularly in exercise
330 training, exhibit a smaller exercise-induced lymphocytosis compared to unfit individuals, which is
331 independent of absolute exercise intensity (16,29). It has previously been reasoned that β 2
332 adrenergic receptors are desensitized due to repeated exercise-induced adrenergic activity (16,29).
333 Also consistent with our findings is the observation that relative to unfit individuals, physically fit
334 individuals tended to show more modest epinephrine responses to stress and exercise tasks (16).
335 Thus, our findings and those of others, suggest that periods of high-intensity exercise training are
336 associated with a reduced CD8⁺TL mobilization, which might be in part mediated by β 2 adrenergic
337 receptor down-regulation, a decline in adrenergic output, or a combination of the two.

338

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

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

350

351 **Paragraph 33.** Although the mobilization of lymphocytes into blood during exercise is well
352 studied, investigation of post-exercise lymphocytopenia and the mechanism behind this process
353 remains unclear. Cortisol is proposed as a neuro-endocrine mediator of lymphocyte extravasation
354 from blood (9,32). Indeed, Dimitrov et al. (7) showed that cortisol infusion causes a selective egress
355 of NA CD8⁺TL from peripheral blood. Consistent with these findings, we observed a smaller NA
356 CD8⁺TL egress occurring in parallel with a reduced cortisol release with high-intensity exercise
357 training. Together, these findings imply that an impaired ability of NA CD8⁺TL to leave blood post-
358 exercise might, at least in part, be mediated by a blunted cortisol response. However, our mediation
359 analyses suggest that a smaller cortisol response to exercise following high-intensity training was
360 not responsible for the reduced NA CD8⁺TL (or any other CD8⁺TL population) lymphocytopenia.
361 Thus, if cortisol does indeed play a role in lymphocytopenia, it might be related to down-stream
362 mechanistic processes. For example, as with lymphocyte β2 adrenergic receptor down regulation
363 with chronic adrenergic stimulation (16), glucocorticoid receptor density might similarly be affected
364 with chronic cortisol stimulation. Likewise, high-intensity exercise training and/or chronic cortisol
365 release might influence other aspects of lymphocytopenia not investigated in this study. For
366 example, lymphocyte migration to bone marrow, as observed with exercise in rodents (20), is

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

372

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

387

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

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

412

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

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

425

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

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

444

445 **Paragraph 38.** When interpreting the results of this study, it should be considered that in humans,
446 the exact destination of cells leaving peripheral blood post-exercise is unknown. Animal studies
447 provide strong evidence for exercise-induced leukocyte migration to peripheral tissues such as the
448 skin, and lungs (6,20). In these models, it is thought that leukocyte tissue migration facilitates the
449 detection and elimination of antigen. Another consideration is that clinical diagnosis of URTI
450 episodes were not made in this study. It therefore remains unclear whether altered redistribution
451 patterns of CD8⁺TLs following high-intensity exercise training translates into increased
452 susceptibility to infection. Future studies are needed to investigate whether altered leukocyte
453 responses to bouts of intense exercise are predictive of clinically diagnosed infectious disease
454 episodes in athletes. Functional measures of lymphocytes (e.g., activation, proliferation and
455 cytokine production) and granulocytes or neutrophils (e.g., chemotaxis, phagocytosis and
456 superoxide production) might provide useful adjunct measurements for follow-up investigations.
457 Further, in light of the present results, mechanistic studies are now warranted to better understand
458 how high-intensity exercise training influences lymphocytosis and lymphocytopenia. For example,
459 relevant to lymphocytosis, cyclic-adenosine monophosphate (cAMP) assays on isoproterenol
460 stimulated T-cell subsets could be conducted *in vitro* to examine whether β 2 adrenergic receptors
461 are indeed desensitized. Relevant to lymphocytopenia, adhesion and tissue homing molecule
462 expression could be investigated *ex vivo* with athletes undertaking very large volumes of exercise.
463 Similar analyses could be made *in vitro*, examining lymphocyte cell-surface molecule expression in
464 response to cortisol exposure.

