

Preliminary evidence of reductive stress in human cytotoxic T-cells 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 ± 5 years; BMI 23.2 ± 2.6 kg·m²; $\dot{V}O_{2max}$ 56.9 ± 6.1 ml·kg⁻¹·min⁻¹) and
33 recreationally active (RA, n=11, mean \pm SD: age 27 ± 6 years; BMI 24.2 ± 3.7 kg·m²; $\dot{V}O_{2max}$
34 45.1 ± 6.4 ml·kg⁻¹·min⁻¹) participants before and after a maximal aerobic exercise tolerance
35 test. Blood samples were taken before (PRE), during (sample acquired at 70% HR_{max}),
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⁺, CD4⁺
38 and CD8⁺) was performed using flow cytometry. A significant increase in cellular F5M
39 fluorescence was observed in CD3⁺ T-cells at POST+0, with changes driven to a greater
40 extent by CD8⁺ 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⁺ T-cells (*CD8⁺T-reduced⁺*) that
42 significantly increased from PRE to POST+0 in RA participants only (RA: +272 cell/ μ L,
43 p<0.05). To further understand these results, *CD8⁺T-reduced⁺* and *CD8⁺T-reduced⁻* cells were
44 analysed for immunophenotype in response to the same exercise protocol (n=6, mean \pm SD:
45 age 24 ± 5 years; BMI 25.7 ± 4.1 kg·m²; $\dot{V}O_{2max}$ 41.33 ± 7.63 ml·kg⁻¹·min⁻¹). *CD8⁺T-*
46 *reduced⁺* 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_{max}: Maximum heart rate, GSH: Glutathione, GSSG:
63 Glutathione disulphide, GTP: Guanidine triphosphate, HBSS: Hanks Balance Salt Solution,
64 H₂O₂: 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, VCO₂: 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⁺ T-cells and Natural Killer cells
78 observed at the expense of CD4⁺ 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 (H₂O₂) 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 H₂O₂ 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⁺ and CD8⁺ 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 ± 5 years; BMI 23.2 ± 2.6 kg.m²; $\dot{V}O_{2max}$ 56.9 ± 6.1
138 ml.kg⁻¹.min⁻¹) 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⁻¹.
141 min⁻¹(2). Recreationally active individuals (RA, n=11, mean \pm SD: age 27 ± 6 years; BMI
142 24.2 ± 3.7 kg.m²; $\dot{V}O_{2max}$ 45.1 ± 6.4 ml.kg⁻¹.min⁻¹) 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 ± 6394 ; RA $4932 \pm$
145 2555) and vigorous physical activity (VPA, mean \pm SD: TR 800 ± 1008 ; RA 264 ± 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 ± 5 years; BMI $25.7 \pm$
148 4.1 kg.m²; $\dot{V}O_{2max}$ 41.33 ± 7.63 ml.kg⁻¹.min⁻¹) were recruited to characterise the CD8⁺T-

149 *reduced*⁺ 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% CO₂, 3.5% O₂ and 5.0% N₂), 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⁻¹min⁻¹) 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 ≥ 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% HR_{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 2×10^6 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 μM) in the dark at 4°C, followed by two washes with FACS buffer. The concentration of
200 F5M was determined through titrations (0-10 μM) 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, 4×10^6 /ml cells from each time point were incubated with 50 μM
216 N-ethylmaleimide (NEM, oxidised glutathione, GSSG), and an additional 4×10^6 /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 μ 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 λ 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⁺ CD4⁺ (T-helper) or CD3⁺ CD8⁺ (T-cytotoxic). The CD4:CD8 ratio was
263 determined by determining the percentage of cells within the CD4⁺ and CD8⁺ regions. Within
264 each T-cell region, a distinct population of cells with high F5M fluorescence were determined
265 (*CD4⁺T-Reduced⁺* and *CD8⁺T-Reduced⁺*). Changes in these populations were adjusted for
266 total lymphocyte count and events within the CD3⁺, CD4⁺ and CD8⁺ gates and thus
267 expressed as cells/ μ 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⁺ CD8⁺ cells identified. *CD8⁺T-Reduced⁺* cells were
271 distinguished from *CD8⁺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⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, CD3⁺CD4⁺T-Reduced⁺,
283 CD3⁺CD4⁺T-Reduced⁻, CD3⁺CD8⁺T-Reduced⁺ and CD3⁺CD8⁺T-Reduced⁻). 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 ± 203 (n=3) to 1569 ± 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 ± 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*⁺ 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 ± 20 to post: -206 ± 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 ± 20 to post: -206 ± 16 . Ehc/
399 mV). We performed further analysis as part of a proof of concept approach to label CD8⁺ 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⁺ T-cells. It must be
405 emphasized that the results of the current study do not definitively pinpoint whether increased
406 CD8⁺T-*reduced*⁺ cells are a result of cysteine thiol mobilisation or shifts in CD8⁺ 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⁺ T-cells have higher basal thiol concentration than naive
412 CD8⁺ T-cells (45). Late differentiated CD8⁺ 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⁺T-*reduced*⁺ cells could relate to an altered composition of CD8⁺ T-cells,
415 rather than altered redox balance intracellularly. Only extensive immunophenotypic and
416 functional analysis of CD8⁺ 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⁺ T-cells in
419 peripheral blood during and in the minutes following exercise (4), with the relative increase

