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1 **High dietary protein restores overreaching induced impairments in leukocyte**
2 **trafficking and reduces the incidence of upper respiratory tract infection in elite cyclists**

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4 OLIVER C. WITARD^{1*}, JAMES E. TURNER^{2*}, SARAH R. JACKMAN³, ARIE K. KIES⁴,
5 ASKER E. JEUKENDRUP⁵, JOS A. BOSCH^{6,7} & KEVIN D. TIPTON¹

6 * These authors contributed equally.

7

8 ¹ Health and Exercise Sciences Research Group, University of Stirling, Stirling FK9 4LA.

9 ² Department for Health, University of Bath, Bath, BA2 7AY.

10 ³Sport & Health Sciences, College of Life and Environmental Sciences, St Luke's Campus,
11 University of Exeter, Exeter, EX1 2LU, Devon, UK.

12 ⁴DSM Biotechnology, Delft, The Netherlands.

13 ⁵Gatorade Sports Science Institute, Chicago, USA

14 ⁶Department of Psychology, University of Amsterdam, The Netherlands.

15 ⁷Mannheim Institute of Public Health, Social and Preventive Medicine (MIPH), Mannheim
16 Medical Faculty, University of Heidelberg, Germany.

17

†**Corresponding author:** Dr Oliver C. Witard, Health and Exercise Science Research Group,
University of Stirling, Stirling, Scotland, FK9 4LA. Tel: +44(0)1786 466 298. Fax:
+44(0)1786 464 551. E-mail: oliver.witard@stir.ac.uk

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19 conflicts of interest.

Abstract:

The present study examined whether a high protein diet prevents the attenuated leukocyte redistribution in response to acute exercise caused by a large volume of high-intensity exercise training. Eight cyclists (VO_{2max} : $64.2 \pm 6.5 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) undertook two separate weeks of high-intensity training while consuming either a high protein diet ($3 \text{ g}\cdot\text{kg}^{-1}\text{ protein}\cdot\text{BM}^{-1}\cdot\text{day}^{-1}$) or an energy and carbohydrate-matched control diet ($1.5 \text{ g}\cdot\text{kg}^{-1}\text{ protein}\cdot\text{BM}^{-1}\cdot\text{day}^{-1}$). High-intensity training weeks were preceded by a week of normal-intensity training under the control diet. Leukocyte and lymphocyte sub-population responses to acute exercise were determined at the end of each training week. Self-reported symptoms of upper-respiratory tract infections (URTI) were monitored daily by questionnaire. Undertaking high-intensity training with a high protein diet restored leukocyte kinetics to similar levels observed during normal-intensity training: $CD8^+TL$ mobilization (normal-intensity: $29,319 \pm 13,130 \text{ cells}/\mu\text{L} \times \sim 165 \text{ min}$ vs. high-intensity with protein: $26,031 \pm 17,474 \text{ cells}/\mu\text{L} \times \sim 165 \text{ min}$, $P > 0.05$), $CD8^+TL$ egress (normal-intensity: $624 \pm 264 \text{ cells}/\mu\text{L}$ vs. high-intensity with protein: $597 \pm 478 \text{ cells}/\mu\text{L}$, $P > 0.05$). This pattern was driven by effector-memory populations mobilizing (normal-intensity: $6,145 \pm 6,227 \text{ cells}/\mu\text{L} \times \sim 165 \text{ min}$ vs. high-intensity with protein: $6,783 \pm 8,203 \text{ cells}/\mu\text{L} \times \sim 165 \text{ min}$, $P > 0.05$) and extravasating from blood (normal-intensity: $147 \pm 129 \text{ cells}/\mu\text{L}$ vs. high-intensity with protein: $165 \pm 192 \text{ cells}/\mu\text{L}$, $P > 0.05$). High-intensity training while consuming a high protein diet was associated with fewer symptoms of URTI compared to performing high-intensity training with a normal diet ($P < 0.05$). To conclude, a high protein diet might reduce the incidence of URTI in athletes potentially mediated by preventing training-induced impairments in immune-surveillance.

Key words: Dietary protein, CD8+ T lymphocytes, Immune-surveillance, Infection risk, Overtraining.

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Introduction

Cross-sectional and longitudinal studies show that large volumes of high-intensity exercise training (often resulting in overreaching; a short-term decrement in performance capacity that may take days or weeks to restore) increases the risk of upper respiratory tract infection (URTI) (Gleeson, 2007; Walsh et al, 2011). This increased risk of URTI is thought to be explained in part, by suppression of cell-mediated immunity (Bishop and Gleeson, 2009; Foster, 1998; Gleeson, 2007; Lancaster et al, 2004; Robson et al, 1999; Walsh et al, 2011). Until recently, studies examining exercise-induced immune suppression have assessed aspects of lymphocyte function (e.g., activation, cytokine secretion, proliferation and target killing) that are important once a body cell, that has become infected by a virus for example, has been detected and located. However, the process by which lymphocytes traffic to potential sites of infection has received little attention in the context of infection risk in athletes.

During exercise, the number of lymphocytes circulating in peripheral blood increases two- or three-fold, and 1-2 hours later, falls to half of normal levels (Gleeson and Bishop, 2005; McCarthy and Dale, 1988) - a process whereby cells capable of initiating rapid effector responses, extravasate to peripheral tissue (e.g., skin, lungs, gut) where they are more likely to detect and eliminate infected cells (Dhabhar et al, 1996; Dhabhar, 2000; Kruger and Mooren, 2007). We have recently shown that the redistribution of specialized effector CD8⁺ T lymphocytes (CD8⁺ TL) that primarily target viral antigens (van Lier et al, 2003), a functionally relevant and readily observable cell-mediated immune response (Dhabhar et al, 1996), is impaired by a period of high-intensity exercise training (Witard et al, 2012). Thus, if the ability to detect virus-infected cells is compromised in athletes by the immunomodulatory effect of high-intensity training, then this process of impaired immune surveillance might explain the increased risk of URTI during demanding training regimens.