465

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

474

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

479

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483

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

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

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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 \pm 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 \pm 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 \pm 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 \pm 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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1 **Table 1**2
3 **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 ^{+*}	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 ^{+*}	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	$F_{(1,7)} = 13.2; p < 0.05$

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24
25 Values are means ± SD. Main effect of time combines both normal- and high-intensity training values. [†]significantly different from baseline in
26 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 [*]	<i>n/a</i>
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

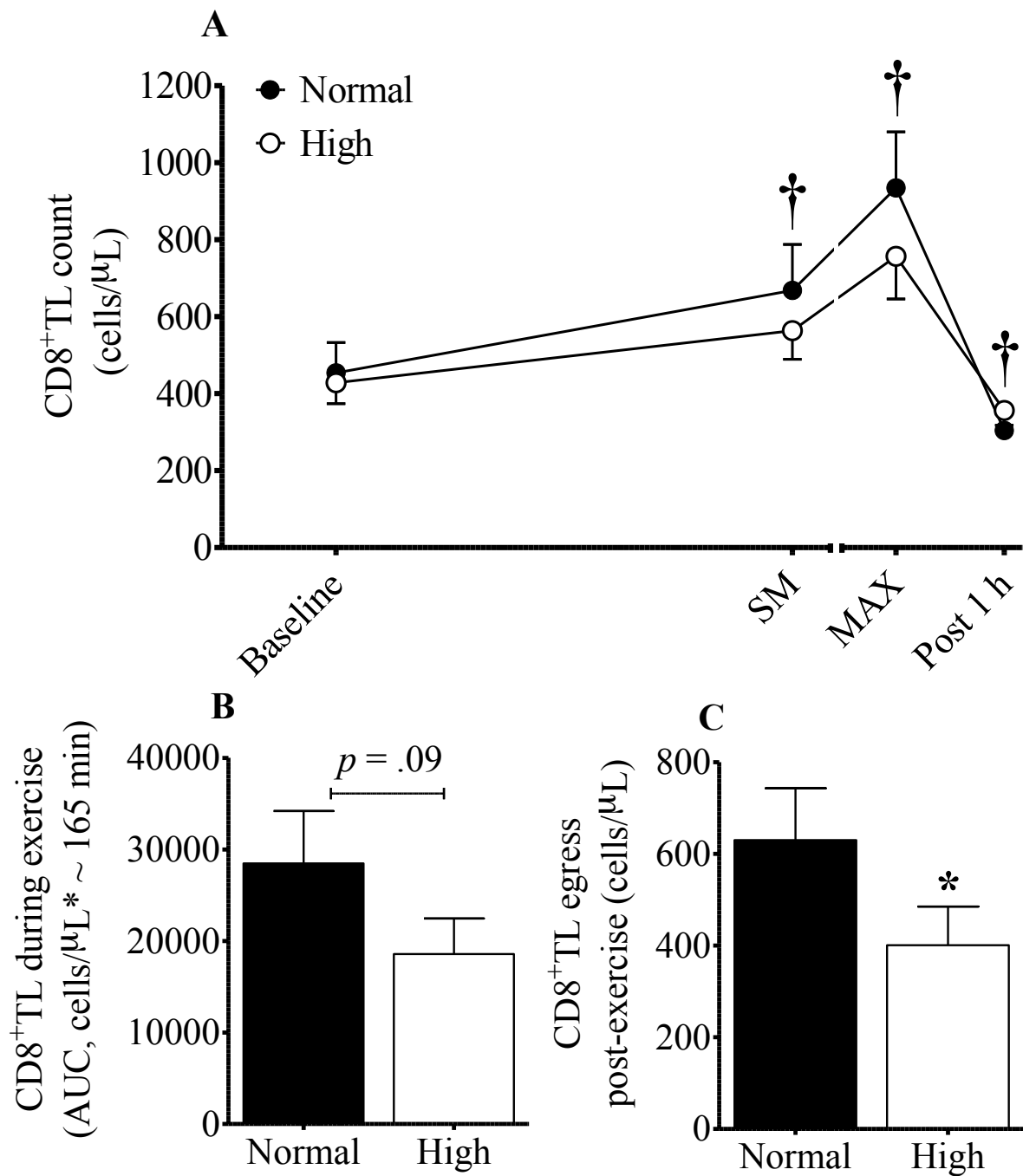


Figure 2

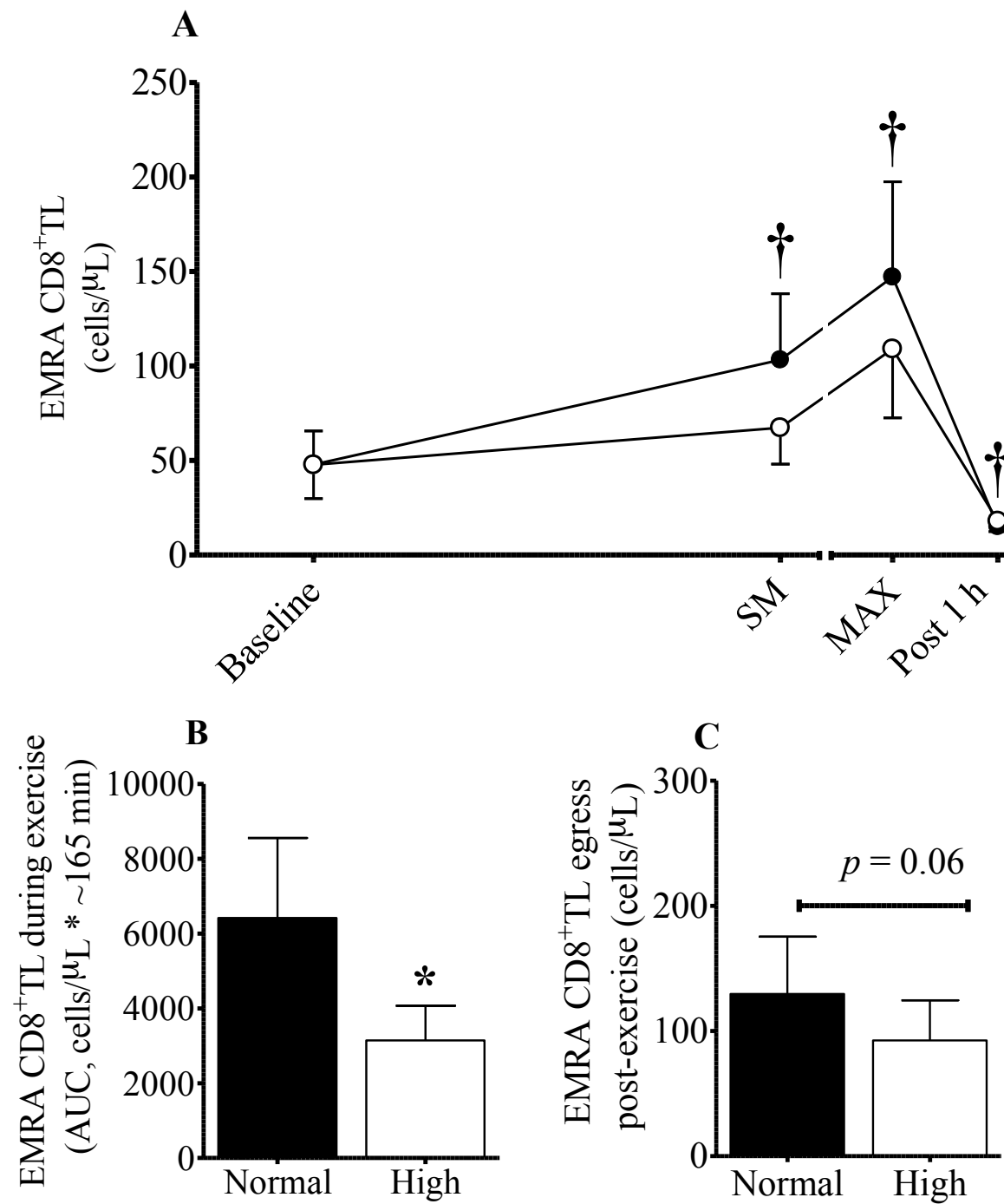


Figure 3

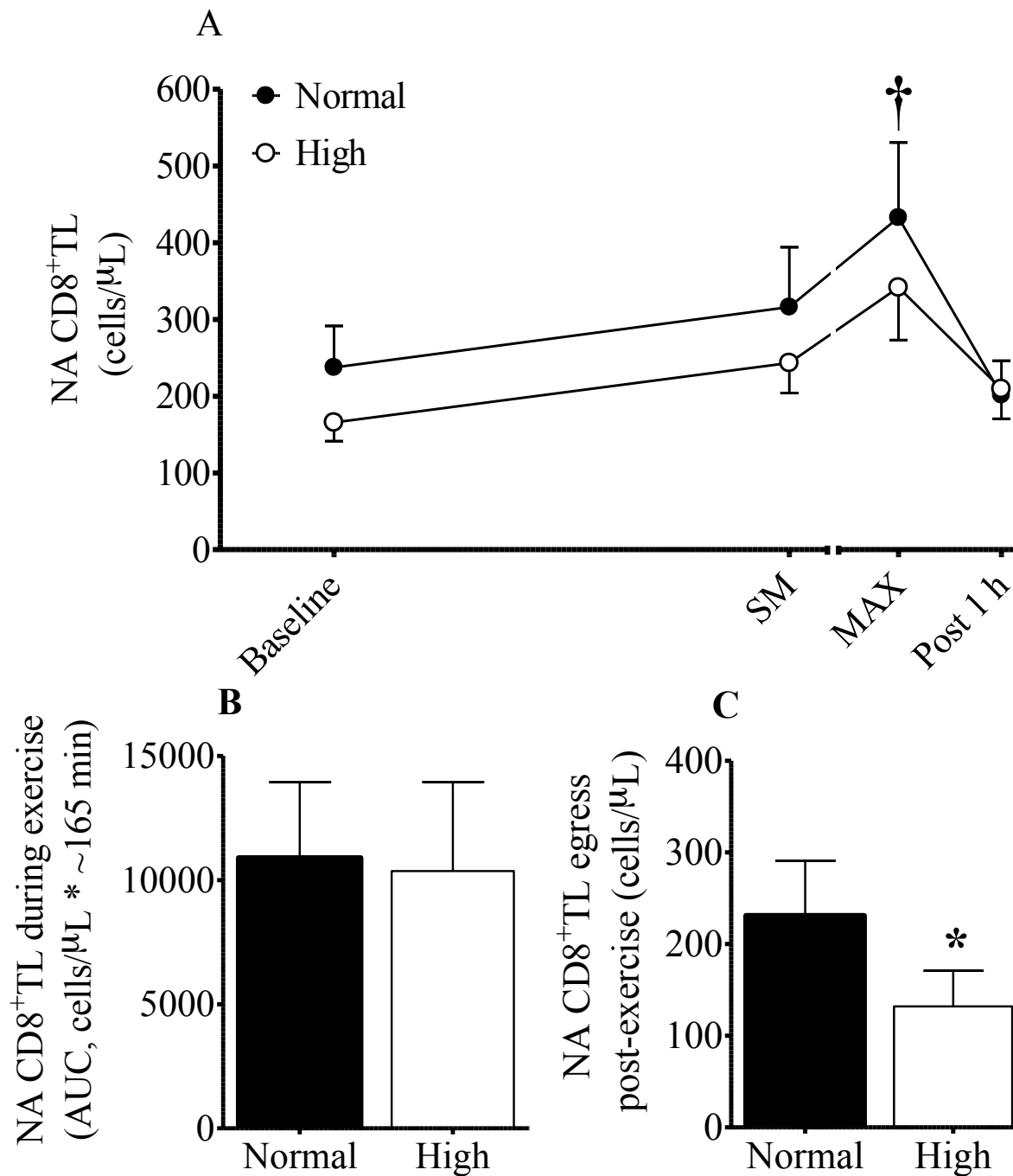


Figure 4

