

## ***Human T lymphocyte migration towards the supernatants of Human Rhinovirus infected airway epithelial cells: Influence of exercise and carbohydrate intake.***

Nicolette C. Bishop<sup>1</sup>, Gary J. Walker<sup>1</sup>, Michael Gleeson<sup>1</sup>, Fiona A. Wallace<sup>2</sup> and Colin R.A. Hewitt<sup>3</sup>

<sup>1</sup> School of Sport and Exercise Sciences, Loughborough University, Loughborough LE11 3TU, UK

<sup>2</sup> GlaxoSmithKline Consumer Healthcare R&D, Slough, SL1 3NW, UK

<sup>3</sup> Department of Genetics, University of Leicester, Leicester LE1 9HN, UK

### **ABSTRACT**

*Physical stress induces a marked redistribution of T lymphocytes that may be influenced by carbohydrate (CHO) availability, yet the effect of these on T lymphocyte migration towards infected tissue is unknown. Therefore, the aim of this study was to determine the effect of strenuous exercise and CHO ingestion on subsequent ex vivo lymphocyte migration towards the supernatants of a Human Rhinovirus (HRV)-infected bronchial epithelial cell line. In a randomised, cross-over, double-blind design, 7 trained males ran for 2 h at 60%  $\dot{V}O_{2peak}$  on two occasions with regular ingestion of either a 6.4% w/v glucose and maltodextrin solution (CHO trial) or placebo solution (PLA trial). Plasma glucose concentration was higher on CHO than PLA after exercise ( $P < 0.05$ ). Migration of CD4+ and CD8+ cells and their CD45RA+ and CD45RO+ subpopulations towards supernatants from HRV-infected cells decreased following exercise (main effect for exercise,  $P < 0.01$  for CD4+, CD4+CD45RA+ and CD4+CD45RO+;  $P < 0.05$  for CD8+, CD8+CD45RA+ and CD8+CD45RO+). Migration of CD4+ cells and CD4+CD45RA+ cells was ~35% and ~30% higher, respectively, on CHO than PLA at 1 h post-exercise (interaction,  $P < 0.05$  for both) and was higher on CHO than PLA for all other subpopulations ( $P < 0.05$ , main effect for trial). There was little effect of exercise or CHO on migration of these cells towards uninfected (control) cell supernatants or on the proportion of these cells within the peripheral blood mononuclear cell population. The findings of this study suggest that physical stress reduces T cell migration towards HRV-infected cell supernatants and that ingestion of CHO can lessen this effect.*

**Key words:** T lymphocyte, exercise, carbohydrate, upper respiratory tract infection

*Address for correspondence:*

Dr Nicolette C Bishop, School of Sport and Exercise Sciences,  
Loughborough University, Loughborough, Leicestershire,  
LE11 3TU, UK, Email: N.C.Bishop@lboro.ac.uk  
Tel: +44 1509 226385

## INTRODUCTION

It is well established that intensive exercise in humans induces significant changes in the distribution of lymphocytes within the peripheral blood compartment (29). Evidence to date suggests that this redistribution can be attributed to a mobilisation of specific populations of T lymphocytes characterised by the expression of low levels of the adhesion molecule L-selectin and high levels of the  $\beta$ -integrin lymphocyte-function associated molecule-1 (LFA-1) and its ligand, intercellular cell adhesion molecule 1 (ICAM-1) (18,29). These cells are not apparent during recovery from intensive exercise, suggesting extravasation from the peripheral circulation. Enhanced sympathetic activity and elevations in plasma glucocorticoids are thought to be at least partly responsible for this altered cellular distribution (17,24,29).

The specific origins and destinations of lymphocytes mobilised by intense exercise in humans is still not clear, although a recent study in mice found that exhaustive exercise caused a release of T lymphocytes from the spleen with lung, bone marrow and Peyer's patches serving as target organs (17). However, studies investigating the effect of physical stress on the migratory potential of mobilised blood lymphocytes towards *infected* tissue are scant. Such information is of potential importance given that evidence suggests that highly active individuals suffer a higher incidence of respiratory infection episodes than their less active counterparts (8,30) with Human Rhinovirus (HRV), the predominant cause of the common cold, the most common respiratory pathogen identified in this population (30).

Lymphocyte migration from the peripheral circulation into the tissue is a highly regulated process, determined by tissue-specific signals and receptors, the expression of which are in turn dependent on the release of a precise cocktail of chemokines and cytokines (16). In the presence of HRV, infected airway epithelial cells produce several chemokines and proinflammatory cytokines resulting in increased airway inflammation. This inflammation is thought to trigger or exacerbate cold symptoms (25). Migration of recirculating memory and effector T lymphocytes through the blood vessel wall into the airway submucosa is essentially regulated by a gradient of these epithelium-derived chemokines radiating from the infected airway epithelia. In order to migrate towards the infected tissue, T lymphocytes must also express an array of receptors for the epithelial chemokines complementary to those produced by the infected epithelial cells. Substantial lymphocyte recruitment to the airways has been demonstrated by experimental HRV infection in human subjects, with symptom severity directly related to the percentage (19) and numbers (31) of lymphocytes recovered in nasal secretions. Furthermore, human airway epithelial cells infected with HRV following both nasal inoculation and exposure *in vitro* produced several chemokines known to attract T lymphocytes (25,31). However, any effect of exercise on the ability of T cells to migrate towards HRV infected tissue is unknown.

Analysis of the factors that control lymphocyte migration in hyper- and hypoglycaemic conditions suggests that the physiological effects of prolonged intensive

exercise may change the migratory properties of T lymphocytes in a manner that is influenced by carbohydrate (CHO) ingestion during exercise. Work from the literature describing diabetic nephropathy suggests that high glucose levels are able to induce chemokine (26,27) and chemokine receptor expression (14) in PBMCs and mesangial cells as a result of metabolic activity and not hyperosmolarity (12). Exposure of human endothelial cells to hyperglycaemic conditions is also associated with enhanced T cell migration compared with normoglycaemic conditions (10). Furthermore, insulin-induced hypoglycaemia in both healthy humans (4,33) and in patients with Type 1 diabetes (9) is associated with marked alterations in the mobilisation of T lymphocyte subpopulations that may be mediated by the stress hormone response to the hypoglycaemia. Intensive exercise is associated with marked increases in cortisol concentrations that can be attenuated by exogenous CHO ingestion (13,20). Studies in humans report that exposure to post-exercise elevations in plasma cortisol increased T lymphocyte migration towards the airway epithelial cell-derived chemokine CXCL12 and induced expression of CXCR4, the receptor for CXCL12, on T lymphocytes (24). Higher levels of glucocorticoid receptors have also been reported on human effector T cells following acute psychological stress (2). Taken together, this literature suggests that CHO ingestion during exercise could impact on T cell migration towards infected tissue following intensive exercise.

Therefore, the aim of the present study was two-fold; (i) to determine the effect of prolonged intensive exercise on the *ex vivo* migratory potential of naïve (CD45RA+) and memory (CD45RO+) CD4+ and CD8+ lymphocytes towards supernatants of a bronchial epithelial cell line infected with the common respiratory pathogen Human Rhinovirus (HRV), and (ii) to determine any influence of exogenous CHO ingestion on this response.