One intervention by which the immunomodulatory effects of high-intensity training might be countered is by manipulating dietary protein intake (Calder and Kew, 2002; Gleeson et al, 2004; Nieman, 2008). Amino acids are important metabolic substrates for lymphocytes (Costa et al, 2009; Costa et al, 2011; Matsumoto et al, 2009). Multiple *in vitro* studies demonstrate that lymphocyte proliferation, cytokine secretion, and cytotoxicity are in part, dependent on amino acid availability (Calder and Jackson, 2000; Calder and Yaqoob, 1999; Li et al, 2007; Waithe et al, 1975). Moreover, numerous studies have shown a mechanistic role for amino acid-induced intracellular signalling, in particular leucine, via the mammalian target of rapamycin (mTOR) signalling cascade that governs lymphocyte function (Nicklin et al, 2009; Peng et al, 2002; Sinclair et al, 2008). A few studies have already examined whether increased dietary protein prevents the immunomodulatory effects of high-intensity exercise training, but these studies have largely showed beneficial effects on cells of the innate immune system (i.e., neutrophils) (Costa et al, 2009; Costa et al, 2011; Nelson et al, 2013). Considering that exercise training increases the amino acid requirements of athletes (Friedman and Lemon, 1989), and amino acids are important signalling molecules governing lymphocyte function, then increasing dietary protein intake of athletes undertaking high-intensity training is an attractive and theoretically justified intervention that might prevent immune impairment and potentially reduce the incidence of URTI.

Given our previous report that the magnitude of exercise-induced lymphocyte redistribution is impaired during high-intensity training (Witard et al, 2012), the present study investigated whether a high protein diet reverses this observation. The results of our previous work (Witard et al, 2012) serve as the control condition (i.e., high-intensity exercise training when consuming a normal diet) in the current report. On the basis of studies examining the effects of protein or amino acids on lymphocyte function (Calder and Jackson, 2000; Calder

and Yaqoob, 1999; Daly et al, 1990; Peng et al, 2002), we hypothesized that a high protein diet would prevent the impaired exercise-induced lymphocyte redistribution that occurs with high-intensity exercise training (Witard et al, 2012), restoring responses towards normal, and subsequently result in fewer reported URTI symptoms in athletes.

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Methods

Participants

Eight well-trained healthy male cyclists (Mean \pm SD; age 27 ± 8 yr; body mass index 22.8 ± 2.2 kg·m⁻² maximal oxygen uptake ($\dot{V}O_2$ max) 64.2 ± 6.5 mL·kg⁻¹·min⁻¹) took part in this study as previously described (Witard et al, 2012). All participants provided informed written consent and the study was approved by the Research Ethics Committee of the School of Sport and Exercise Sciences, University of Birmingham, United Kingdom. Before participation, the health status of each participant was assessed using a general health questionnaire. All participants were free from infection at the beginning of the study.

Experimental design

Participants were engaged in two separate investigative weeks of high-intensity exercise training (see Figure 1). Participants received either a high protein diet (protein-diet) or an energy and carbohydrate-matched normal diet (control-diet) in a single blinded randomised crossover design. The energy and macronutrient content of the control- and protein-diet is shown in Table 1.

Each week of high-intensity exercise training was preceded by one week of normal-intensity training in which the control-diet was provided (see *Dietary Control*). The study design did not include a condition whereby participants received a high-protein diet during normal-intensity training. If a high-protein diet had been provided during one of the normal-intensity training weeks, then comparisons between each period of high-intensity training (i.e., control-diet vs. protein-diet) would be complicated by a two-week period of increased protein consumption. Thus, the purpose of the normal-intensity training weeks was three-fold: first, to control for training intensity, second to control for dietary intake, and third, to

compare immune responses observed during high-intensity training (under the control- and protein-diet) to responses during normal-intensity training. As each week of normal-intensity exercise training was similar in terms of energy expenditure, diet received, and immune cell responses to exercise, values from these two weeks were averaged. Importantly, results remained the same if data were analysed separately (data not shown).

On the final day of each exercise training week (i.e., normal- or high-intensity training under the protein- or control-diet) and at least 18 h after the last exercise session, participants undertook a standardized exercise trial (see *Exercise trials*), in which immune cell and neuroendocrine responses to exercise were quantified. To eliminate known effects of other macronutrients (e.g., carbohydrate) on aspects of immune function (Bishop et al, 2001; Scharhag et al, 2006), and in accordance with the study design of other similar investigations (Achten et al, 2004; Halson et al, 2004), exercise trials in the present study were performed in a fasted state.

Preliminary exercise testing and familiarization

Participants visited the laboratory to have maximal oxygen uptake ($\dot{V} O_{2\max}$) assessed and for a familiarisation exercise trial under fasted conditions (Witard et al, 2012). $\dot{V} O_{2\max}$ data were used to prescribe the intensity of each acute exercise trial. Training conditions (normal- and high-intensity exercise training) were prescribed using percentages of maximal heart rate (HR_{\max}) (see *Exercise training conditions*).

Exercise training conditions

During the normal-intensity training condition, participants engaged in their usual level of regular exercise (one session/day, 4-5 training days/wk). The high-intensity training condition required athletes to markedly increase training load: exercise volume, (i.e., duration

of each training session) and exercise intensity (i.e., difficulty of each training session) were increased by ~70% relative to normal-intensity training (1-2 sessions/day, 7 training days/wk). To rule out immunological differences during exercise trials as a result of possible changes in $\dot{V}O_{2\max}$ following the normal- and high-intensity training conditions, a $\dot{V}O_{2\max}$ test was conducted on day 6 of each training condition. Body mass was recorded daily to ensure energy balance was maintained throughout both normal and high-intensity training weeks.

