

## Preliminary evidence of reductive stress in human cytotoxic T-cells following exercise

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1 **Preliminary evidence of *reductive stress* in human cytotoxic T-cells**  
2 **following exercise**

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28

## 29 **Abstract**

30 This study investigated immunophenotypic differences in intracellular thiol redox  
31 state of peripheral blood mononuclear cells (PBMCs) isolated from trained (TR, n=9, mean  $\pm$   
32 SD: age  $28 \pm 5$  years; BMI  $23.2 \pm 2.6$  kg·m<sup>2</sup>;  $\dot{V}O_{2max}$   $56.9 \pm 6.1$  ml·kg<sup>-1</sup>·min<sup>-1</sup>) and  
33 recreationally active (RA, n=11, mean  $\pm$  SD: age  $27 \pm 6$  years; BMI  $24.2 \pm 3.7$  kg·m<sup>2</sup>;  $\dot{V}O_{2max}$   
34  $45.1 \pm 6.4$  ml·kg<sup>-1</sup>·min<sup>-1</sup>) participants before and after a maximal aerobic exercise tolerance  
35 test. Blood samples were taken before (PRE), during (sample acquired at 70% HR<sub>max</sub>),  
36 immediately (POST+0) and 15 minutes post-exercise (POST+15). PBMCs were isolated and  
37 reduced thiol analysis (fluorescein-5 maleimide (F5M)) by immunophenotype (CD3<sup>+</sup>, CD4<sup>+</sup>  
38 and CD8<sup>+</sup>) was performed using flow cytometry. A significant increase in cellular F5M  
39 fluorescence was observed in CD3<sup>+</sup> T-cells at POST+0, with changes driven to a greater  
40 extent by CD8<sup>+</sup> T-cells (fold change in both groups CD4: +2.3, CD8: +2.8; p<0.05). Further  
41 analysis revealed a population of highly reduced CD8<sup>+</sup> T-cells (*CD8<sup>+</sup>T-reduced<sup>+</sup>*) that  
42 significantly increased from PRE to POST+0 in RA participants only (RA: +272 cell/ $\mu$ L,  
43 p<0.05). To further understand these results, *CD8<sup>+</sup>T-reduced<sup>+</sup>* and *CD8<sup>+</sup>T-reduced<sup>-</sup>* cells were  
44 analysed for immunophenotype in response to the same exercise protocol (n=6, mean  $\pm$  SD:  
45 age  $24 \pm 5$  years; BMI  $25.7 \pm 4.1$  kg·m<sup>2</sup>;  $\dot{V}O_{2max}$   $41.33 \pm 7.63$  ml·kg<sup>-1</sup>·min<sup>-1</sup>). *CD8<sup>+</sup>T-*  
46 *reduced<sup>+</sup>* had significantly less lymphoid homing potential (CCR7) POST+0 compared to  
47 PRE. This study is the first to demonstrate that lymphocyte populations become more  
48 *reductive* in response to acute exercise.

49

50 **New & Noteworthy**

51 The study presented provides the first evidence to suggest that cytotoxic T-cells become  
52 transiently reductive in healthy individuals following a single bout of cycling. Detection of  
53 these cells was enabled via the use of a flow cytometric assay that incorporates the thiol  
54 reactive probe Fluorescein-5 maleimide. Using this method, transient *reductive stress* in  
55 viable T-cells is permissible and provides the basis for further research in the area of exercise  
56 immunology.

57

58 **Abbreviations**

59 ANOVA: Analysis of Variance, BMI: Body mass index, CV: Coefficient of variance, CD:  
60 Cluster of differentiation, CCR7: Chemokine receptor type 7, DTNB: 5,5'-dithiobis (2-  
61 nitrobenzoic) acid, EDTA: ethylene diaminetetraacetic acid, FCS: Fetal Calf Serum, F5M:  
62 Fluorescein-5 maleimide, HR<sub>max</sub>: Maximum heart rate, GSH: Glutathione, GSSG:  
63 Glutathione disulphide, GTP: Guanidine triphosphate, HBSS: Hanks Balance Salt Solution,  
64 H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide, IL: Interleukin, IPAQ: International Physical Activity  
65 Questionnaire, NAC: N-acetylcysteine, NADPH: Nicotinamide adenine dinucleotide  
66 phosphate, NEM: N-ethylmaleimide, PD-1: Programmed death receptor-1, PBMC: Peripheral  
67 Blood Mononuclear Cell, PBS: Phosphate buffered saline, PRDX: Peroxiredoxin, Pre: Blood  
68 sample taken before exercise, Post+0: Blood sample taken immediately post-exercise,  
69 Post+15: Blood sample taken 15 minutes post-exercise, RONS: Reactive oxygen and  
70 nitrogen species, RPM: repetitions per minute, RPMI: Roswell Park Memorial Institute, SD:  
71 Standard deviation, -SH: Sulphur-hydryl, TNB: 5-thio-2-nitrobenzoic acid, TR: Trained,  
72 VPA: Vigorous physical activity, VCO<sub>2</sub>: Carbon dioxide consumption,  $\dot{V}O_2$ : Oxygen uptake,  
73  $\dot{V}O_{2\text{ MAX}}$ : Maximum oxygen uptake.

74

75 **Introduction**

76           Alterations in peripheral blood immune cell subpopulations during exercise are well  
77 characterised, with transient increases in effector CD8<sup>+</sup> T-cells and Natural Killer cells  
78 observed at the expense of CD4<sup>+</sup> T-cells and B lymphocytes (4). However, the precise  
79 mechanisms driving mobilization and tissue extravasation of these cell types are unclear.  
80 Studies have demonstrated that global protein oxidation increases after acute exercise, with  
81 redox enzyme activity and protein content (i.e. superoxide dismutase, glutathione peroxidase  
82 and peroxiredoxin) concurrently increased (8, 43–45, 48). This suggests that redox balance  
83 may have a role in the regulation of the immune system after exercise.