## METHODS

*Ethical Approval.* The study was approved by the Loughborough University Ethical Advisory Committee and conformed to the standards set by the *Declaration of Helsinki* (2004). All subjects were informed of the rationale and protocol for the study before providing written informed consent. Subjects were required to complete a comprehensive health-screening questionnaire prior to each exercise test. None reported any symptoms of infection and had not taken any medication in the four weeks prior to the study, nor were they currently on medication.

*Overall Experimental Design.* Seven male endurance trained runners (mean (SD) age: 27 (4) years, body mass: 72.3 (3.6) kg, peak aerobic capacity ( $\dot{V}O_{2peak}$ ): 55.9 (5.6) ml.kg<sup>-1</sup>.min<sup>-1</sup>) performed two exercise trials, separated by at least 7 days, in a randomised, double blind, cross-over design. For each trial, subjects ran on a motorised treadmill (0% gradient) at 60%  $\dot{V}O_{2peak}$  for 2 h. Mean running speed was 10.0 (1.3) km.h<sup>-1</sup>. For one trial, subjects ingested a CHO beverage (6.4 % w/v glucose and maltodextrin) before, during and after the exercise (CHO trial). For the other trial, subjects ingested a water-based artificially sweetened placebo drink before, during and after the exercise (PLA trial). Each drink was

identical in flavour (orange) and appearance and the neither the subjects nor the investigators were aware of the identity of the drinks in each trial.

*Preliminary testing.* Approximately 10 days prior to the main exercise trials, each subject performed a two-part incremental treadmill test to volitional fatigue to determine  $\dot{V}O_{2\text{peak}}$  and allow determination of the running speed equivalent to 60%  $\dot{V}O_{2\text{peak}}$  for the main trials. Initially subjects performed a 16-min submaximal test from which the running speed for the subsequent  $\dot{V}O_{2\text{peak}}$  test was determined. The treadmill gradient during this initial submaximal test was 0° and the test started at 8 km.h<sup>-1</sup> increasing by 1.5 km.h<sup>-1</sup> every 4 min. Samples of expired gas (to determine  $\dot{V}O_2$ ) were collected during the final minute of each stage and heart rate was also recorded. The  $\dot{V}O_{2\text{peak}}$  test was then performed at the running speed that elicited a heart rate of 150-160 beats.min<sup>-1</sup> during the submaximal test. This consisted of 3 min incremental stages until volitional exhaustion with subjects running at an initial gradient of 3.5°, increasing by 2.5° with each stage. Expired gas and heart rate were taken during the final minute of each stage and during the final minute of exercise.  $\dot{V}O_{2\text{peak}}$  was taken as the highest  $\dot{V}O_2$  achieved. The running speed that would elicit an exercise intensity of 60%  $\dot{V}O_{2\text{peak}}$  at 0° gradient was interpolated from the  $\dot{V}O_2$ -running speed relationship determined during the submaximal test.

Approximately 1 week before their first main trial, each subject returned to the laboratory to perform 2 h of treadmill running at 60%  $\dot{V}O_{2\text{peak}}$  in order to familiarise themselves fully with the exercise protocol. During this test heart rates were recorded every 15 min and samples of expired gas were collected after 15 min and every 30 min thereafter in order to ensure that they were exercising at the required intensity.

*Main experimental trials.* Subjects were asked to refrain from training during the 24 h before each main experimental trial and were asked to ensure that they followed the same diet during the 24 h prior to each main experimental trial in an effort to standardise nutritional status.

For each trial, subjects arrived at the laboratory at 07.45 am following an overnight fast of approximately 12 h. Each subject was asked to empty his bladder before body mass (in shorts only) was recorded. Subjects then sat quietly for 15 min before a pre-exercise venous blood sample (40 ml) was obtained by venepuncture from an antecubital forearm vein. Subjects then ingested 5 ml.kg<sup>-1</sup> body mass of either a beverage comprising 6.4% (w/v) glucose and maltodextrin (CHO trial) or a placebo that was similar in taste and appearance but with zero carbohydrate content (PLA trial). Five min after drink ingestion subjects began running on a motorised treadmill for 2 h at 60%  $\dot{V}O_{2\text{peak}}$ . During the exercise, subjects consumed 2 ml.kg<sup>-1</sup> body mass of the prescribed drink at 15-min intervals, consuming a further 5 ml.kg<sup>-1</sup> body mass of the prescribed drink at 5 min post-exercise. Mean fluid intake per trial was 1.6 (0.2) l per subject and mean CHO intake on the CHO trial was 109 (13) g per subject.

During the exercise heart rate was recorded at 15-min intervals using short-range telemetry. Samples of expired gas were collected at 15 min of exercise and every

30 min thereafter in order to determine relative exercise intensity. Further blood samples (40 ml) were obtained immediately after exercise and at 1 h post-exercise. No further drinks or food were consumed until after the 1 h post-exercise sample had been collected. Laboratory conditions were 23.6 (0.6)°C and 37 (6)% relative humidity.

*Total and differential leukocyte counts and plasma volume.* The collected blood sample was aliquoted into two separate monovette tubes (evacuated blood collection tubes, Sarstedt, Leicester, UK), one (2.7 ml) containing K<sub>3</sub>EDTA (1.6 mg EDTA·ml<sup>-1</sup> blood) and one (7.5 ml) containing lithium heparin (1.5 IU heparin·ml<sup>-1</sup> blood). The remaining sample (29.8 ml) was added to a universal centrifuge tube containing 1.5 IU heparin·ml<sup>-1</sup> blood for subsequent separation of peripheral blood mononuclear cells (PBMCs). Blood taken into the K<sub>3</sub>EDTA monovette was used for haematological analysis including haemoglobin, haematocrit and total and differential leukocyte counts using an automated cell-counter (A<sup>c</sup>.T<sup>TM</sup>5diff haematology analyser, Beckman Coulter, High Wycombe, UK). All cell counts were corrected for plasma volume changes relative to the first blood sample for each bout of exercise; these were estimated from the haemoglobin and haematocrit values according to Dill & Costill (7).

#### *Preparation of HRV stocks*

Human H1HeLa cells (ATCC, CRL-1958) were cultured in MEM + Earle's salts supplemented with 10% foetal calf serum (FCS), 0.1 mM non essential amino acids and GlutaMAX I. HRV 16 (ATCC, VR-283) was propagated in H1HeLa cells infected at a multiplicity of infection (moi) of 1 in serum free medium for two hours at 33°C. Infected cells were incubated with medium containing 10% FCS until cytopathic effect (CPE) progressed no further. Cells and supernatants underwent two rapid freeze thaw cycles, were separated by centrifugation and frozen in aliquots at -80°C. Supernatant and lysate from uninfected H1HeLa cells were similarly prepared for use as controls. The TCID<sub>50</sub>·ml<sup>-1</sup> of the virus stock was assessed by CPE on H1HeLa cells 7 days after infection with serial dilutions of the virus stock.