Exercise trials

Exercise trials took place on the seventh day of each exercise training condition as previously described (Witard et al, 2012). Trials started at ~06:30 after an overnight fast. Following a 15 min rest, a baseline blood sample was collected. Each exercise trial was identical, and consisted of two stages completed in short succession. First, participants cycled for 120 min at a fixed exercise intensity equivalent to ~60% $\dot{V}O_{2\max}$ (sub-maximal effort cycling). Second, participants undertook a maximal-effort time trial lasting ~45 min (~85-100% $\dot{V}O_{2\max}$). The time trial required an individualized target amount of work to be completed in as short a time as possible (Jeukendrup et al, 1996). Cyclists could monitor task progress (i.e., kilojoules of energy expended), however performance time, power output or cadence (RPM) data were not made available. In addition to the resting sample before exercise, blood was collected in the final min of each exercise stage and also 1 h post-exercise. During exercise trials, participants consumed water *ad libitum*, but no food was provided.

Dietary control

Assessment of habitual diet

A three-day weighed food record was completed prior to each fourteen-day period of prescribed exercise training. Participants weighed all food items consumed during two weekdays and one weekend day using digital scales. Diets were analysed using internet-based nutrition analysis software (www.weightlossresources.co.uk). The habitual energy intake of the participants was 2882 ± 172 kcal/day and consisted of 1.6 ± 0.1 g protein·kg body mass⁻¹·day⁻¹, 5.4 ± 0.3 g carbohydrate·kg body mass⁻¹·day⁻¹ and 1.3 ± 0.1 g fat·kg body mass⁻¹·day⁻¹. A food questionnaire was used to assess food preferences for the study period.

Dietary manipulation

In the control-diet condition, participants were provided with an energy-balanced diet based on a macronutrient intake equivalent to 1.5 g protein·kg body mass⁻¹·day⁻¹ and 6 g carbohydrate·kg body mass⁻¹·day⁻¹ with the remainder of energy derived from fat. In the protein-diet condition, protein intake was doubled to 3 g protein·kg body mass⁻¹·day⁻¹. The carbohydrate content of the diet remained constant at 6 g carbohydrate·kg body mass⁻¹·day⁻¹ with the remainder of energy derived from fat. In both dietary conditions, carbohydrate-rich fruit drinks were consumed daily. In the protein-diet condition, 60 g (3×20 g) of casein protein hydrolysate (PeptoPro®, DSM Food Specialties, Delft, The Netherlands) was disguised in each fruit drink. Fruit drinks were ingested on completion of training sessions.

To rule out possible immunological changes during exercise that might be caused by a differential energy expenditure between the training- (i.e., normal- and high-intensity exercise training) or the dietary- (i.e., control- or protein-diet) conditions respectively, energy intake was matched to the energy expenditure of exercise training (Witard et al, 2011). Food and drinks were provided as three main meals together with a collection of snacks. Participants did not consume alcohol for the duration of the study.

Flow cytometry

Blood was collected into K₃EDTA Vacutainers and processed within 5 h as previously described (Witard et al, 2012). Briefly, whole blood was incubated with fluorescently conjugated antibodies at room temperature. Erythrocytes were lysed using FACS lysing solution. Cells were washed by centrifugation and re-suspended in paraformaldehyde phosphate buffered saline. Cells were read on a FACS calibur flow cytometer (Becton Dickinson, San Jose, USA). Cytotoxic CD8⁺TL subsets were identified by the expression of the cell-surface molecules CD27 and CD45RA (Hamann et al, 1997; Turner et al, 2010). Briefly, four CD8⁺TL sub-populations exist, which differ along a continuum of antigen experience and specificity as well as the capacity to exert immediate effector function (e.g., proliferation, cytokine secretion and target killing). First, a population of antigen inexperienced CD8⁺ naïve cells exist (NA; CD27+CD45RA+). These cells show the smallest responses to exercise and have a low effector potential. Next, three populations of memory cells exist, each of which exhibits a greater propensity for cytotoxicity as they differentiate along the memory continuum: central-memory cells (CM; CD27+CD45RA-), effector-memory cells (EM; CD27-CD45RA-), and terminally differentiated effector-memory cells (EMRA; CD27-CD45RA+). This latter population are largely responsible for exercise-induced lymphocytosis and lymphocytopenia and are capable of rapidly eliminating virus-infected cells (Campbell et al, 2009; Hamann et al, 1997; Sallusto et al, 1999; Turner et al, 2010). Data were analyzed using FlowJo v5.2 (Tree Star Inc., Aston, Oregon). Whole blood cell counts (i.e., total leukocytes, lymphocytes, granulocytes and monocytes) were determined based on the Coulter principle using an automated haematology analyzer (Coulter GEN-S, Beckman-Coulter, Miami, USA).

Biochemical measurements

Cortisol and epinephrine concentrations were measured in plasma as described previously (Witard et al, 2012) using commercially available kits (Cortisol, IDS, Tyne and Wear, UK; CAT-COMBO, IDS, Tyne and Wear, UK, respectively). Enzymatic analysis for plasma urea concentration (urea; ABX Diagnostics) was measured in duplicate using a semi-automated analyser (COBAS MIRA S-plus; ABX Diagnostics).

Symptoms of upper respiratory tract infection (URTI)

Participants completed the Daily Analysis of Life Demands for Athletes (DALDA) questionnaire (Rushall, 1990) on a daily basis to monitor symptoms indicative of URTI, including sore throat, bronchial congestion and running nose. Symptoms of URTI were classified as 'worse than normal', 'normal' or 'better than normal.' The number of 'worse than normal' symptoms reported by each participant was summated on a weekly basis.