84           Exercise is known to induce the production of reactive oxygen and nitrogen species  
85 (RONS), a diverse group of ubiquitous reactive molecules that are widely implicated in  
86 mediating cell and tissue function during exercise (11, 19, 23, 29, 30). *In vitro* evidence  
87 suggests that cell permeable RONS such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) can reversibly oxidise  
88 nucleophilic cysteine thiol groups that are conserved within various cellular proteins (51).  
89 These include antioxidant enzymes such as thioredoxin and peroxiredoxin (39, 52) as well as  
90 various signaling proteins, such as GTPases, phosphatases and transcription factors (33). The  
91 oxidation (i.e. sulfenylation/ sulfinylation) of thiol-containing proteins has been previously  
92 demonstrated in total lymphocyte pools following acute exercise (44, 48). This could be a  
93 potential mechanism by which H<sub>2</sub>O<sub>2</sub> triggers redox-sensitive signaling pathways and/ or  
94 elicits oxidative stress, which may in turn influence immune cell mobilization and  
95 immunophenotype. Interestingly, recent studies have demonstrated that supplementation with  
96 the thiol donor, N-acetylcysteine (NAC) can blunt immune cell mobilization (i.e.  
97 macrophages and lymphocytes) patterns in response to both muscle-damaging (24, 31) and  
98 exhaustive aerobic exercise (28). This drives the hypothesis that shifts in thiol redox state  
99 may regulate immunoregulation.

100 Previous studies which have evaluated redox changes in protein thiols following acute  
101 exercise have relied on isolating all immune cells from peripheral blood (44, 48), through  
102 which downstream analysis is performed using lysates from a heterogeneous cell populations.  
103 These techniques, whilst informative, do not permit evaluation of cellular thiol redox state  
104 within individual cells of a specific immunophenotype. Furthermore, these analytical  
105 procedures require cell lysis for their proteomic approaches, which could lead to artificial  
106 thiol oxidation, making data interpretation a challenge. Previous studies have utilized the  
107 thiol specific probe, fluorescein-5 maleimide (F5M) to label and identify intracellular  
108 proteins with solvent accessible reduced cysteines in cell line models using immunoblotting  
109 techniques (35, 53). In this respect, a loss of fluorescent signal relative to background  
110 controls is indicative of thiol oxidation i.e. an increased cellular *oxidative stress*, whereas a  
111 gain in signal would indicate *reductive stress*. Interestingly, F5M is excited at  $\lambda 488$  nm and  
112 emits at  $\lambda 525$  nm (38), offering the potential for F5M to be incorporated into flow cytometric  
113 immunophenotyping assays. Thus by adapting previous protocols that have used F5M to  
114 monitor intracellular thiol redox changes (7, 35, 53), flow cytometry would represent a  
115 powerful option to monitor *oxidative* and *reductive* stress by immunophenotype in a variety  
116 of contexts, e.g. following exercise. For example, cells of the adaptive immune system i.e. T-  
117 helper ( $CD4^+$ ) and T-cytotoxic ( $CD8^+$ ) lymphocytes are important to evaluate in this context,  
118 since oxidative stress has been documented in these cells post-exercise (8, 43–45, 48). Both  
119 types of T-lymphocyte are antigen specific, where  $CD4^+$  T-cells serve broadly to orchestrate  
120 the immune response via cytokine production and  $CD8^+$  T-cells kill infected cells through the  
121 release of cytotoxic granules (50). Whilst the importance of *oxidative stress* in  $CD4^+$  and  
122  $CD8^+$  T-cells following exercise is appreciated, the role of *reductive stress* in this context is  
123 currently unknown. The impact of chronic *reductive* stress is now being appreciated in the  
124 literature (1, 20, 22), however the potential immunological significance of this response is

125 unclear, specifically in relation to transient changes which may take place in response to  
126 exercise.

127 The aim of the present study was to monitor thiol-mediated redox changes in viable  
128 CD4<sup>+</sup> and CD8<sup>+</sup> T-cells isolated from individuals before and after a cycling ramp test to  
129 exhaustion. Following optimization of a flow cytometric protocol, thiol mediated redox  
130 changes in these lymphocytes were quantified, and further studies carried out to characterise  
131 these cells with regards to immunophenotype and lymphoid homing potential.

132

### 133 **Materials and methods**

#### 134 **Participants**

135 Following ethical approval from the University of Worcester Research Ethics  
136 Committee, twenty healthy males were initially recruited to take part in the study. Trained  
137 individuals (TR, n=9, mean  $\pm$  SD: age  $28 \pm 5$  years; BMI  $23.2 \pm 2.6$  kg.m<sup>2</sup>;  $\dot{V}O_{2max}$   $56.9 \pm 6.1$   
138 ml.kg<sup>-1</sup>.min<sup>-1</sup>) were recruited from local athletics, cycling and triathlon clubs. The criterion  
139 for "trained" was regularly completing in a minimum of 3 hours of endurance training per  
140 week, for a minimum of 2 years and a maximal oxygen consumption of at least 50 ml.kg<sup>-1</sup>.  
141 min<sup>-1</sup>(2). Recreationally active individuals (RA, n=11, mean  $\pm$  SD: age  $27 \pm 6$  years; BMI  
142  $24.2 \pm 3.7$  kg.m<sup>2</sup>;  $\dot{V}O_{2max}$   $45.1 \pm 6.4$  ml.kg<sup>-1</sup>.min<sup>-1</sup>) were recruited through adverts at the  
143 University of Worcester. All participants completed questionnaires addressing health history,  
144 and habitual levels of weekly physical activity (mean  $\pm$  SD: TR  $10235 \pm 6394$ ; RA  $4932 \pm$   
145  $2555$ ) and vigorous physical activity (VPA, mean  $\pm$  SD: TR  $800 \pm 1008$ ; RA  $264 \pm 271$ ) were  
146 assessed by the International Physical Activity Questionnaire (IPAQ). Based on the results, a  
147 further six recreationally active participants (n=6, mean  $\pm$  SD: age  $24 \pm 5$  years; BMI  $25.7 \pm$   
148  $4.1$  kg.m<sup>2</sup>;  $\dot{V}O_{2max}$   $41.33 \pm 7.63$  ml.kg<sup>-1</sup>.min<sup>-1</sup>) were recruited to characterise the CD8<sup>+</sup>T-

149 *reduced*<sup>+</sup> cell population.

150 All participants gave their written informed consent and the study was carried out in  
151 accordance with the Declaration of Helsinki (2008). Participants were non-smokers and  
152 reported that they had not taken any antioxidant vitamin supplements or anti-inflammatory  
153 drugs for 4 weeks prior to the laboratory visit. In addition, participants reported to be free  
154 from any viral or bacterial infections for at least 4 weeks prior to taking part. Participants  
155 were also required to refrain from any strenuous physical activity, consumption of alcoholic  
156 beverages or caffeine for two days prior to the experimental session.