#### *Preparation of HRV-infected BEAS-2B supernatants.*

Human BEAS-2B airway epithelial cells (ATCC, CRL-9609) were cultured in 50% F-12 Nutrient Mixture (Ham), (Invitrogen), 50% MEM + Earle's salts supplemented with GlutaMAX I, 0.1mM non essential amino acids (Invitrogen) and 2% Ultrosor G (Pall BioPharmaceuticals, St-Germain-en-Laye, France). Sub confluent BEAS-2B cells were infected with HRV containing supernatant/lysate stock at an moi of 0.1; mock infected cells were treated with the same volume of control supernatant/lysate. Twenty-four hours after infection, supernatants from HRV-infected cells and non-infected cells were harvested, aliquoted and frozen.

*Assessment of ex vivo lymphocyte migration towards supernatants of HRV infected BEAS-2B cells.* PBMCs were separated from heparinised whole blood by density gradient centrifugation. The cells were washed in sterile isotonic salt solution and resuspended in culture medium (RPMI 1640 containing 10% FCS, 100 U·ml<sup>-1</sup> penicillin, 100 mg·ml<sup>-1</sup> streptomycin, 50 mM HEPES and 24 mM Na<sub>2</sub>CO<sub>3</sub>). The

PBMCs were counted and the cell suspensions diluted to achieve a final concentration of  $3.3 \times 10^6$  cells.ml<sup>-1</sup>.

The ability of lymphocytes to migrate towards supernatants of HRV infected BEAS-2B cells was determined using 5 µm pore, 96 well Neuro Probe ChemoTx migration plates (Receptor Technologies, Adderbury, Oxon, UK). A concentration of 50% of the undiluted, HRV infected or control BEAS-2B supernatants was found to give optimal migration rates as determined by titration prior to the main experiment (data not shown). 300 µl of each supernatant containing the released chemokines or corresponding supernatant control were added to the microplate wells. The semi-permeable membrane was then placed on top of the plate so that surface tension held the membrane in contact with the supernatant; 75 µl (250,000 cells.well<sup>-1</sup>) of purified PBMCs were then placed on the upper side of the membrane. Six replicate wells were used for each supernatant providing a total of  $1.5 \times 10^6$  PBMCs for analysis with each set of conditions. The cells were allowed to migrate for 90 min at 37°C in a 5% CO<sub>2</sub>, 100% humidity atmosphere. Following incubation, cells remaining on top of the membrane (the stationary cells) were aspirated and the membrane removed from the plate. The plate was then centrifuged for 5 min at 400 g to collect the migrated cells; 90% of the supernatants were removed, and the cells in replicate wells pooled into labelled tubes which were centrifuged again at 5000 g for 2 min.

*Assessment of lymphocyte subpopulations by flow cytometry.* The original PBMC and the cells that had migrated were analysed for expression of CD4, CD8 and their CD45RA and CD45RO subsets by flow cytometry, using Pharmingen monoclonal antibodies and isotype control antibodies (Becton Dickinson Biosciences, Oxford, UK). Cells were labelled with cocktails of the following monoclonal antibodies against human lymphocyte cell surface markers: fluorescein isothiocyanate (FITC) conjugated anti-CD4 (clone RPA-T4), phycoerythrin (PE) conjugated anti-CD45RA (clone HI100), peridinin chlorophyll protein (PerCP) conjugated anti-CD8 (clone SK1) and allophycocyanin (APC) conjugated anti-CD45RO (clone UCHL1). Labelling was carried out for 30 min on ice and the cells subsequently washed once in 1% RPMI containing 1% FCS. Four-colour flow cytometric analysis was carried using a FACScalibur flow cytometer with CellQuest Pro analysis software (Becton Dickinson, Oxford, UK). Side scatter versus forward scatter plots were used to gate on the lymphocyte population and 30,000 lymphocyte events were acquired per analysis. Cells incubated with appropriate fluorescent-labelled isotype control antibodies were used to define the thresholds of positive staining for each marker. Subsequent quadrant analysis on dot plots was applied to assess the proportion of cells expressing CD4 and CD8 and co-expressing CD45RA and CD45RO in both the original PBMC suspension and in the population of migrated cells. The CD8+ population was taken as the bright population only (typically 100% CD3+), rather than including the small percentage of CD8dim cells, which typically contain a high proportion of CD3- cells.

*Plasma glucose and cortisol.* Heparinised plasma was obtained from blood collected into the lithium heparin monovette. This was spun at 1500 g for 10 min in a

refrigerated centrifuge (4°C) within 5 min of sampling. The plasma obtained was immediately stored at -80°C for later analysis. Plasma cortisol and glucose concentrations were determined in duplicate using ELISA (EIA-1887, IDS, Boldon, UK) and colorimetric (GOD-PAP method, Randox Laboratories, Co. Antrim, N. Ireland) methods, respectively. For each assay all samples were analysed on the same day. The intra-assay co-efficient of variation was 1.7% for cortisol and 1.6% for glucose.

*Statistical Analysis.* Data were analysed using a two-factor (trial x time) ANOVA with repeated measures design. Assumptions of homogeneity and sphericity in the data were checked and, where appropriate, adjustments in the degrees of freedom for the ANOVA were made using the Huynh-Feldt method of correction. Any significant F ratios subsequently shown were assessed using Student's paired t-tests with Holm-Bonferroni correction for multiple comparisons applied to the unadjusted P value. P values in the text are those from the post-hoc tests. Single comparisons between trials for overall exercise intensity were made using Student's paired t-tests. Values in the text, table and figures are means (SD).

## RESULTS

*Physiological and metabolic measurements.* Relative exercise intensity was similar in both trials; %  $\dot{V}O_{2peak}$  during exercise was 60.9 (4.8)% and 62.4 (4.9)% in the CHO and PLA trials, respectively. Likewise, heart rates were similar throughout the exercise (CHO:  $148 \pm 13$  beat·min<sup>-1</sup>, PLA:  $149 \pm 13$  beat·min<sup>-1</sup>). After exercise body mass losses (corrected for fluid intake) were similar in both trials ( $2.5 \pm 0.2$  kg and  $2.3 \pm 0.3$  kg on CHO and PLA, respectively). There was no significant time x trial interaction for changes in plasma volume relative to the first blood sample; after exercise plasma volume had decreased by  $7.6 \pm 2.7\%$  and  $8.6 \pm 3.7\%$  in CHO and PLA, respectively. Plasma

Table 1: Plasma glucose, cortisol and total and differential blood leukocyte responses to 2 h of treadmill running at 60%  $\dot{V}O_{2peak}$  with and without CHO ingestion. Values are means (SD). n=7.