Statistical analysis

All data were confirmed as being normally distributed using the Kolmogorov Smirnov test. To examine differences in the redistribution of leukocyte subsets and blood metabolite concentrations between the training and dietary conditions over the defined exercise period (measurements collected at baseline, sub-maximal exercise and maximal exercise, only), data were expressed as incremental area under the curve (iAUC) as described previously (Witard et al, 2012). iAUC is routinely used to detect differences across multiple time-points (Pruessner et al, 2003). An advantage of using this technique is that both the magnitude of response (increases in immune cell counts above baseline) and changes over time (i.e., time intervals between consecutive measurements of immune cell counts) are accurately captured using iAUC, irrespective of non-uniform time intervals between measurements (Fekedulegn

et al, 2007; Pruessner et al, 2003). iAUC was calculated using PRISM software (v4 Graph pad INC., San Diego, CA) with baseline set as the basal immune cell sub-population/subset count or metabolite concentration measured in the corresponding training condition. Post-exercise lymphocytopenia (egress or extravasation) was expressed as absolute change in cell number (i.e., cell count upon immediate cessation of time trial minus cell count 1 h post-exercise).

Differences in mobilization or egress of cells between training and dietary conditions were examined with paired samples *t*- tests consistent with our previous analysis (Witard et al, 2012). *A priori*, we made the following analytical decision to test the hypothesis that addition of protein to the diet would restore lymphocyte responses towards normal values: comparisons were made between normal-intensity training (control diet; black bars in figures) and high-intensity training (protein diet; grey bars in figures). For illustrative purposes, we have also presented comparisons made in our original report (Witard et al, 2012) between normal-intensity training (control diet; black bars) vs. high-intensity training (control diet; white bars).

Given that there were no significant changes in plasma volume between normal-intensity training (control diet; black bars) and high-intensity training (protein diet; grey bars) (data not shown), and that making adjustments to immune cell numbers has a negligible effect on results (Bosch et al, 2005), we did not adjust our values for minor fluctuations in plasma volume. All data were expressed as means \pm SEM, unless otherwise stated. Data were analysed using SPSS 18.0 for Windows (SPSS Inc., USA). Significance was set at the $P < 0.025$ level to allow for multiple comparisons.

Results

Diet

The energy and macronutrient content of the control- and protein-diet is shown in Table 1. The protein-diet provided participants with significantly more protein than the control-diet (Paired samples *t*-test; $t_{(7)} = -16.1$, $P < 0.001$). However, to control for potential immunological changes caused by a different caloric content of the two diets, energy content of the control- and protein-diet was the same during each week of high-intensity training (Paired samples *t*-test; $t_{(7)} = 0.4$, $P > 0.05$). To maintain energy balance between the normal- and high-intensity training conditions, energy content of the control- and protein-diets was increased by ~23% during high-intensity training (Paired samples *t*-test; $t_{(7)} > 5.2$, $P < 0.05$). All participants maintained body mass throughout both normal and high-intensity training conditions (data not shown). While the majority of this extra energy was derived from protein in the protein-diet condition, the fat content of the control-diet during high-intensity training was increased (Paired samples *t*-test; $t_{(7)} = 10.1$, $P < 0.05$). Carbohydrate intake remained the same between the control- and protein-diets (Paired samples *t*-test; $t_{(7)} = -0.9$, $P > 0.05$).

Exercise training conditions

Training volume (total duration of exercise per week; min) and training intensity (difficulty of exercise training each week; average bpm of training session) were increased during high-intensity training compared with the normal-intensity condition (data not shown). During high-intensity training, there were no differences in training volume or training intensity between the control- and protein-diet conditions (Volume: 1067 ± 234 min/wk (control-diet) vs. 1034 ± 179 min/wk (protein-diet); paired-samples *t*-test, $t_{(7)} = -1.0$, $P >$

0.05; Intensity: 140 ± 7 bpm (control-diet) vs. 141 ± 2 bpm (protein-diet); paired-samples *t*-test, $t_{(7)} = -1.3$, $P > 0.05$).

Physiological and psychological responses to exercise

There were no major differences in physiological (e.g., power output) or psychological (e.g., perceived exertion) variables during acute exercise between the normal- and high-intensity training weeks. Importantly, these variables did not influence the impaired immune response to exercise caused by high-intensity training. As reported below, dietary protein supplementation largely reversed the impaired immune response to exercise caused by high-intensity training. Analysis of co-variance showed that physiological and psychological variables were not significant covariates mediating this protective effect of dietary protein supplementation (supplementary Table 1). In other words, a high protein diet prevented the impaired immune response to exercise brought about by high-intensity training when consuming a normal diet, and this effect was not influenced by changes in physiological or psychological variables.

Effects of increased dietary protein on leukocyte responses to exercise

Total leukocyte and leukocyte sub-set responses to exercise are shown in supplementary Table 2. Exercise resulted in the characteristic mobilization of total leukocytes, lymphocytes, monocytes and granulocytes. Following exercise, lymphocyte numbers fell below baseline levels, whereas total leukocyte numbers increased, driven by granulocytes exhibiting the typical secondary mobilization.

When consuming a normal diet (i.e., the control-diet), high-intensity training is associated with a smaller exercise-induced mobilization of leukocytes compared to normal-

intensity training (Fig. 2A, white bar; paired samples t -test; $t_{(7)} = 2.4$, $P < 0.05$) (Witard et al, 2012). A high protein diet restored this impaired mobilization of leukocytes: there was no difference between normal-intensity training (control-diet) and high-intensity training (protein-diet) (Fig. 2A; grey bar, paired samples t -tests; $t_{(7)} = 0.6$, $P > 0.05$).

When consuming a normal diet, high-intensity training tended to be associated with a smaller exercise-induced mobilization of granulocytes compared to normal-intensity training, (Fig. 2B, white bar, paired-samples t -test; $t_{(7)} = 2.3$, $P = 0.07$) (Witard et al, 2012). A high protein diet restored this impaired mobilization of granulocytes: there was no difference between normal intensity training (control-diet) and high-intensity training (protein-diet) (Fig. 2B, grey bar, paired samples t -tests; $t_{(7)} = 0.5$, $P > 0.05$).