157

158 Experimental session

159 All experimental sessions took place at the University of Worcester in the morning  
160 (7.00-7.30am start time), following at least a 10 hour fast. After a thirty-minute period of rest,  
161 resting heart rate and blood pressure (*Omron Healthcare, Hoofddorp, The Netherlands*),  
162 height (*Seca Alpha, Hamburg, Germany*) and mass (*Tanita, Tokyo, Japan*) were determined.  
163 Cardiorespiratory fitness ( $\dot{V}O_{2\max}$ ) was then measured using a ramp exercise test to  
164 exhaustion on an electromagnetically braked cycle ergometer (*Lode Excalibur Sport,*  
165 *Groningen, Netherlands*). Workload commenced at 50 Watts and was increased by 1 Watt  
166 every 6 seconds, until volitional exhaustion or until cadence fell below 60 revolutions per  
167 minute. Oxygen uptake was assessed continuously using a breath-by-breath system (*Oxycon*  
168 *Prx, Jaeger, Wuerzberg, Germany*) and heart rate monitored using a Polar Vantage heart rate  
169 monitor (*Polar Vantage, Kempele, Finland*). The breath-by-breath system was calibrated  
170 daily or before each test to verify gas levels using a fixed volume cylinder (3L), a gas canister  
171 of known composition (4.5% CO<sub>2</sub>, 3.5% O<sub>2</sub> and 5.0% N<sub>2</sub>), as well as adjusting for any subtle  
172 atmospheric changes. A final obtained value of rate of oxygen consumption relative to body  
173 mass was accepted as  $\dot{V}O_{2\max}$  (ml.kg<sup>-1</sup>.min<sup>-1</sup>) if two of the following criteria were met in



174 conjunction with a plateau in oxygen consumption with an increase in work load: volitional  
175 exhaustion; a respiratory exchange ratio of  $\geq 1.15$ ; heart rate within  $10 \text{ beats} \cdot \text{min}^{-1}$  of the age-  
176 predicted maximal heart rate  $(220 - \text{age})$  (16). Upon completion of the exercise test,  
177 participants rested in a seated position for 15 minutes before the final blood sample was  
178 obtained.

179

#### 180 Blood sampling

181 A catheter (*Appleton Woods, Birmingham, UK*) was inserted into the antecubital vein  
182 of the arm prior to exercise to obtain a baseline sample after thirty minutes of rest (Pre). A  
183 second blood sample was taken when participants reached predicted 70%  $\text{HR}_{\text{max}}$  during the  
184 exercise test. Subsequent blood samples were taken immediately (Post+0) and 15 minutes  
185 post-exercise (Post+15). At each time point, 12 ml of blood was drawn into two separate  
186 vacutainer tubes containing potassium ethylene diaminetetraacetic acid (EDTA) (*Becton,*  
187 *Dickson & Company, Oxford, UK*). The catheter was flushed every 30 minutes with isotonic  
188 saline solution (0.9% sodium chloride) to prevent blood clotting. For the characterisation of  
189  $\text{CD8}^+ \text{T-reduced}^+$  (n=6), two blood samples were obtained (12ml) via venepuncture to the  
190 antecubital vein of the arm prior to (Pre) and immediately following exercise (Post+0) only.

191

#### 192 Flow cytometry method validation

193 Jurkat immortalised T-cell line (*ATCC, Middlesex, UK*) was maintained in RPMI-  
194 1640 adjusted to contain 10% (v/v) foetal calf serum, 2 mM L-glutamine and 100 U/ml  
195 penicillin /100 mg/ml streptomycin. Redox balance was perturbed by adding 0.1 mU/mL  
196 glucose oxidase to  $2 \times 10^6$  Jurkat cells (14). The cells were incubated for four hours with  
197 samples taken at the start (T0), after 1 hour (T1) and after 4 hours (T4). The samples were  
198 washed with phosphate buffered saline (PBS) and then incubated for 20 minutes with F5M

199 (0.1  $\mu\text{M}$ ) in the dark at 4°C, followed by two washes with FACS buffer. The concentration of  
200 F5M was determined through titrations (0-10  $\mu\text{M}$ ) during pilot analysis to optimise the  
201 fluorescent signal on FL-1 FITC (data not shown). The F5M-labelled cells were subjected to  
202 flow cytometric analysis (*Guava easyCyte, Millipore UK Ltd, Hertfordshire, UK*). F5M MFI  
203 was monitored by the  $\lambda 488$  nm laser and  $\lambda 525/30\text{nm}$  detector (FL1 channel). Cell viability  
204 was evaluated using trypan blue.

205

#### 206 Blood cell isolation and sample preparation

207 Whole blood from each time point was used to isolate peripheral blood monocular  
208 cells (PBMCs) using density gradient centrifugation. Blood was diluted 1:1 with Hanks  
209 Balance Salt Solution (HBSS), and then layered carefully on top of Ficoll paque PLUS (*GE*  
210 *Healthcare, Buckinghamshire, UK*), before centrifuging at 400g for 40 minutes at 21°C. The  
211 PBMC layer was aspirated and then washed three times with HBSS, by centrifuging steps at  
212 300g for 10 minutes.

213 Approximately one million cells per time point were used for flow cytometry analysis  
214 of intracellular reduced thiol content using F5M. As part of the immunophenotypic  
215 characterisation analysis,  $4 \times 10^6$  /ml cells from each time point were incubated with 50  $\mu\text{M}$   
216 N-ethylmaleimide (NEM, oxidised glutathione, GSSG), and an additional  $4 \times 10^6$  /ml cells  
217 with PBS (total glutathione) for 20 minutes at 4°C to determine reduced: oxidised glutathione  
218 ratios. Cells were washed twice with FACS buffer (PBS supplemented with 2% (v/v) foetal  
219 calf serum, 0.02% sodium azide (v/v) and 5 mM EDTA) and then stored at -80 °C.