	pre-exercise	post-exercise	1 h post-exercise
<i>plasma glucose (mmol.l<sup>-1</sup>)</i>			
CHO	5.0 (0.4)	5.7 (0.9)*	4.4 (0.8)
PLA	5.2 (0.5)	5.0 (0.9)	5.0 (0.6)
<i>plasma cortisol (nmol.l<sup>-1</sup>)</i>			
CHO	521 (151)	398 (137)	339 (156)
PLA	518 (130)	550 (157)	453 (98)
<i>circulating cell count (x10<sup>9</sup>.l<sup>-1</sup>)</i>			
<i>leukocytes</i>			
CHO	4.9 (0.9)	6.2 (2.0)*	6.6 (2.0)
PLA	4.7 (0.8)	8.2 (2.9)†	9.8 (3.7)†
<i>lymphocytes</i>			
CHO	2.0 (0.5)	2.2 (0.6)	1.7 (0.2)†
PLA	1.8 (0.3)	2.7 (0.9)†	1.4 (0.3)†
<i>monocytes</i>			
CHO	0.6 (0.2)	0.8 (0.3)	0.8 (0.3)
PLA	0.6 (0.2)	0.8 (0.3)	0.7 (0.2)

\*significantly different from PLA, P<0.05

†significantly different to pre-exercise within trial, P<0.05

glucose was higher in CHO than PLA at post-exercise (interaction  $F_{2,12}=7.8$ ,  $P<0.05$ ; Table 1). Plasma cortisol concentration decreased in line with diurnal variations yet was higher in PLA than CHO (main effect for trial,  $F_{1,6}=10.5$ ,  $P<0.05$ ). The interaction between trial and time approached significance ( $F_{2,12}=3.7$ ,  $P=0.057$ , Table 1).

*Total leukocyte and PBMC counts.* Total leukocyte and PBMC counts are shown in Table 1. Total numbers of circulating leukocytes were lower in CHO than PLA after exercise (interaction  $F_{2,12}=8.2$ ,  $P<0.05$ ). Circulating lymphocyte count did not differ between trials at any specific time before or after exercise. However, compared with pre-exercise values within each trial, a lymphocytosis was apparent after exercise in PLA only and a lymphopenia was evident at 1 h post-exercise in both CHO and PLA trials (interaction,  $F_{2,12}=7.5$ ,  $P<0.05$  for all). Monocyte numbers increased after exercise (main effect for time,  $F_{2,12}=4.3$ ,  $P<0.05$ ) but there was no interaction effect or main effect for trial.

*Migration of T lymphocytes towards HRV infected BEAS2-B supernatants.* All data relating to T lymphocyte migration are expressed as a percentage change relative to the corresponding pre-exercise value. Data for all migration towards HRV infected BEAS2-B supernatants are for  $n=6$  owing to apparent contamination in the samples from one subject on the CHO trial. For all samples T lymphocyte migration towards the HRV infected BEAS2-B supernatants was consistently higher than migration towards

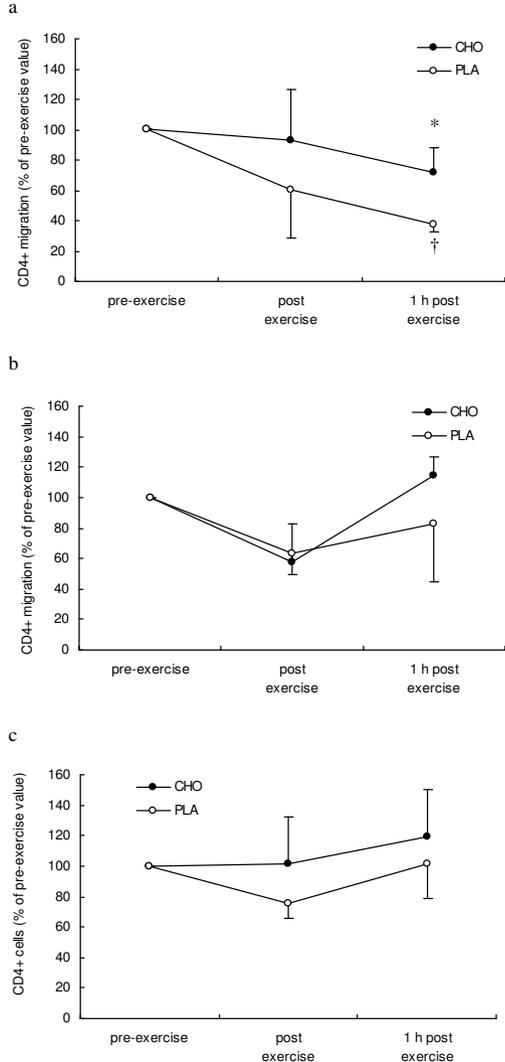


Fig. 1. Migration of CD4+ cells towards HRV (a) infected ( $n=6$ ) and (b) uninfected ( $n=7$ ) BEAS2-B supernatants and (c) proportion of CD4+ cells in PBMC suspension prior to migration ( $n=7$ ). Values are means  $\pm$  SD. \* significantly higher on CHO than PLA at that time,  $P<0.05$ ; † significantly lower than resting value,  $P<0.01$

the uninfected (control) BEAS2-B supernatants (range 64-88% higher for CD4+ and their CD45 RA and RO subsets, 56-170% higher for CD8+ and their CD45 RA and RO subsets).

*Migration of CD4+, CD4+CD45RA+ and CD4+CD45RO+ cells towards HRV infected and uninfected BEAS2-B supernatants.* Migration of CD4+ cells towards HRV infected BEAS2-B supernatants was 35% higher 1 h after exercise when

subjects ingested CHO during exercise than when they ingested PLA (interaction,  $F_{2,10}=4.6$ ,  $P<0.05$ ). At this time, a significant decrease in migration from pre-exercise values was observed in PLA only ( $P<0.01$ ) (Fig. 1a). Migration towards uninfected cell supernatants demonstrated no effect of trial, but was lower immediately post-exercise compared with pre-exercise (main effect for time,  $F_{2,12}=27.0$ ,  $P<0.001$ ) (Fig. 1b). There was no significant interaction effect or main effects for trial or time for the proportion of CD4 cells within the PBMC population (i.e. the proportion of CD4+ cells that were available to migrate) relative to pre-exercise values (Fig. 1c).

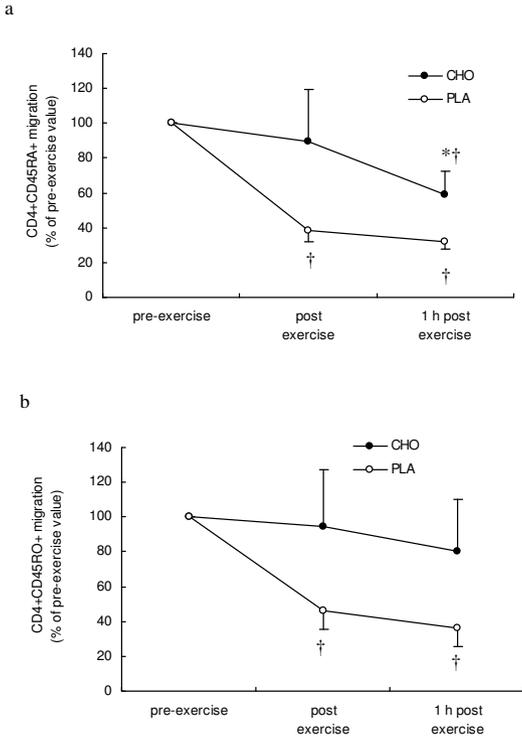


Fig. 2. Migration of (a) CD4+CD45RA+ and (b) CD4+CD45RO+ cells towards HRV infected BEAS2-B supernatants (n=6). Values are means ± SD. \* significantly higher on CHO than PLA at that time,  $P<0.05$ ; † significantly lower than resting value,  $P<0.01$

yet values were ~30% higher on CHO than PLA at this time ( $P<0.05$ ). Migration of this subset was also lower at post-exercise compared with pre-exercise in PLA only ( $P<0.01$ ) (Fig. 2a). There was no interaction effect or main effects for trial or time for CD4+CD45RA+ cells migrating towards uninfected BEAS2-B supernatants or for the proportion of these cells within the PBMC population (data not shown).