Effects of increased dietary protein on total CD8⁺ T lymphocyte mobilization during exercise

When consuming a normal diet (i.e., the control-diet), high-intensity training tended to be associated with a smaller exercise-induced mobilization of CD8⁺TL compared to normal-intensity training (Fig. 3A, white bar, paired-samples t -test; $t_{(7)} = 1.4$, $P = 0.08$) (Witard et al, 2012). A high protein diet restored this training-induced reduction in the mobilization of CD8⁺TL. There was no significant difference between high-intensity exercise training (protein-diet) and normal-intensity training (control-diet) (Fig. 3A, grey bar, paired-samples t -test; $t_{(7)} = -0.4$, $P > 0.05$).

Effects of increased dietary protein on total CD8⁺ T lymphocyte egress post exercise

Undertaking high-intensity exercise training when consuming a normal protein diet (i.e., the control-diet) is associated with a reduced egress of total CD8⁺TL 1 h post-exercise

compared to normal-intensity training (Fig. 3B, white bar, paired samples t -test; $t_{(7)} = 2.9$; $P < 0.05$) (Witard et al, 2012). A high protein diet restored this training-induced reduction in the egress of CD8⁺TL: when high-intensity exercise training was repeated with the protein-diet, this observation was reversed. There was no significant difference in the egress of CD8⁺TL 1 h post-exercise between normal-intensity training (control-diet) and high-intensity training (protein-diet) (Fig 3B, grey bar, paired samples t -test; $t_{(7)} = 0.3$; $P > 0.05$).

Effects of increased dietary protein on EMRA CD8⁺ T lymphocyte mobilisation during exercise

High intensity exercise training when consuming a normal diet (i.e., the control-diet) is associated with a smaller exercise-induced mobilization of EMRA CD8⁺TL (Fig. 4A, white bar, paired-samples t -test; $t_{(7)} = 2.1$, $P < 0.05$) (Witard et al, 2012). When exercise was repeated during high-intensity training with a high protein-diet, this reduced mobilization was prevented; i.e., there was no difference in EMRA CD8⁺TL mobilization between high-intensity training (protein-diet) and normal-intensity training (control-diet) (Fig. 4A, grey bar, paired-samples t -test; $t_{(7)} = -0.7$, $P > 0.05$).

Effects of increased dietary protein on CD8⁺ T lymphocyte EMRA subset egress

When consuming a normal diet (i.e., the control diet), during high-intensity exercise training, there was a tendency for the egress of CD8⁺TL EMRA 1 h post-exercise to be smaller than with normal-intensity exercise training (Fig. 4B, white bar, paired samples t -tests; $t_{(7)} = 1.8$; $P = 0.06$) (Witard et al, 2012). When exercise was repeated during high-intensity training when consuming a high protein-diet, this tendency for a smaller cell egress was prevented; there was no difference in EMRA CD8⁺TL egress between high-intensity

training (protein-diet) and normal-intensity training (control-diet) (Fig. 4B, grey bar, paired samples t -tests; $t_{(7)} = -0.6$; $P > 0.05$).

Effects of increased dietary protein on CD8⁺ T lymphocyte naïve (NA) subset kinetics

Performing high-intensity exercise when consuming a normal diet appears not to impair the mobilization of NA CD8⁺ T cells (Witard et al, 2012). As expected, there were no diet-induced alterations in NA CD8⁺ T cell mobilization during exercise between normal- and high-intensity training (data not shown). Unlike mobilization, the egress of NA CD8⁺ T cells 1 h post-exercise is impaired by high-intensity exercise training when consuming a normal diet (Witard et al, 2012). When exercise was repeated during high-intensity training with a high protein diet, the smaller egress of NA CD8⁺ T cells returned to normal levels (Paired samples t -tests iAUC between training conditions; $t_{(7)} = 0.2$; $P > 0.05$; data not shown).

Effects of increased dietary protein on neuro-endocrine responses to exercise

As previously reported (Witard et al, 2012), when consuming a normal diet, high-intensity training is associated with lower plasma epinephrine and cortisol concentrations during exercise compared to normal-intensity training. A high protein diet had no effect on the epinephrine or cortisol response to exercise (data not shown). Thus, high-intensity exercise training resulted in smaller neuroendocrine responses to exercise, even when the diet was supplemented with protein.

Effects of increased dietary protein on plasma urea concentration

As the protective effects of a high protein diet appeared to be unrelated to neuroendocrine activation, we next examined plasma urea concentrations following exercise.

Plasma urea concentrations indirectly represent the rate of gluconeogenesis. We hypothesised that increased protein intake would elevate urea concentrations, indicating higher production of glucose from nitrogen containing sources (i.e., amino acids). Indeed, when the high-protein diet was provided, plasma urea concentrations were increased above normal concentrations both at baseline (data not shown) and during exercise (Fig. 5, grey bar, paired-samples t -test; $t_{(7)} = <2.4, P < 0.05$).

Effects of increased dietary protein on self-reported symptoms of upper respiratory tract infection (URTI)

The number of 'worse than normal' scores for symptoms indicative of URTIs were greater during high-intensity training when consuming a normal diet (control-diet) compared with normal-intensity training (Fig. 6, white bar, paired-samples t -test; $t_{(7)} = -5.4, P < 0.05$). When a high protein diet was consumed during high-intensity training, there was no difference in the number of symptoms of URTI compared to normal-intensity training (control-diet) (Fig. 6, grey bar, paired-samples t -test; $t_{(7)} = -1.8, P > 0.05$).

Discussion

We recently demonstrated that the redistribution of CD8⁺TL in response to acute exercise tended to be impaired by a period of high-intensity exercise training (Witard et al, 2012). Given the anti-viral properties of CD8⁺TL (van Lier et al, 2003), reduced CD8⁺TL trafficking might explain the increased incidence of URTI reported by athletes during high-intensity training regimens (Foster, 1998; Gleeson, 2007). The results of the present study show most prominently, that the tendency for an impaired exercise-induced mobilization and egress of CD8⁺ TL (and also total leukocytes and granulocytes), during high-intensity training is restored by increasing dietary protein intake. Intriguingly, the number of self-reported symptoms of URTI was lower when high-intensity training was performed when consuming a high-protein diet. These data provide preliminary evidence for a dietary strategy to prevent URTI in athletes.