220

#### 221 Flow cytometry and confocal microscopy analysis

222 Approximately 200,000 viable PBMCs were used for identification of reduced  
223 cellular thiols in specific lymphocyte populations using four-colour flow cytometry (*Guava*

224 *easyCyte*, Millipore UK Ltd, Hertfordshire, UK). Cells were incubated with fluorescently  
225 conjugated antibodies CD3-PE (clone: HIT3b), CD3-APC (clone: HIT3a) CD4-APC (clone:  
226 RPA-T4), CD8-APC (clone: HIT8a), CD8-PE (clone: SK1), CD279-PerCP (clone:  
227 EH12.2H7), CD197- PerCP (clone: G043H7) and CD28-APC (clone: CD28.2) (*Biologend*,  
228 *Cambridge, UK*) for 30 minutes at 4°C followed by intermittent washes with PBS for 5  
229 minutes at 300 x g. Cells were then incubated for 20 minutes with F5M in the dark (0.1 µM)  
230 at 4°C, followed by two washes with FACS buffer. NEM (20 µM) was used as an  
231 experimental control to confirm thiol-binding specificity. Confocal microscopy analysis was  
232 performed to confirm that F5M had acquiesced within viable T-cells. Cells treated with F5M  
233 (0.1 µM) were stained with CD3-APC (as above). Fluorescent cells were visualised using the  
234 resonant scanning head of a TCS SP2 confocal laser microscope (*Leica, Buckinghamshire*,  
235 *UK*), under a x 63 oil immersion objective NA 1.32 (HCX-PL-APO). Isotype-matched  
236 control and NEM treated cells were used to determine the fluorescence threshold for the  
237 helium-neon (CD3-APC) and argon (F5M) laser respectively.

238

#### 239 Glutathione ratio assay

240 Reduced: oxidised ratios of GSH:GSSG in PBMCs were quantified using a  
241 commercially available kit (*Sigma Aldrich, CS0260, Dorset, UK*), following minor  
242 modifications. Samples were prepared as described above (n=6). On the day of analysis,  
243 NEM (GSSG) and PBS-stained pellets (total glutathione) were thawed on ice and then 50 µL  
244 of Sulfosalicylic acid (5%) added to each pellet. Cells were rapidly freeze-thawed in liquid  
245 nitrogen and a 37°C water bath twice respectively, followed by centrifugation at 10,000 g for  
246 10 minutes. The supernatant was extracted and then loaded (10 µL) onto a 96-well microtiter  
247 plate with known reduced glutathione (GSH) standards (0-50 µM) in duplicate. A working  
248 solution (150 µL) of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 1.5 mg/ml) and glutathione

249 reductase (6 units/ ml) was added to each well and left for 5 minutes at room temperature.  
250 Nicotinamide adenine dinucleotide phosphate (NADPH, 50  $\mu$ L) was then added to each well,  
251 and the rate of DTNB reduction to 2-nitro-5-thiobenzoate (TNB) monitored by recording the  
252 change in absorbance every minute for 5 minutes at  $\lambda$ 412. Nanomoles of GSH in each sample  
253 were determined for both NEM and PBS pre-incubated samples to determine GSSG and total  
254 glutathione concentrations respectively. GSH concentration was determined by subtracting  
255 GSSG from total glutathione concentration, and a subsequent ratio of GSH: GSSG used to  
256 determine half-cell reduction potential (Ehc/ mV) using the Nernst equation (36).

257

## 258 **Data analysis**

259 Flow cytometry data were analysed using GuavaSoft 3.1 (*Millipore UK Ltd,*  
260 *Hertfordshire, UK*). Background fluorescence was established by isotope-matched controls.  
261 Lymphocytes were identified based on forward versus side scatter and T-cells determined as  
262 being either CD3<sup>+</sup> CD4<sup>+</sup> (T-helper) or CD3<sup>+</sup> CD8<sup>+</sup> (T-cytotoxic). The CD4:CD8 ratio was  
263 determined by determining the percentage of cells within the CD4<sup>+</sup> and CD8<sup>+</sup> regions. Within  
264 each T-cell region, a distinct population of cells with high F5M fluorescence were determined  
265 (*CD4<sup>+</sup>T-Reduced<sup>+</sup>* and *CD8<sup>+</sup>T-Reduced<sup>+</sup>*). Changes in these populations were adjusted for  
266 total lymphocyte count and events within the CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> gates and thus  
267 expressed as cells/ $\mu$ L. For each lymphocyte population, F5M mean fluorescence intensity  
268 (MFI) was normalised relative to pre-exercise (i.e. pre = 1), indicative of basal redox state.  
269 For the immunophenotypic characterisation analysis, lymphocytes were gated on forward  
270 versus side scatter and CD3<sup>+</sup> CD8<sup>+</sup> cells identified. *CD8<sup>+</sup>T-Reduced<sup>+</sup>* cells were  
271 distinguished from *CD8<sup>+</sup>T-Reduced* cells, based on F5M binding and MFI changes in  
272 programmed cell death protein-1 (PD-1), CD28 and chemokine receptor-7 (CCR7)  
273 determined.

274

## 275 **Statistical analysis**

276 The Kolmogorov Smirnov test was used to test for normality in scale data at all time  
277 points and variables were accordingly log transformed if necessary. Differences between  
278 participant characteristics and the physiological responses to exercise were assessed using  
279 unpaired samples T-tests. The influence of training status (Trained and Recreationally active)  
280 on CD4:CD8 ratio and F5M signal were assessed over time (Pre, During, Post+0 and  
281 Post+15) by a 2\*4 repeated-measures analysis of variance (ANOVA) for each of the different  
282 T-cell pools (Lymphocytes, CD3<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>T-Reduced<sup>+</sup>,  
283 CD3<sup>+</sup>CD4<sup>+</sup>T-Reduced<sup>-</sup>, CD3<sup>+</sup>CD8<sup>+</sup>T-Reduced<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup>T-Reduced<sup>-</sup>). Post hoc  
284 analysis of any interaction effects (Group\*Time) was performed by a test of simple effects by  
285 pairwise comparisons, with Bonferroni correction. GSH:GSSG ratios and MFI changes in  
286 CCR7 and CD28 over time (Pre and Post) were assessed by paired samples T-tests. MFI  
287 changes for PD-1 over time were assessed by the Wilcoxon Signed Rank Test.

288 All values are presented as means  $\pm$  standard deviation or error (indicated throughout  
289 manuscript). Data in figures are not reported as log transformed values. Statistical  
290 significance was accepted at the  $p < 0.05$  level. Statistical analyses were performed using  
291 SPSS (*PASW Statistics, release 23.0, SPSS Inc., Chicago, IL, USA*).

292

## 293 **Results**

### 294 *Flow cytometric assay optimization*

295 Jurkat cells exposed to 0.1 mU/ml glucose oxidase showed a F5M MFI increase from  
296  $1059 \pm 203$  (n=3) to  $1569 \pm 380$  (n=3) after 1 hour (T1) compared with the start (T0). After 4  
297 hours of incubation (T4) the F5M MFI decreased to  $788 \pm 211$  (n=3) (Figure 1A). Normalised  
298 values ( $T_n/T_0$ ) illustrate an increase in F5M at T1 compared to T0 and a decrease at T4

299 (Figure 1B). Since F5M reacts specifically with reduced thiols, these data indicate *reductive*  
300 stress at T1 and oxidative stress at T4 (Figure 1B). Treatment with glucose oxidase had no  
301 overall impact on cell viability (data not shown).