Migration of CD4+CD45RO+ cells on the PLA trial towards HRV infected cell supernatants fell immediately post-exercise and at 1 h post-exercise relative to

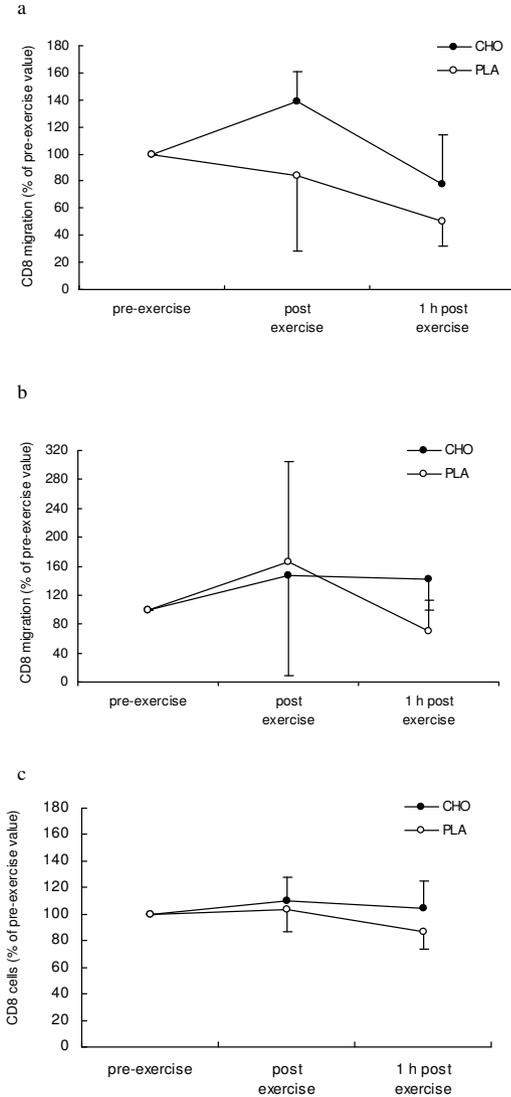


Fig. 3. Migration of CD8+ cells towards HRV (a) infected (n=6) and (b) uninfected (n=7) BEAS2-B supernatants and (c) proportion of CD8+ cells in PBMC suspension prior to migration (n=7). Values are means  $\pm$  SD.

pre-exercise values (interaction,  $F_{2,10}=6.1$ ,  $P<0.01$  for both trials). Migration of CD4+CD45RO+ cells on the CHO trial did not change over time but was higher on CHO than PLA (main effect for trial,  $F_{1,5}=10.8$ ,  $P<0.05$ ) (Fig. 2b). There was no interaction effect or main effects for trial or time for CD4+CD45RO+ cells migrating towards uninfected BEAS2-B supernatants or for the proportion of these cells within the PBMC population (data not shown).

*Migration of CD8+, CD8+CD45RA+ and CD8+CD45RO+ cells towards HRV infected and uninfected BEAS2-B supernatants.* There was no significant interaction effect for migration of CD8+ cells towards HRV infected BEAS2-B supernatants, but migration was lower at 1 h post-exercise compared with pre-exercise (main effect for time,  $F_{2,10}=13.9$ ,  $P<0.05$ ). In addition, migration was higher on CHO than PLA (main effect for trial,  $F_{1,5}=8.0$ ,  $P<0.05$ ) (Fig. 3a). There was no interaction effect or main effects for trial or time for CD8+ cells migrating towards uninfected BEAS2-B supernatants (Fig. 3b) or for the proportion of these cells within the PBMC population (Fig. 3c).

There were no significant interaction effects for migration of CD8+CD45RA+ or CD8+CD45RO+ cells towards HRV infected BEAS2-B supernatants. For both T cell subsets migration fell by 1 h post-exercise compared with pre-exercise values (main effect for time, CD8+CD45RA+  $F_{2,10}=7.9$ ,  $P<0.05$ ; CD8+CD45RO+  $F_{2,10}=5.0$ ,  $P<0.05$ ). In addition, migration was higher on CHO than PLA for both

subsets (main effect for trial,  $CD8+CD45RA+F_{1,5}=29.6$ ,  $P<0.01$ ;  $CD8+CD45RO+F_{1,5}=7.6$ ,  $P<0.05$ ) (Fig. 4a and b). There were no interaction effects or main effects for trial or time for  $CD8+CD45RA+$  or  $CD8+CD45RO+$  cells migrating towards uninfected BEAS2-B supernatants or for the proportion of these cells within the PBMC population (data not shown).

To summarise, there was a decrease in migration of  $CD4+$  and  $CD8+$  cells and their  $CD45RA+$  and  $CD45RO+$  subpopulations towards supernatants from HRV-infected airway epithelial cells following exercise. Migration of  $CD4+$  cells and  $CD4+CD45RA+$  cells was  $\sim 35\%$  and  $\sim 30\%$  higher, respectively, on CHO than PLA at 1 h post-exercise. For all other subpopulations, migration on was greater

on the CHO trial compared with PLA (main effect of trial only). There was little effect of exercise or CHO on migration of these cell types towards uninfected (control) cell supernatants or on the proportion of these cells within the peripheral blood mononuclear cell population.