A possible mechanism underpinning the protective effects of a high protein diet on CD8⁺TL redistribution is the stimulatory effects of amino acids on the intracellular signalling pathway mTOR. Activation of the mTOR cascade is essential for regulating lymphocyte protein synthesis and therefore cell function. Although mTOR signaling can be initiated by classical T cell activation signals (i.e., T cell receptor engagement with antigen, co-stimulation and cytokines), amino acids also are a potent stimulus for mTOR activation in lymphocytes (Fumarola et al, 2005). Indeed, mTOR associated signaling molecules have been described as key nutrient sensors that serve to match the metabolic demands of lymphocyte function to the availability of energy substrates (Powell and Delgoffe, 2010; Sinclair et al, 2008). In the context of the present results, amino acid-induced mTOR signaling, which in turn up-regulates a variety of lymphocyte genes associated with cell function, alters the expression of adhesion molecules implicated in lymphocyte trafficking (Finlay et al, 2012;

Peng et al, 2002; Sinclair et al, 2008). Thus, acute protein malnutrition, caused by high-intensity exercise training, might prevent lymphocytes down-regulating molecules associated with transit to lymphoid tissue (e.g., CD62L) and impair up-regulation of molecules associated with extravasation to peripheral tissue (e.g., cutaneous lymphocyte antigen; CLA) (Finlay et al, 2012; Sinclair et al, 2008), thus potentially impairing trafficking into and out of the bloodstream. We speculate that the effects of high-intensity exercise training on lymphocyte trafficking is reversed by a high protein diet, due to amino acid induced mTOR activation and efficient adhesion molecule regulation.

An alternative explanation for the restored lymphocyte redistribution with a high protein diet might be related to energy substrate supply, in particular glucose availability (Shephard and Shek, 1995). This hypothesis is supported by studies showing that carbohydrate supplementation prevents impaired lymphocyte function post-exercise (Henson et al, 1998; Nieman, 1998). In the present study, carbohydrate intake was controlled at $6 \text{ g}\cdot\text{kg}^{-1} \text{ body mass}\cdot\text{day}^{-1}$ which is less than the $8\text{-}10 \text{ g}\cdot\text{kg}^{-1} \text{ body mass}\cdot\text{day}^{-1}$ recommended for athletes (Burke, 2004). With suboptimal carbohydrate intake, increased dietary protein may indirectly increase carbohydrate availability or muscle glycogen stores via gluconeogenic pathways during high-intensity training. This hypothesis is appealing considering that gluconeogenesis has been shown to be elevated by 40% with a high-protein diet (Linn et al, 2000). Although lymphocyte substrate use was not assessed in the present study, unsurprisingly, plasma urea concentrations measured at rest, and in response to exercise, were markedly higher when high-intensity training was performed while consuming a high protein diet. This result implies that more amino acids were made available for deamination and transamination, and ultimately, the rate at which carbon skeletons could be used for gluconeogenesis or oxidation in lymphocytes was increased. Thus, higher dietary protein *per se* might not have been the

only factor responsible for restoring CD8⁺TL redistribution during high-intensity exercise training. Instead, the increased protein may have partially countered inadequate availability of carbohydrate during high-intensity training and thus may have restored the availability of glucose to fuel the homing of effector CD8⁺TL to target tissues.

Considering the potent effects of β 2 adrenergic receptor stimulation on lymphocyte mobilization and egress (Landmann, 1992), an intuitive explanation for the present findings might be that a high protein diet causes a larger neuroendocrine response to exercise. Together with our previous findings (Witard et al, 2012), we now suggest that altered neuroendocrine responses to exercise are unlikely to be the cause of post-exercise immunosuppression during high-intensity training. First, although in our previous work, we showed that the reduced lymphocyte trafficking in response to exercise caused by high-intensity training occurred in parallel with a smaller epinephrine and cortisol response to exercise, our subsequent analyses showed that these parameters were not driving the impaired immune response to exercise (Witard et al, 2012). Second, while the present study shows that providing a high protein diet restores lymphocyte responses to exercise, the high protein diet did not restore neuroendocrine responses to those observed with normal-intensity training. Hence, the restored trafficking of CD8⁺TL with a high protein diet appears not to be caused by altered neuroendocrine activation, and is perhaps more likely explained, by alterations in intracellular signalling, that might affect adhesion molecule expression.

In the present study, the protective effect of protein ingestion was not limited to cells of the adaptive immune system. For example, increased dietary protein intake during high-intensity training restored the redistribution of total leukocytes to levels that were similar to those observed during normal-intensity training – an observation largely driven by granulocytes. In peripheral blood, granulocytes largely comprise of neutrophils, which are

recruited to skeletal muscle in the hours after exercise. Here, neutrophils in part regulate tissue repair by phagocytosis of damaged cells, modulate the function of macrophage infiltrators, and eliminate microbial infection (Tidball and Villalta, 2010). Amino acids, and in particular glutamine, are important substrates for neutrophil function (Li et al, 2007; Newsholme and Parry-Billings, 1990). In the present study, rather than supplementing the diet with individual amino acids, the whole protein content of the diet was increased. However, *in vitro*, protein increases neutrophil anti-bacterial mechanisms, including production of reactive oxygen species and degranulation (Ruso et al., 2009; Rusa et al. 2010). *Ex vivo* studies have shown that protein ingestion counters exercise-induced impairments in neutrophil function (Costa et al, 2009; Nelson et al, 2013) and the present results add to this literature, by showing that exercise-induced granulocytosis, which is impaired by high intensity exercise training, can be reversed by consuming a high protein diet. The implications of this finding are unclear, but conceivably, larger or at least normal granulocyte kinetics in response to exercise, might aid detection and elimination of bacterial infection.