302

### 303 *Physiological responses to the exercise test*

304 To assess the physiological responses of TR and RA groups to exercise,  $\dot{V}O_{2\max}$ ,  
305 power output, heart rate and test duration were measured and reported in Table 1. All  
306 participants achieved  $\dot{V}O_{2\max}$  with regards to our criteria. TR had a significantly greater  $\dot{V}O_2$   
307  $\max$  ( $p=0.001$ ) and maximal power output relative to body mass ( $p=0.003$ ) than RA. No  
308 significant differences in test duration, timing of the blood sample during exercise or peak  
309 heart rate were noted between TR and RA. There were no statistically significant differences  
310 in the physiological responses between the two RA cohorts when characterising lymphocyte  
311 thiols ( $n=20$ ) and subsequent immunophenotyping ( $n=6$ ).

312

### 313 *Analysis of the lymphocyte composition of peripheral blood in response to exercise*

314 Changes in the ratios of  $CD4^+$  to  $CD8^+$  T-cells are reported in Figure 2. A significant  
315 reduction in CD4:CD8 ratio was observed during and immediately post-exercise only in RA  
316 (Figure 2, Group\*Time effect:  $F_{3,54} = 3.77$ ,  $p=0.016$ ). The ratio decreased at all time points  
317 relative to Pre ( $p<0.0001$ ), and was lower at Post+0 relative to During ( $p<0.0001$ ) in RA. In  
318 TR, the ratio was significantly lower only at Post+0 relative to Pre ( $p=0.004$ ). The ratio  
319 increased Post+15, relative to Post+0 (TR  $p=0.045$ ; RA  $p=0.041$ ) in all participants, but was  
320 only significantly lower than Pre in RA ( $p<0.0001$ ). At rest, CD4:CD8 ratio was higher in  
321 RA, relative to TR ( $p=0.016$ ).

322

323 *Analysis of intracellular reduced thiol content in lymphocytes by immunophenotype*

324           Following this, we applied our flow cytometry method to determined intracellular  
325 *reduced* cysteine thiol fluorescence in viable human T-populations (Figure 3) in response to  
326 exercise (Figures 4 and 5). Using a hierarchical gating strategy, it was first determined that  
327 exercise caused an increase in the normalised F5M ratio at Post+0 in gated lymphocyte (time  
328 effect:  $F_{3,54} = 4.2$ ,  $p=0.010$ ) and  $CD3^+$  pools (time effect:  $F_{3,54} = 4.1$ ,  $p=0.011$ ) in all  
329 participants. These changes were driven by  $CD8^+$  (time effect:  $F_{3,54} = 3.5$ ,  $p=0.029$  – Figure  
330 5A) and not  $CD4^+$  T-cells (time effect:  $F_{3,54} = 2.0$ ,  $p=0.132$  – Figure 4A). There were no  
331 differences in F5M normalised ratios between RA and TR groups in either T-cell population.

332           Alterations in the  $CD4^+T-Reduced^+$  and  $CD8^+T-Reduced^+$  concentration are reported  
333 in Figures 4C and 5C. A significant increase in the concentration of  $CD8^+T-Reduced^+$  cells at  
334 Post+0, relative to Pre ( $p=0.003$ ) and During ( $p=0.009$ ) were noted in recreationally active  
335 participants only (time\*group interaction effect:  $F_{3,54} = 3.821$ ,  $p=0.015$ ). At Post+15, the  
336 concentration of  $CD8^+T-Reduced^+$  cells was not greater than Pre ( $p=0.216$ ). No differences  
337 were noted in the concentration of  $CD4^+T-Reduced^+$  cells in either group.

338

339 *Characterization of  $CD8^+T-Reduced^+$*

340           To further understand these observations, immunophenotypic characterization of the  
341  $CD8^+T-Reduced^+$  cells was carried out in a different sample of 6 individuals. Markers of T-  
342 cell activation (CD28), suppression (PD-1) and lymphoid homing (CCR7) were stratified  
343 according to the F5M signal before and after exercise. MFI changes in CD28, PD-1 and  
344 CCR7 are reported in Figure 6, with the exercise test data for this sample reported in Table 2.  
345 A significant decrease in CCR7 MFI was observed immediately after exercise in  $CD8^+T-$

346 *Reduced* ( $CD8^+T\text{-Reduced}^+$ :  $p=0.173$ ;  $CD8^+T\text{-Reduced}$ :  $p=0.027$ ). However,  $CD8^+T\text{-}$   
347 *Reduced*<sup>+</sup> exhibited a significantly lower CCR7 MFI relative to  $CD8^+T\text{-Reduced}$  after  
348 exercise ( $p=0.042$ ). PD-1 expression in gated  $CD8^+$  cells was unchanged in response to  
349 exercise ( $CD8^+T\text{-Reduced}^+$ :  $p=0.655$ ;  $CD8^+T\text{-Reduced}$ :  $p=0.273$ ). PD-1 expression was  
350 lower in  $CD8^+T\text{-Reduced}^+$ , compared to  $CD8^+T\text{-Reduced}$  at both time points but did not  
351 reach statistical significance (Pre-Exercise:  $p=0.068$ ; Post-Exercise:  $p=0.109$ ). No differences  
352 were noted in CD28 expression between the two populations.

353

#### 354 *Glutathione analysis*

355 To identify a redox couple driving the observed *reductive* shift in cellular redox state,  
356 changes in the most abundant cellular redox couple, GSH:GSSG, was first quantified. The  
357 concentrations of GSH and GSSG were used to calculate an Ehc/ mV for the pool of isolated  
358 PBMCs using Nernst equations (36). No difference in PBMC Ehc/ mV was observed in  
359 response to exercise (pre:  $-209 \pm 20$  to post:  $-206 \pm 16$ . Ehc/ mV).