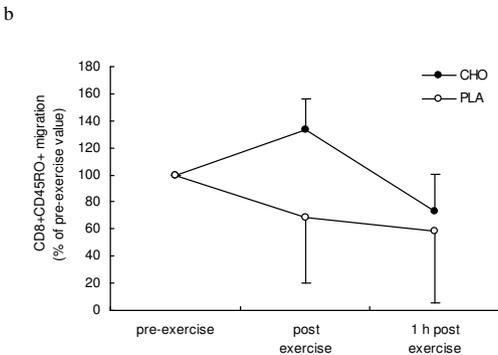
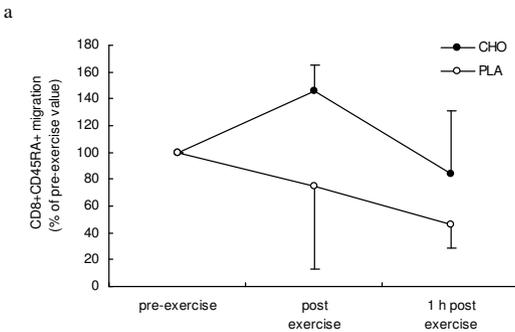


Fig. 4. Migration of (a)  $CD8+CD45RA+$  and (b)  $CD8+CD45RO+$  cells towards HRV infected BEAS2-B supernatants ( $n=6$ ). Values are means  $\pm$  SD.

and trial evident for all subpopulations. These effects were apparent despite little effect of exercise or CHO ingestion on migration towards supernatants from uninfected bronchial epithelial cells or on the proportion of these cells within the PBMC suspension. Therefore, these effects could not be accounted for by spontaneous movement of T cells or by the availability of  $CD4+$  and  $CD8+$  cells and their naïve and memory subpopulations in the PBMC suspension.

## DISCUSSION

The present study demonstrated that prolonged physical stress was associated with reduced *ex vivo* migration of  $CD4+$  and  $CD8+$  cells and their  $CD45RA+$  and  $CD45RO+$  subpopulations towards the supernatants of a HRV-infected human bronchial epithelial cell line and this decrease was attenuated by CHO ingestion. An interaction between exercise and trial was evident for  $CD4+$ ,  $CD4+CD45RA+$  and  $CD4+CD45RO+$  cells with main effects for both exercise

Migration of T lymphocytes is a tightly-regulated process involving both the release of chemokines and the expression of chemokine receptors on T cells, and expression of adhesion molecules on both lymphocytes and tissue cells. Altered adhesion molecule expression and/or recruitment of T lymphocytes expressing specific adhesion molecules is one important factor influencing the marked redistribution of these cells within lymphoid and non-lymphoid tissue in response to intensive physical stress (16) and infection/inflammation (21). However, because there were no endothelial cells on the migration membrane in the present study, our findings suggest that physical stress reduces migration of T cells towards HRV-infected bronchial epithelial cells in a manner that is independent of adhesion molecule expression. Under conditions of tissue infection, T cell migration into airway submucosa is regulated by a gradient of chemokines derived from the infected cells. Therefore, in the present study, T lymphocyte expression of an array of chemokine receptors complementary to the chemokine cocktail produced by the HRV-infected bronchial epithelial cell line (25) would have been required in order for the T cells to migrate at a rate above that occurring spontaneously. Consequently, in the absence of any involvement of cellular adhesion, the findings of the present study appear to suggest that physical stress acts to downregulate or inhibit the expression of chemokine receptors complimentary to the chemokines secreted by the infected airway epithelial cells, thereby reducing T cell migration. Alternatively, strenuous exercise may desensitise chemokine receptors with little effect on the actual level of chemokine receptor expression.

Exercise-induced elevations in plasma cortisol levels have been implicated in the modifications of T lymphocyte trafficking and function associated with intensive physical stress (13,22). In the present study, plasma cortisol concentrations decreased in line with expected circadian fluctuations but were higher when subjects ingested PLA than when CHO was ingested, suggesting some exercise-induced cortisol release. Recent work suggests that 72 h exposure to plasma cortisol concentrations induced by 90 min cycling at 70%  $\dot{V}O_{2max}$  increased CD4+ and CD8+ cell migration towards stromal cell-derived factor-1 $\alpha$ /CXCL12 (24), which is constitutively expressed by human airway epithelial cells. Post-exercise plasma cortisol concentrations were positively related to expression of the receptor for CXCL12, CXCR4, on CD4+ and CD8+ T lymphocytes (24). These observations contrast with our finding of reduced migration of CD4+ and CD8+ cells towards the supernatants of HRV-infected bronchial epithelial cells following 2 h exercise at 60%  $\dot{V}O_{2peak}$ . However, it is difficult to make comparisons between these studies as recent work suggests that human epithelial cell production of CXCL12 is not substantially induced by HRV infection *in vitro* or *in vivo*, in contrast to that of several chemokines known to be involved in T cell migration (25). This aside, it is also likely that differences in both the duration of exposure to cortisol and the circulating concentrations of the hormone would influence any potential effect of cortisol here. In the study of Okutsu *et al.* (24) migration towards CXCL12 was determined after T cells had been exposed to elevated cortisol concentrations for 72 h *in vitro*; it is unlikely that circulating lymphocytes would be exposed to elevated plasma cortisol concentrations for 72 h *in vivo*. The effect of a shorter period of exposure was not determined by those authors. In the present study, the effects of the exercise (and therefore exposure to altered physi-

ological concentrations of cortisol) was observed immediately and 1 h post-exercise. This was because analysis of previous studies investigating the effect of both intensive exercise and CHO on lymphocyte function suggested that the major alterations in circulating lymphocyte populations and function would occur during this time in response to our protocol (5,13,15,28). It is unlikely that a longer period of observation, for example 2-4 h post-exercise, would have revealed a different migratory response because cortisol levels did not rise above resting values throughout either trial.

Physical stress activates the sympathetic nervous system. T lymphocytes express both  $\alpha$ - and  $\beta$ -adrenoreceptors on their surface and elevations in circulating concentrations of catecholamines have been implicated in altered lymphocyte subset distribution observed within the peripheral circulation with strenuous exercise (16). A role for adrenaline-mediated T lymphocyte migration with physical stress is supported by a recent report that intensive exercise in mice induced a release of T cells from the spleen and an accumulation of T lymphocytes throughout lung tissue that was completely abolished following administration of either  $\alpha$ - and  $\beta$ -adrenoreceptor antagonists (17). The authors suggested that the exercise-induced homing to lung tissue might reflect enhanced immune vigilance following physical stress, particularly because increased lung ventilation rates at higher exercise intensities could potentially increase exposure to airborne pathogens. However, our findings suggest that strenuous exercise actually impairs T cell migration towards HRV- infected airway epithelial cells. Therefore, any involvement of adrenergic mechanisms is unlikely in our *ex vivo* migration model. We assessed migration of T cells towards HRV-infected bronchial epithelial cells in a manner that is independent of adhesion molecule expression. Therefore, consideration of our findings with those of Krüger *et al.* (17) may suggest that adrenergic mechanisms act on cellular adhesion rather than chemokine receptor expression.

In the absence of any obvious neuroendocrine involvement in the observed exercise-induced decrease in migration of CD4+ and CD8+ cells and their CD45RA+ and CD45RO+ subpopulations towards HRV-infected bronchial epithelial cell supernatants, the potential mechanisms underlying this effect remain unclear. However, it is known that the potent T cell chemoattractant RANTES is capable of downregulating the expression of chemokine receptor CCR5 on T cells, via receptor internalisation (1). This may serve as a negative feedback mechanism to terminate accumulation of T cells in infected or inflamed tissue. With this in mind, it is tempting to consider that downregulation of chemokine receptor expression via negative feedback mechanisms may be contributing to the overall effect of exercise on T cell migration observed here. Indeed, increased ventilation rates with strenuous exercise are responsible for airway hyperosmolarity which is known to stimulate the release of RANTES by bronchial epithelial cells and this is suggested to contribute to exercise-induced lymphocyte migration to the airways from the peripheral circulation (3).