When interpreting the results of this study, it should be considered that URTI was not diagnosed clinically. Instead, we used a self-reported measure that inferred URTI by assessing symptoms, including sore throat, bronchial congestion and running nose. Future studies that employ high-intensity training models should combine measurements of immune-surveillance simultaneously with clinically diagnosed symptoms of infection. Another consideration is that the dietary intervention provided $3.0 \text{ g}\cdot\text{kg}^{-1} \text{ body mass}\cdot\text{day}^{-1}$ of protein which is above current recommendations of $1.6 \text{ g}\cdot\text{kg}^{-1} \text{ body mass}\cdot\text{day}^{-1}$ for endurance athletes (Tarnopolsky, 2004). These levels however, are not uncommon among athletes; in the Tour de France cycling race for example, protein intake by athletes is regularly reported to be as high as $3 \text{ g}\cdot\text{kg}^{-1} \text{ body mass}\cdot\text{day}^{-1}$ (Saris et al, 1989). Moreover, because energy intake was increased during high-

intensity training to maintain energy balance, $3.0 \text{ g}\cdot\text{kg}^{-1} \text{ body mass}\cdot\text{day}^{-1}$ of protein was equivalent to ~20% of total energy intake which is not an unfeasible percentage contribution to total energy intake. Thus, future studies could identify the minimal effective dose of protein that is necessary to replicate these results, as well as examining other dietary strategies in combination, such as carbohydrate supplementation (Burke, 2004; Jeukendrup and McLaughlin, 2011). It should also be noted that, with the exception of salivary immunoglobulin A, there are no established biomarkers to predict URTI in athletes (Gleeson et al, 1999; MacKinnon, 2000) and it remains to be established whether lymphocyte kinetics to standardized exercise bouts are predictive of infection. Although the present results show that restored/normal lymphocyte responses to acute exercise during times of demanding training occur in parallel with a decreased incidence of URTI, potentially mediated by dietary protein intake, it might be that this dietary intervention confers protection by modulating some other aspect of immunity. Indeed, it might be questioned whether the restoration of lymphocyte kinetics to “normal values” (i.e., responses we observed on two occasions, under a normal training load) has direct clinical relevance. We maintain however, that the observed change in this immune response occurs simultaneously with fewer self-reported incidents of infection – thus providing the first data on a potentially novel predictor of infection risk in athletes.

In summary, we have shown that by increasing dietary protein during high-intensity training, it is possible to restore, most prominently $\text{CD8}^+\text{TL}$ (but also total leukocyte and granulocyte) redistribution, to levels observed during normal-intensity training. Although the mechanisms behind this observation remain unclear, given that the number of reported symptoms of URTI was reduced, it is possible that immune surveillance might be maintained during high-intensity training by consuming a high protein diet.

Acknowledgments

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

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Table captions

Table 1: Energy and macronutrient intake during the protein- and control-diet conditions.

Table 2: Total leukocyte and leukocyte sub-population responses to exercise following normal-intensity training and high-intensity training in the control condition and high-intensity training in the protein condition.

Figure legends

Figure 1: Schematic representation of study design. In this crossover design, participants engaged in a 7-d period of normal-intensity exercise training on a normal protein diet (1.5 g / kg body mass / day) followed by a 7-d period of high-intensity training on a normal protein diet (1.5 g / kg body mass / day) or a 7-d period of high-intensity training on a high protein diet (3.0 g / kg body mass / day). Numbers 1-7 represent days of the week.

Figure 2: Mobilization (expressed as incremental area curve, iAUC) of total leukocyte (A) and granulocyte (B) cells during exercise following normal- and high-intensity training in the control trial and high-intensity training in the high protein trial. Values are means \pm SEM (n = 8). *significant difference ($p < 0.05$).

Figure 3: Total CD8⁺TL responses to exercise following normal-intensity training and high-intensity training in the control trial and high-intensity training in the high protein trial. A) Changes in cell number over time; B) mobilization of cells expressed as incremental area curve (iAUC) during exercise; C) egress of cells 1 hour post-exercise. SM: sub-maximal exercise, MAX: maximal exercise, Post 1 h: 1 hour following maximal exercise. Values are means \pm SEM (n = 8). †significantly different from baseline in corresponding training period ($p < 0.05$). *significant difference between training weeks ($p < 0.05$).

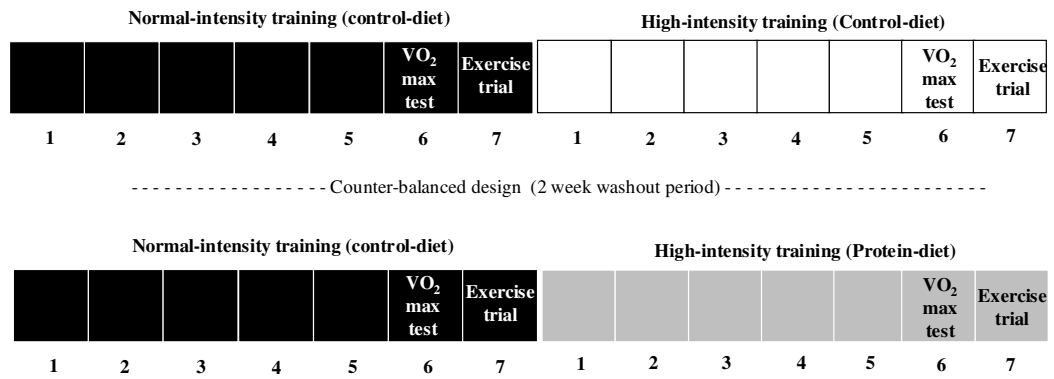
Figure 4: CD45RA⁺ effector memory CD8⁺TL (EMRA) responses to exercise following normal-intensity training and high-intensity training in the control trial and high-intensity training in the high protein trial. A) Mobilization of cells expressed as incremental area curve

(iAUC) during exercise; B) Egress of cells 1 hour post-exercise. SM: sub-maximal exercise, MAX: maximal exercise, Post 1 h: 1 h following maximal exercise. Values are means \pm SEM (n = 8). *significant difference between training weeks ($p < 0.05$).