360

#### 361 **Discussion**

362 The present study represents the first to investigate changes in lymphocyte solvent  
363 accessible reduced thiols in response to exhaustive exercise in healthy young males. Using  
364 the thiol-specific and fluorescent probe, F5M, we identified that the lymphocyte pool  
365 becomes transiently *reductive* in response to exercise. Within the lymphocyte pool,  $CD8^+$  T-  
366 cells demonstrated a greater F5M fluorescence than  $CD4^+$  T-cells immediately after exercise  
367 (Figures 4 and 5). At this time point, a population of  $CD8^+$  T-cells was identified with high  
368 F5M binding capacity ( $CD8^+T\text{-reduced}^+$ ), and it was demonstrated that the concentration of  
369 these cells was greater in recreationally active than aerobically trained participants. We



370 carried out further analysis to characterise immunophenotype specific exercise-induced  
371 changes in  $CD8^+T\text{-reduced}^+$  in a separate group of recreationally active individuals.  
372 Collectively, the present data suggests that immediately after a single bout of exercise,  
373 peripheral blood is composed of more  $CD8^+T\text{-reduced}^+$  cells that have a lower expression of  
374 CCR7, relative to  $CD8^+T\text{-reduced}^-$ .

375 It is well documented that exercise of sufficient intensity and duration can induce a  
376 transient increase in markers of *oxidative stress* (42, 43). As a result, multiple studies have  
377 reported elevated indices of oxidative stress following exercise in skeletal muscle (34, 46),  
378 blood cells (erythrocytes (3) and leukocytes(43, 48)) and extracellular fluids (plasma (49),  
379 saliva (6) and urine (37, 40)). The results of our study using an optimised flow cytometric  
380 approach provide evidence that the immune cell compartment of peripheral blood undergoes  
381 transient *reductive stress* after exercise. Of the 20 participants studied, 17 demonstrated a  
382 reductive shift in  $CD8^+$  cell thiol redox state either immediately or 15 minutes after exercise  
383 (Figure 5). To our knowledge, reports of cellular *reductive stress* in humans have only been  
384 observed in chronic neurological (1, 20) and cardiovascular disease (25). Margaritelis et al  
385 (22) made the observation that many studies reporting mean trends in a specific extracellular  
386 marker of oxidative stress dismissed potential ‘reductive’ outliers as part of the dataset. In  
387 support of this, the present data supports the notion of acute intracellular *reductive stress* after  
388 exercise.

389 Determining the role of protein thiols as regulators of exercise modified redox balance is  
390 an evolving area of investigation (5). *Reductive stress* implies increased electron availability  
391 within the cell or a subcellular compartment whereby an abundant redox couple accepts more  
392 electrons. Although less abundant, signaling protein thiols may also become reduced in this  
393 context, altering key signaling pathways after exercise (47). Indeed, previous evidence has  
394 shown that the increased availability of low molecular weight *reduced* protein thiols such as

395 Glutathione (GSH) within T-cells facilitates their proliferation and secretion of Interleukin-2  
396 (13). We quantified GSH:GSSG ratio to estimate changes in global thiol redox balance within  
397 PBMC lysates in response to exercise. Using Nernst equations based on Schafer et al (36), no  
398 changes were observed in PBMC mV after exercise (pre:  $-209 \pm 20$  to post:  $-206 \pm 16$ . Ehc/  
399 mV). We performed further analysis as part of a proof of concept approach to label CD8<sup>+</sup> T-  
400 cells with F5M and assess exercise-induced changes in solvent accessible cysteine thiol redox  
401 state, using mass spectrometry (see supplementary information). These preliminary data  
402 indicate that specific proteins can be identified (e.g. including transcription factors), and  
403 alterations in thiol redox state interpreted after exercise. Further analysis is required to  
404 identify the redox couples involved in the *reductive* shift in CD8<sup>+</sup> T-cells. It must be  
405 emphasized that the results of the current study do not definitively pinpoint whether increased  
406 CD8<sup>+</sup>T-*reduced*<sup>+</sup> cells are a result of cysteine thiol mobilisation or shifts in CD8<sup>+</sup> cell  
407 immunophenotype. The former would imply higher intracellular reductive capacity that may  
408 result from an altered flux of glucose through the pentose phosphate pathway during exercise  
409 (41), thus increasing the cellular pool of NADPH, a cofactor for thiol reducing enzymes such  
410 as thioredoxin reductase (15). With regards to the immunophenotype, there is evidence to  
411 suggest that late differentiated CD8<sup>+</sup> T-cells have higher basal thiol concentration than naive  
412 CD8<sup>+</sup> T-cells (45). Late differentiated CD8<sup>+</sup> T-cells have high effector function and are  
413 known to be highly responsive to exercise-induced mobilization (4). This would suggest that  
414 an increase in CD8<sup>+</sup>T-*reduced*<sup>+</sup> cells could relate to an altered composition of CD8<sup>+</sup> T-cells,  
415 rather than altered redox balance intracellularly. Only extensive immunophenotypic and  
416 functional analysis of CD8<sup>+</sup> cells over a suitable time course would be able to determine the  
417 origin of the observed *reductive stress* in the immune cell compartment of peripheral blood.

418 The current data supports previous work showing an elevation in CD8<sup>+</sup> T-cells in  
419 peripheral blood during and in the minutes following exercise (4), with the relative increase

420 in CD8<sup>+</sup> cells exaggerated in individuals with lower training status (Figure 2). The CD4:CD8  
421 ratio decreased During (70% HR<sub>max</sub>) and Post+0 in RA, but only Post+0 in TR. This indicates  
422 that RA participants were more sensitive to CD8<sup>+</sup> cell mobilization. In conjunction with this  
423 data, a novel finding herein was the identification of a population of CD8<sup>+</sup> T-cells with a high  
424 capacity to bind F5M immediately post-exercise (CD8<sup>+</sup>T-reduced<sup>+</sup>, Figure 5B). The  
425 concentration of CD8<sup>+</sup>T-reduced<sup>+</sup> cells present Post+0 increased from pre-exercise values in  
426 RA, with no change noted in TR individuals (Figure 5C). As a proportion of the CD8<sup>+</sup> pool,  
427 CD8<sup>+</sup>T-reduced<sup>+</sup> cells increased from 16 to 42% post-exercise in RA, with a 4% decrease  
428 noted in TR (data not shown). No differences were noted in CD4<sup>+</sup>T-reduced<sup>+</sup> concentration  
429 or proportional composition between groups or in response to exercise (Figure 4C),  
430 suggesting that the *reductive* shift observed in CD8<sup>+</sup> thiol redox state may relate to the  
431 mobilization patterns of immune cells in response to exercise.