The effect of physical stress on T lymphocyte migration towards HRV-infected cells was similar for both CD4+ and CD8+ cells and their CD45RA+ and CD45RO+ subpopulations, although the responses were more varied for CD8+

cells. The general pattern of a decline in migration occurred despite circulating naïve T cells and memory/effector cells having diverse migratory properties. In addition to CD45RA, naïve T cells can be identified by their expression of the chemokine receptor CCR7 and preferentially migrate towards secondary lymphoid tissues, although their ability to migrate towards non-lymphoid tissue has been recently demonstrated in mice (6). In contrast CD45RO+ T cells can be further subdivided into CCR7- effector and effector memory cells that have immediate effector function and preferentially migrate towards non-lymphoid tissue, and CCR7+ central memory subsets that lack immediate effector function (yet respond rapidly upon subsequent exposure) and preferentially migrate towards lymphoid tissue (21). This may suggest that in the present study there may have been differences in migration rates within the CD45RO+ subpopulation. However, any selective effect of exercise on migration of memory/effector T cells towards infected tissue remains unknown because chemokine receptor expression was not determined here.

An important finding of this study was that CHO ingestion attenuated the exercise-induced impairment of T lymphocyte migration towards the supernatants of a HRV-infected bronchial epithelial cell line. This effect was evident for all T cell subpopulations measured (main effect for trial), but was more distinct for CD4+ and CD4+CD45RA+ cells when migration under conditions of CHO ingestion was around 30% higher at 1 h post-exercise than when placebo had been ingested. However, given that our *ex vivo* model suggests that exercise impairs T cell migration towards infected tissue via altered chemokine receptor expression, it would appear that CHO acts to somehow lessen this effect. The underlying reasons for this are unclear. Any involvement of the hormonal candidates commonly associated with the modulating effects of CHO on other cellular aspects of immune function can not be easily justified. A CHO-influenced attenuation of plasma cortisol concentration has been implicated in several studies reporting that CHO ingestion during physical stress blunts exercise-induced impairments of several immune cell functions (13,23). However, other studies report that CHO ingestion lessens the effect of exercise on antigen (5) and mitogen (11)-stimulated T cell functions independently of any effect on plasma cortisol concentration. Although here plasma cortisol levels fell in line with expected circadian variations, concentrations were lower when CHO was ingested compared with PLA (main effect for trial), suggesting an attenuation of the (albeit limited) endogenous cortisol release. However, without any clear involvement of cortisol in the observed exercise-induced decrease in T cell migration (as discussed above), it is unlikely that this is the mechanism by which CHO is modulating the T cell migratory response. Likewise, exercise-induced elevations in plasma levels of adrenaline are attenuated by CHO ingestion (20) yet are unlikely to play a key role here for similar reasons.

In addition to an indirect effect via altered stress hormone release during exercise, glucose availability could also exert a direct effect on the ability of T lymphocytes to migrate towards infected tissue. Glucose is a key fuel source for T lymphocytes with T cell receptor activation associated with elevated GLUT 1 receptor expression and glucose metabolism (32). Studies of both hyper- and hypoglycaemic

conditions suggest that altered glucose homeostasis can influence the migratory properties of T lymphocytes (10,26,27,33), although any effect on migration towards infected tissue is unknown. In the present study, plasma glucose concentrations were significantly higher under conditions of CHO ingestion during exercise compared with placebo ingestion. However, the glucose response observed under both conditions was still well within the normal physiological range ( $\sim 4\text{-}6\text{ mmol}\cdot\text{l}^{-1}$ ) and therefore it is unlikely that the effect of CHO ingestion on post-exercise T cell migration towards infected tissue can be accounted for by any direct effect of glucose availability.

To summarise, this study demonstrates that physical stress reduces the ability of CD4+ and CD8+ T cells and their CD45RA+ and CD45RO+ subpopulations to migrate towards the supernatants of a HRV-infected bronchial epithelial cell line and this is attenuated by CHO ingestion during exercise. The *ex vivo* model used here suggests that these effects are related to an exercise-induced alteration in T cell chemokine receptor expression, although the precise mechanisms by which physical stress and CHO ingestion are acting remain unclear. While comparison with the available literature suggests that any involvement of cortisol or catecholamines can likely be discounted, it is tentatively suggested that the combination of exercise-induced chemokine exposure and exposure to the same chemokines released from the infected bronchial epithelial cells may induce a downregulation of the receptors for these chemokines on T cells, thereby inhibiting T cell migration into infected tissue. Finally, it should be noted that the outcome of any changes in *ex vivo* migration on *in vivo* anti-viral immunity are unknown but future studies to assess chemokine receptor expression on pre- and post-migrated cells and the antigen-stimulated functional capacity of migrated cells would provide further insight.

## ACKNOWLEDGMENT

This study was supported by a grant from GlaxoSmithKline Nutritional Healthcare.

## REFERENCES

1. Amara, A., S. L. Gall, O. Schwartz, J. Salamero, M. Montes, P. Loetscher, M. Baggiolini, J. L. Virelizier, and F. Arenzana-Seisdedos. HIV coreceptor downregulation as antiviral principle: SDF-1 alpha-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. *J Exp Med.* 186:139-146, 1997.
2. Atanackovic, D., B. Schnee, G. Schuch, C. Faltz, J. Schulze, C. S. Weber, P. Schafhausen, K. Bartels, M. C. Brunner-Weinzierl, and H. C. Deter. Acute psychological stress alerts the adaptive immune response: stress-induced mobilization of effector T cells. *J Neuroimmunol.* 176:141-152, 2006.