Figure 5: Plasma urea concentrations during exercise following normal- and high-intensity training in the control trial and high-intensity training in the high protein trial. Urea concentration expressed as incremental area curve (iAUC) during exercise. Values are means \pm SEM (n = 8). *significant difference between training weeks ($p < 0.05$).

Figure 6: Incidence of upper respiratory tract infections (URTI) following normal- and high-intensity training in the control trial and high-intensity training in the high protein trial. Incidence of URTI included symptoms of sore throat, bronchial congestion and running nose and are expressed as the mean (\pm SEM, n = 8) number of episodes reported over the duration of the week. *significant difference between training weeks ($p < 0.05$).

Figure 1



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Figure 2

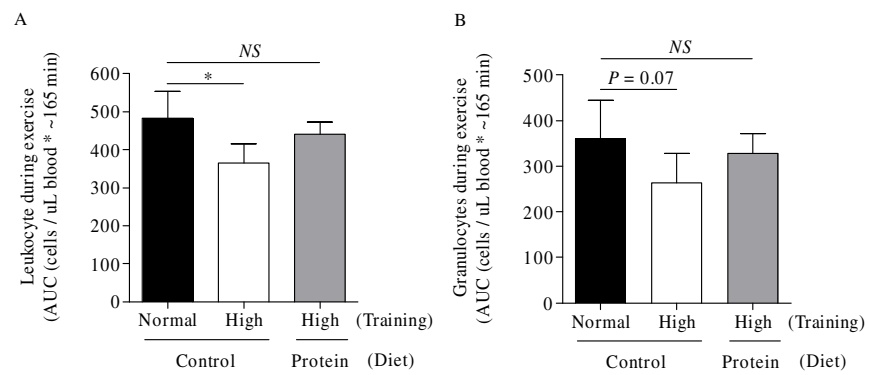


Figure 3

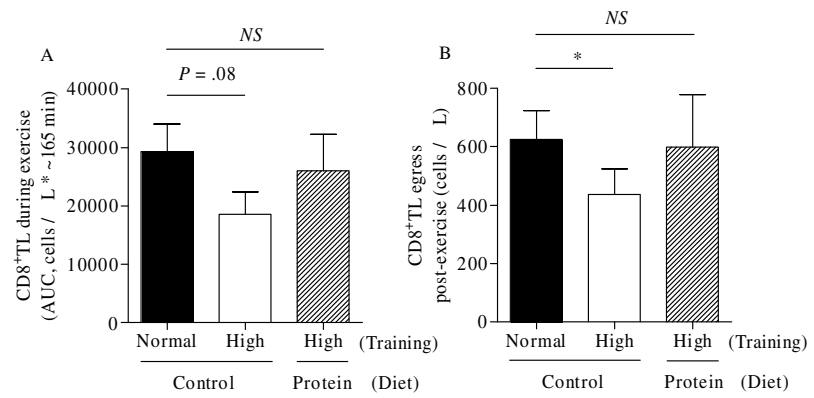


Figure 4

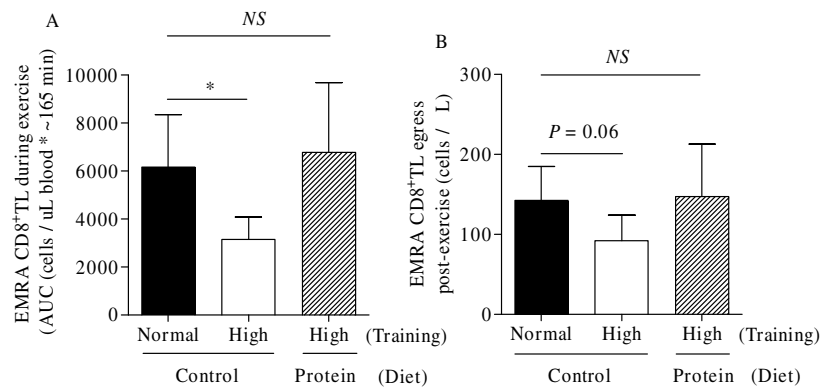
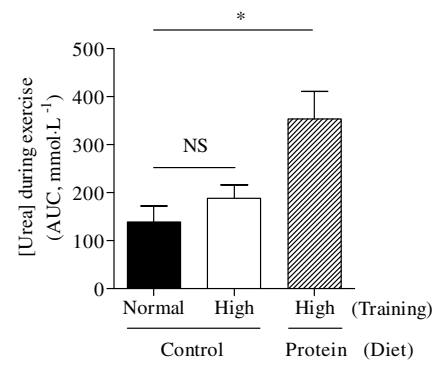
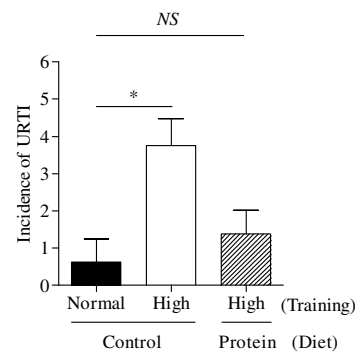


Figure 5



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Figure 6



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Table 1. Energy and macronutrient content of the control- and protein-diet during normal- and high-intensity training.

	Normal-intensity training		High-intensity training	
	Control-diet		Control-diet	Protein-diet
Energy intake (kcal/day)	3511±161		4300±155*	4273±153*
Protein intake (g/day)	110±3		110±3	218±6* [#]
CHO intake (g/day)	436±14		440±12	446±18
Fat intake (g/day)	148±15		240±11* ^{\$}	185±9*

Data are means ± SD. *significantly different from normal-intensity training.
[#]significantly different from high-intensity training (control-diet). ^{\$}significantly different from high-intensity training (protein-diet) ($p < 0.05$).

A high dietary protein intake during high-intensity training may restore immune surveillance in athletes

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