432 To our knowledge, transient differences in intracellular redox state following exercise,  
433 based on training status have not been previously reported. However, there is evidence to  
434 suggest that physical training can upregulate the expression of endogenous antioxidant  
435 enzymes in both skeletal muscle (12, 18) and immune cells (42, 43). We can therefore  
436 hypothesize that enhanced antioxidant protection at rest may have therefore provided  
437 improved tolerance to exercise-induced ROS in the TR group and reduced the need for these  
438 individuals to mobilise CD8<sup>+</sup>T-reduced<sup>+</sup> cells immediately after exercise. It must be noted  
439 that there were no statistical differences in total MFI changes in F5M between groups at  
440 Post+0, indicating that CD8<sup>+</sup> cells in TR were also transiently *reductive*, however these  
441 individuals did not exhibit exercise-induced changes in CD8<sup>+</sup>T-reduced<sup>+</sup> cell concentration.  
442 With regards to CD8<sup>+</sup>T-reduced<sup>+</sup> cells, no correlation was noted between the concentration of  
443 CD8<sup>+</sup>T-reduced<sup>+</sup> and CD8<sup>+</sup> cells present in the circulation at Post+0 in RA (*data not shown*),  
444 suggesting no direct relationship between CD8<sup>+</sup> mobilization and thiol redox state; however,

445 as mentioned above, this may be due to the precise immunophenotype of the CD8<sup>+</sup> cells not  
446 being fully characterized. Nevertheless, our findings strengthen the growing body of evidence  
447 that suggests that *reduced* protein thiols may play an important role in the mobilization of  
448 specific subsets of immune cells after exercise, with training status of the individual being a  
449 factor worthy of further investigation.

450 Based on the above findings, an additional six recreationally active participants were  
451 recruited in order to characterize the immunophenotype of CD8<sup>+</sup>*T-reduced*<sup>+</sup> cells in response  
452 to the same exercise protocol. We coupled F5M and antibody staining to compare alterations  
453 in thiol redox state with changes in cell markers of T-cell: activation (CD28), suppression  
454 (PD-1) and lymphoid homing (CCR7) before and immediately after exercise (Figure 6: B-D).  
455 CCR7 expression facilitates the migration of T-cells to secondary lymphoid organs i.e. lymph  
456 nodes, where interaction with other immune cells takes place. This process plays a central  
457 role in immune cell activation and clonal proliferation of antigen specific T-cells (9). A  
458 significant decrease in CCR7 expression was noted in CD8<sup>+</sup>*T-reduced* cells after exercise  
459 only (Figure 6C). Interestingly, CD8<sup>+</sup>*T-reduced*<sup>+</sup> cells expressed lower levels of CCR7 than  
460 CD8<sup>+</sup>*T-reduced* cells post exercise. CCR7 is a lymphoid homing receptor expressed on naïve  
461 T-cells located in the primary lymphoid organs (i.e. thymus and bone marrow). Lower  
462 expression of CCR7 is known to mediate trafficking of effector T-cells towards secondary  
463 lymphoid organs (32). Therefore, given that CD8<sup>+</sup>*T-reduced*<sup>+</sup> have less lymphoid homing  
464 capacity, but may be mobilised to a greater extent during exercise, these findings propose a  
465 role for redox-mediated driven immune cell mobilization and possibly, extravasation. No  
466 changes in CD28 expression were noted, suggesting that acute cellular *reduction* has little  
467 impact on co-stimulatory marker expression (Figure 6D). A trend for lower expression levels  
468 of the immunosuppressive cell-surface receptor, PD-1 was observed in CD8<sup>+</sup>*T-reduced*<sup>+</sup>  
469 compared to CD8<sup>+</sup>*T-reduced* cells (p=0.068, Figure 6B). PD-1 is a cell surface receptor

470 expressed on activated T-cells that downregulates antigen-mediated T-cell activation.  
471 Interestingly, elevated PD-1 expression has been observed in many cancers (17, 21, 27), with  
472 subsequent T-cell exhaustion promoting poor anti-tumor responses.

473 We have validated a flow cytometry method *in vitro* (Figure 1), indicating that a standard  
474 physiological dose of enzyme-mediated H<sub>2</sub>O<sub>2</sub> can elicit *oxidative* and *reductive* stress in  
475 Jurkat cells. This method has high utility for determining the behavior patterns of specific  
476 immune cell populations in response to exercise in humans. Further research is needed to  
477 expand on our findings regarding CCR7 and PD-1; to more accurately characterise the  
478 relationship between T-cell function, thiol redox state and the physiological relevance to  
479 exercise and immune function. Given that thiol labeling is achieved prior to cell lysis,  
480 advanced proteomic analysis can be used to determine modifications to solvent accessible  
481 thiol proteins that may alter cellular function (see supplementary information).

482 We must acknowledge some potential limitations to the present study. The application of  
483 F5M in highly diverse biological samples such as human PBMCS is subject to some subtle  
484 differences in membrane permeability, cross-reactivity with other nucleophiles (e.g. with  
485 primary and secondary amines) and probe hydrolysis, the latter two of which are pH  
486 dependent (pH>7.5). Decreases in lymphocyte pH (7.4) following a  $\dot{V}O_{2\max}$  test would likely  
487 be minimal (10, 26), however, whereby our method is applied to more prolonged exercise  
488 models, subtle changes in pH should be accounted for. In line with this comment, it must also  
489 be noted that our results only reflect immune thiol redox state changes in response to a  
490  $\dot{V}O_{2\max}$  test, and not bouts of exercise that are more prolonged and sustained at moderate or  
491 high intensity. Future work is needed to clarify our current findings in response to more  
492 conventional types of exercise.

493

494 **Conclusion**

495 The present study provides evidence that the peripheral blood lymphocyte pool  
496 becomes transiently more *reductive* in response to acute exhaustive exercise in healthy males.  
497 Using the fluorescent probe, F5M we have identified a specific population of CD8<sup>+</sup> T-cells  
498 (CD8<sup>+</sup>*T-reduced*<sup>+</sup>) that drives this reductive shift, with training status appearing to be a key  
499 variable differentiating individuals. We provide evidence to suggest that CD8<sup>+</sup>*T-reduced*<sup>+</sup>  
500 may target secondary lymphoid organs post exercise and recommend that further research is  
501 undertaken to validate these observations, and characterise the significance of *oxidative* and  
502 *reductive* shifts in immune cell redox state post exercise.

503  
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514

515 **Conflict of Interest**

516 None of the authors declare a conflict of interest.

517

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## Tables

**Table 1: Exercise physiology data (n=20):** test duration, relative peak power output (Watt/kg), peak heart rate (bpm) and relative maximal oxygen consumption achieved during the exercise test. All values are means (standard deviation).