3. Bermon, S. Airway inflammation and upper respiratory tract infection in athletes: is there a link? *Exerc Immunol Rev.* 13:6-14, 2007.
4. Bhakri, H. L., H. Jones, D. A., Jones, K. W. Pettingale, and T.E. Tee. T cell subpopulation dynamics following insulin-induced hypoglycaemia in normal subjects. *Clin Exp Immunol.* 53:83-87, 1983.
5. Bishop, N. C., G. J. Walker, L. A. Bowley, K. F. Evans, K. Molyneux, F. A. Wallace, and A. C. Smith. Lymphocyte responses to influenza and tetanus toxoid in vitro following intensive exercise and carbohydrate ingestion on consecutive days. *J Appl Physiol.* 99:1327-1335, 2005.
6. Cose, S., C. Brammer, K. M. Khanna, D. Masopust, and L. Lefrançois. Evidence that a significant number of naïve T cells enter non-lymphoid organs as part of a normal migratory pathway. *Eur J Immunol.* 36:1423-1433, 2006.
7. Dill, D. B. and D. L. Costill. Calculation of percentage changes in volumes of blood, plasma and red cells in dehydration. *J Appl Physiol.* 37:247-248, 1974.
8. Fahlman, M. M. and H. Engels. Mucosal IgA and URTI in American college football players: A year longitudinal study. *Med Sci Sports Exerc.* 37:374-380, 2005.
9. Fisher, B. M., and J. G. Smith, D. C. McCrudden, and B. M. Frier. Responses of peripheral blood cells and lymphocyte subpopulations to insulin-induced hypoglycaemia in human insulin-dependent (Type 1) diabetes. *Eur J Clin Invest.* 17:208-213, 1987.
10. Gappa-Fahlenkamp, H. and A. S. Shukla. The effect of short-term, high glucose concentration on endothelial cells and leukocytes in a 3D in vitro human vascular tissue model. *In Vitro Cell Dev Biol Anim.* Epub ahead of print, 2009.
11. Green, K. J., S. J. Croaker, and D. G. Rowbottom. Carbohydrate supplementation and exercise-induced changes in T-lymphocyte function. *J Appl Physiol.* 95:1216-1223, 2003.
12. Haslinger, B., S. Mandl-Weber, A. Sellmayer, S. R. Lederer, and T. Sitter. Effect of high glucose concentration on the synthesis of monocyte chemoattractant protein-1 in human peritoneal mesothelial cells: Involvement of protein kinase C. *Nephron.* 87:46-351, 2001.
13. Henson, D. A., D. C. Nieman, J. C. D. Parker, M. K. Rainwater, D. E. Butterworth, B. J. Warren, A. Utter, J. M. Davis, O. R. Fagoaga, and S.L. Nehlsen-Cannarella. Carbohydrate supplementation and the lymphocyte proliferative response to long endurance running. *Int J Sports Med.* 19:574-580, 1998.
14. Janssen U., E. Sowa, P. Marchand, J. Floege, A. O. Phillips, and H. H. Radeke. Differential expression of MCP-1 and its receptor CCR2 in glucose primed human mesangial cells. *Nephron.* 92:797-806, 2002.
15. Kendall, A., L. Hoffman-Goetz, M. Houston, B. MacNeil, Y. Arumugam. Exercise and blood lymphocyte subset responses: intensity, duration and subject fitness effects. *J Appl Physiol.* 69: 251-260, 1990.
16. Krüger, K. and F. C. Mooren. T cell homing and exercise. *Exerc Immunol Rev.* 13:37-54, 2007.
17. Krüger, K., A. Lechtermann, M. Fobler, K. Völker, and F. C. Mooren. Exercise-induced redistribution of T lymphocytes is regulated by adrenergic mechanisms. *Brain Behav Immun.* 22:324-338, 2008.
18. Kurokawa, Y., S. Shinkai, J. Torii, S. Hino, and P. N. Shek. Exercise-induced changes in the expression of surface adhesion molecules on circulating granulocytes

- and lymphocytes subpopulations. *Eur J Appl Physiol Occup Physiol.* 71:245-252, 1995.
19. Levandowski, R. A., C. W. Weaver, G. G. Jackson. Nasal secretion leukocyte populations determined by flow cytometry during acute rhinovirus infection. *J Med Virol.* 25:423-432, 1988.
  20. Li T. L. and M. Gleeson. The effects of carbohydrate supplementation during the second of two prolonged cycling bouts on immunoendocrine responses. *Eur J Appl Physiol.* 95:391-399, 2005.
  21. Marelli-Berg, F. M., L. Cannella, F. Dazzi, and V. Mirenda. The highway code of T cell trafficking. *J Pathol.* 214:179-189, 2008.
  22. Nieman, D. C., A. R. Miller, D. A. Henson, B. J. Warren, G. Gusewitch, R. L. Johnson, J. M. Davis, D. E. Butterworth, J. L. Herring, and S. L. Nehlsen-Cannarella. Effect of high- versus moderate-intensity exercise on lymphocyte subpopulations and proliferative response. *Int J Sports Med.* 15:199-206, 1994.
  23. Nieman, D. C., S. L. Nehlsen-Cannarella, O. R. Fagoaga, D. A. Henson, A. Utter, J. M. Davis, F. Williams, and D. E. Butterworth. Effects of mode and carbohydrate on the granulocyte and monocyte response to intensive, prolonged exercise. *J Appl Physiol.* 84:1252-1259, 1998.
  24. Okutsu, M., K. Ishii, K. J. Niu, and R. Nagatomi. Cortisol-induced CXCR4 augmentation mobilizes T lymphocytes after acute physical stress. *Am J Physiol Regul Integr Comp Physiol.* 288:R591-R599, 2005.
  25. Proud D., R. B. Turner, B. Winther, S. Wiehler, J. P. Tiesman, T. D. Reichling, K. D. Juhlin, A. W. Fulmer, B. Y. Ho, A. A. Walanski., C. L. Poore, H. Mizoguchi, L. Jump, M. L. Moore, C. K. Zukowski and J. W. Clymer. Gene expression profiles during in vivo human Rhinovirus infection: insights into the host response. *Am J Respir Crit Care Med.* 178:962-968, 2008.
  26. Ruster, C. and G. Wolf. The role of chemokine receptors in diabetic nephropathy. *Front Biosci.* 13:944-955, 2008.
  27. Shek, P. N., B. H. Sabiston, A. Buguet and M. W. Radomski. Strenuous exercise and immunological changes: a multiple-time-point analysis of leukocyte subsets CD4/CD8 ratio, immunoglobulin production and NK cell response. *Int J Sports Med* 16:466-474, 1995.
  28. Shanmugam, N., M. A. Reddy, M. Guha and R. Natarajan. High glucose-induced expression of proinflammatory cytokine and chemokine genes in monocytic cells. *Diabetes.* 52:1256-1264, 2003.
  29. Shephard, R. J. Adhesion molecules, catecholamines and leucocyte redistribution during and following exercise. *Sports Med.* 33:261-284, 2003.
  30. Spence, L., W. J. Brown, D. B. Pyne, M. D. Nissen, T. P. Sloots, J. G. McCormack, A. S. Locke, and P.A. Fricker. Incidence, etiology, and symptomatology of upper respiratory illness in elite athletes. *Med Sci Sports Exerc.* 39:577-586, 2007.
  31. Spurrell, J. C. L., S. Wiehler, R. S. Zaheer, S. P. Sanders and D. Proud. Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection. *Am J Physiol Lung Cell Mol Physiol* 289:L85-L95, 2005.
  32. Swainson, L., S. Kinet, N. Manel, J.-L. Battini, M. Sitbon, and N. Taylor. Glucose transporter-1 expression identifies a population of cycling CD4+CD8+ human thymocytes with high CXCR4-induced chemotaxis. *Proc Natl Acad Sci USA.* 102:12867-12872, 2005.

144 • T lymphocyte migration following exercise and carbohydrate intake

33. van Tits, L. J., A. Daul, H. J. Bauch, H. Grosse-Wilde, M. Happel, M. C. Michel, and O.E. Brodde. Effects of insulin-induced hypoglycemia on beta 2-adrenoceptor density and proliferative responses of human lymphocytes. *J Clin Endocrinol Metab.* 71:187-192, 1990.