420 in CD8⁺ cells exaggerated in individuals with lower training status (Figure 2). The CD4:CD8
421 ratio decreased During (70% HR_{max}) and Post+0 in RA, but only Post+0 in TR. This indicates
422 that RA participants were more sensitive to CD8⁺ cell mobilization. In conjunction with this
423 data, a novel finding herein was the identification of a population of CD8⁺ T-cells with a high
424 capacity to bind F5M immediately post-exercise (CD8⁺T-reduced⁺, Figure 5B). The
425 concentration of CD8⁺T-reduced⁺ 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⁺ pool,
427 CD8⁺T-reduced⁺ 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⁺T-reduced⁺ concentration
429 or proportional composition between groups or in response to exercise (Figure 4C),
430 suggesting that the *reductive* shift observed in CD8⁺ 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⁺T-reduced⁺ 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⁺ cells in TR were also transiently *reductive*, however these
441 individuals did not exhibit exercise-induced changes in CD8⁺T-reduced⁺ cell concentration.
442 With regards to CD8⁺T-reduced⁺ cells, no correlation was noted between the concentration of
443 CD8⁺T-reduced⁺ and CD8⁺ cells present in the circulation at Post+0 in RA (*data not shown*),
444 suggesting no direct relationship between CD8⁺ mobilization and thiol redox state; however,

445 as mentioned above, this may be due to the precise immunophenotype of the CD8⁺ 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⁺*T-reduced*⁺ 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⁺*T-reduced* cells after exercise
459 only (Figure 6C). Interestingly, CD8⁺*T-reduced*⁺ cells expressed lower levels of CCR7 than
460 CD8⁺*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⁺*T-reduced*⁺ 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⁺*T-reduced*⁺
469 compared to CD8⁺*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 H₂O₂ 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⁺ T-cells
498 (CD8⁺*T-reduced*⁺) that drives this reductive shift, with training status appearing to be a key
499 variable differentiating individuals. We provide evidence to suggest that CD8⁺*T-reduced*⁺
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).

	Trained (n=9)	Recreationally Active (n=11)
Test Duration (min:sec)	27:31 (07:03)	22:44 (06:04)
Timing of During Exercise Sample (min:sec)	14:03 (03:35)	12:27 (04:06)
Relative Watt Max (Watt·kg ⁻¹)	4.24 (0.48) ^{\$\$}	3.45 (0.53)
Peak Heart Rate (beats·min ⁻¹)	185.89 (11.76)	189.36 (5.85)
Relative VO₂max (ml·kg ⁻¹ ·min ⁻¹)	56.93 (6.09) ^{\$}	45.10 (6.44)

Table 1 Legend: ^{\$} indicates a significant difference between trained and recreationally active groups (Group effect): ^{\$} p<0.05; ^{\$\$} 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
Test Duration (min:sec)	18:07 (03:02)
Relative Watt Max (Watt·kg ⁻¹)	2.98 (0.60)
Peak Heart Rate (beats·min ⁻¹)	180.5 (9.67)

Relative VO_{2max}
(ml·kg⁻¹·min⁻¹) 41.33 (7.63)

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 (λ 488nm; λ 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⁺ cells. Lymphocytes were gated (left panel) on forward light scatter (FSC) vs. side light scatter (SSC) and then stained for CD3⁺ (PE) and CD8⁺ (APC, right panel). (B) Intracellular reduced thiols were measured by a gain of F5M binding and increase in FL1-H (λ 488nm; λ 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⁺ cells in response to acute exercise (n=20). (A) Bars represent normalised MFI on FL1-H (λ 488nm; λ 525/30nm) in gated CD4⁺ 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⁺ cells Pre (grey fill) and Post+0 (white fill) treated with 0.1 μ M F5M. CD4⁺T-Reduced⁺ 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⁺T-Reduced⁺ cells per μ 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⁺ cells in response to acute exercise (n=20). (A) Bars represent normalised MFI on FL1-H (λ 488nm; λ 525/30nm) in gated CD8⁺ 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⁺ cells Pre (grey fill) and Post+0 (white fill) treated with 0.1 μ M F5M. CD8⁺T-Reduced⁺ 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⁺T-Reduced⁺ cells per μ 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⁺ 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⁺ (white bars) and $CD8^+T$ -Reduced⁻ (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⁺ and $CD8^+T$ -Reduced⁻ (Time*Group effect; pairwise comparison): # p<0.05.

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