	<b>Trained (n=9)</b>	<b>Recreationally Active (n=11)</b>
<b>Test Duration</b> (min:sec)	27:31 (07:03)	22:44 (06:04)
<b>Timing of During Exercise Sample</b> (min:sec)	14:03 (03:35)	12:27 (04:06)
<b>Relative Watt Max</b> (Watt·kg <sup>-1</sup> )	4.24 (0.48) <sup>\$\$</sup>	3.45 (0.53)
<b>Peak Heart Rate</b> (beats·min <sup>-1</sup> )	185.89 (11.76)	189.36 (5.85)
<b>Relative VO<sub>2</sub>max</b> (ml·kg <sup>-1</sup> ·min <sup>-1</sup> )	56.93 (6.09) <sup>\$</sup>	45.10 (6.44)

*Table 1 Legend:* <sup>\$</sup> indicates a significant difference between trained and recreationally active groups (Group effect): <sup>\$</sup> p<0.05; <sup>\$\$</sup> p<0.001.

**Table 2: Participant demographics and physiological data from participants taking part in the immunophenotyping analysis arm of the study (n=6):** Values are means (standard deviation).

	n=6
<b>Test Duration</b> (min:sec)	18:07 (03:02)
<b>Relative Watt Max</b> (Watt·kg <sup>-1</sup> )	2.98 (0.60)
<b>Peak Heart Rate</b> (beats·min <sup>-1</sup> )	180.5 (9.67)



**Relative VO<sub>2max</sub>**  
(ml·kg<sup>-1</sup>·min<sup>-1</sup>)                      41.33 (7.63)

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**Fig 1: Optimisation of the F5M flow cytometric assay using Jurkat cells (n=3).** (A) Representative flow cytometric plot illustrating Jurkat cells labelled with F5M following glucose oxidase treatment. T0 = start, T1 = 1 hour incubation with glucose oxidase and T4 = 4 hours incubation with glucose oxidase. Intracellular thiol redox status was measured by F5M binding and a change in FL1-H ( $\lambda$ 488nm;  $\lambda$ 525/30nm) intensity. A cell sample unstained with F5M is also represented. (B) Summarised data illustrating normalised F5M signal ( $T_n/T_0$ ). Indicated is ‘reduction’ i.e. more free cys-thiol relative to T0 and ‘oxidation’ i.e. less free cys-thiol relative to T0.

**Fig 2: Changes in T-cell populations in response to acute exercise (n=20).** Bars represent CD4:CD8 ratio changes in RA (grey bars) and TR (white bars) Pre, During, Post+0 and Post+15. Values are means  $\pm$  standard deviation. \* indicates a significant difference relative to Pre: \* p<0.05, \*\*\* p<0.0001. + indicates a significant difference relative to During: +++ p<0.0001. # indicates a significant difference relative to Post: # p<0.05. \$ indicates a significant difference between RA and TR (all Time\*Group effects; pairwise comparisons).

**Fig 3 Immunophenotypic assessment of reduced intracellular thiol content in viable T-cell populations (n=20).** (A) Representative gating strategy for CD8<sup>+</sup> cells. Lymphocytes were gated (left panel) on forward light scatter (FSC) vs. side light scatter (SSC) and then stained for CD3<sup>+</sup> (PE) and CD8<sup>+</sup> (APC, right panel). (B) Intracellular reduced thiols were measured by a gain of F5M binding and increase in FL1-H ( $\lambda$ 488nm;  $\lambda$ 525/30nm) intensity. Unstained and an experimental control (NEM) were used to confirm thiol binding specificity

(C) A representative PBMC confocal image illustrating intracellular F5M signal (left image) and surface staining for T-cells with anti-human CD3-APC (right image).

**Fig 4: Flow cytometric analysis of intracellular thiol redox status in CD4<sup>+</sup> cells in response to acute exercise (n=20).** (A) Bars represent normalised MFI on FL1-H ( $\lambda$ 488nm;  $\lambda$ 525/30nm) in gated CD4<sup>+</sup> cells for RA (grey bars) and TR (white bars) participants. Data are normalised relative to pre-exercise fluorescence (B) Panel shows a representative overlaid FL1 histogram of CD4<sup>+</sup> cells Pre (grey fill) and Post+0 (white fill) treated with 0.1  $\mu$ M F5M. CD4<sup>+</sup>T-Reduced<sup>+</sup> was identified based on a highly reduced population of cells on the far right of the histogram (C) Bars represent changes in the concentration of CD4<sup>+</sup>T-Reduced<sup>+</sup> cells per  $\mu$ L in RA (grey bars) and TR (white bars) participants in response to acute exercise. All values are means  $\pm$  standard error.

**Fig 5: Flow cytometric analysis of intracellular thiol redox status in CD8<sup>+</sup> cells in response to acute exercise (n=20).** (A) Bars represent normalised MFI on FL1-H ( $\lambda$ 488nm;  $\lambda$ 525/30nm) in gated CD8<sup>+</sup> cells for RA (grey bars) and TR (white bars) participants. Data are normalised relative to pre-exercise fluorescence (B) Panel shows a representative overlaid FL1 histogram of CD8<sup>+</sup> cells Pre (grey fill) and Post+0 (white fill) treated with 0.1  $\mu$ M F5M. CD8<sup>+</sup>T-Reduced<sup>+</sup> was identified based on a highly reduced population of cells on the far right of the histogram (C) Bars represent changes in the concentration of CD8<sup>+</sup>T-Reduced<sup>+</sup> cells per  $\mu$ L in RA (grey bars) and TR (white bars) participants in response to acute exercise. All values are means  $\pm$  standard error. \* indicates a significant difference relative to Pre: \*\* p<0.001. + indicates a significant difference relative to During: ++ p<0.001 (all Time\*Group effect; pairwise comparisons).

**Fig 6: Immunophenotypic analysis of  $CD8^+T$ -Reduced<sup>+</sup> activation, suppression and lymphoid homing following exhaustive exercise (n=6).** (A) Chart illustrates changes in MFI for F5M in response to exercise (B-D) Bars represent MFI for PD-1 (B), CCR7 (C) and CD28 in  $CD8^+T$ -Reduced<sup>+</sup> (white bars) and  $CD8^+T$ -Reduced<sup>-</sup> (black bars) before and after exercise. Values are means  $\pm$  standard deviation. \* indicates a significant difference relative to Pre (Time\*Group effect; pairwise comparison): \* p<0.05. # indicates a significant difference between  $CD8^+T$ -Reduced<sup>+</sup> and  $CD8^+T$ -Reduced<sup>-</sup> (Time\*Group effect; pairwise comparison): # p<0.05.

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