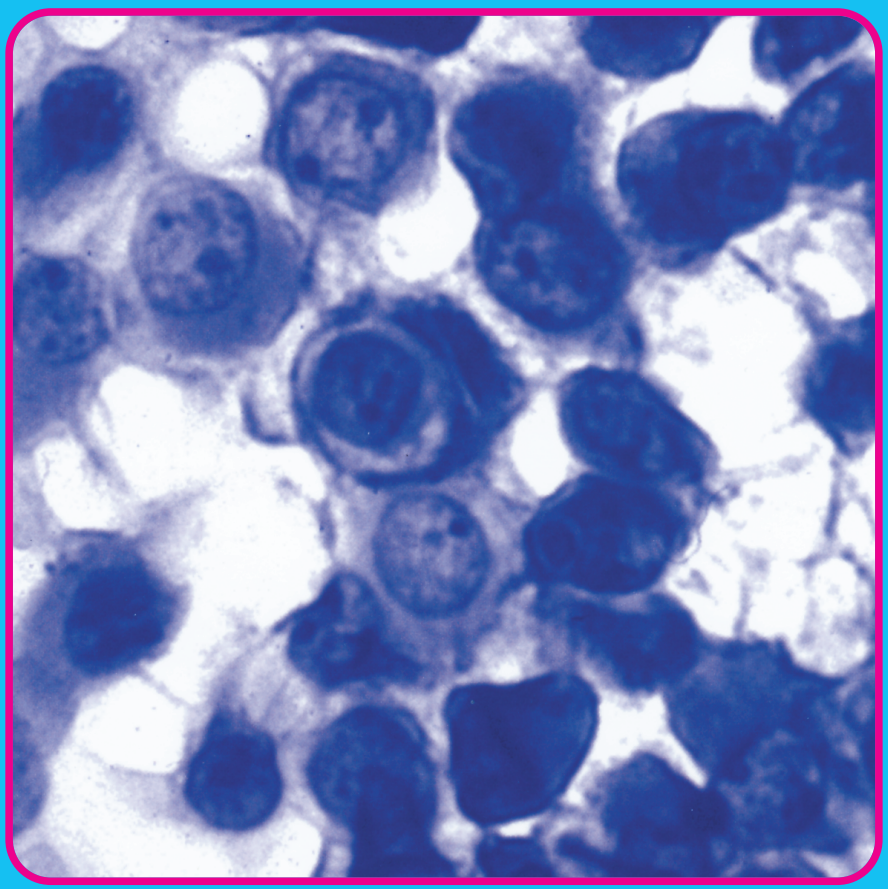
EXERCISE IMMUNOLOGY REVIEW



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EXERCISE IMMUNOLOGY REVIEW

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Hinnak Northoff
for the editorial team and all our readers

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Editorial Statement

Exercise Immunology Review, an official publication of the International Society of Exercise Immunology and of the German Society of Sports Medicine and Prevention, is committed to developing and. enriching knowledge in all aspects of immunology that relate to sport, exercise, and regular physical ativity. In recognition of the broad range of disciplines that contribute to the understanding of immune function, the journal has adopted an interdisciplinary focus. This allows dissemination of research findings from such disciplines as exercise science, medicine, immunology, physiology, behavioral science, endocrinology, pharmacology, and psychology.

Exercise Immunology Review publishes review articles that explore: (a) fundamental aspects of immune function and regulation during exercise; (b) interactions of exercise and immunology in the optimization of health and protection against acute infections: (c) deterioration of immune function resulting from competitive stress and overtraining; (d) prevention or modulation of the effects of aging or disease (including HIV infection; cancer; autoimmune, metabolic or transplantation associated disorders) through exercise. (e) instrumental use of exercise or related stress models for basic or applied research in any field of physiology, pathophysiology or medicine with relations to immune function.

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From the editors

This year´s issue of EIR starts with 3 reports on cytokine production following exercise, each approaching the subject from a different perspective. The first by Abbasi et al describes exercise effects on LPS-stimulated ex vivo cultures and finds significant differences between sexes. The second by Sugama et al provides evidence that plasma and urinary cytokine concentrations differ markedly and raises the possibility that renal damage may be involved in part. The third by Vaisberg et al compares cytokines in sera and nasal mucosa and finds links to upper airway inflammation for nasal cytokines.

The next two reports share a common focus on neutrophils and their functions. In the work by Sasaki et al, active subjects, while showing signs of elevated oxidative stress, also presented elevated IgM antibodies against reactive carbonyl derivates, a possible mechanism of vascular protection by exercise. Kanda et al extensively describe the effects of eccentric exercise / DOMS on cytokine production and neutrophil mobilization and migration.

The more clinical part of this issue starts with two reports which share a common interest in nutritive aspects of exercise immunology. He et al present results from a huge study on endurance athletes showing that a vitamin D deficient status was significantly associated with increased susceptibility to URTI. On the other hand no effects on immunity were found by West et al in physically active individuals taking a butylrilated starch supplement.

The last two papers are clinical reviews. Kruijsen-Jaarsma et al present a systematic review, evaluating the effects of exercise on immunity in cancer patients and in cancer survivors. The review by Leicht et al describes what is known on the immune response to exercise in persons with spinal cord injury - to my knowledge the first of its kind.

Finally, we publish an open letter from Bortolini et al to Brazilian authorities of physical education, calling for a higher level of attention to Exercise Immunology. We happily endorse these activities and congratulate the country for hosting the first Brazilian Symposium on Sport Immunology and for the foundation of a national society of Exercise Immunology. Bravo!

For the editors

Hinnak Northoff

Changes in Spontaneous and LPS-induced ex vivo Cytokine Production and mRNA expression in Male and Female Athletes Following Prolonged Exhaustive Exercise

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Abstract

Purpose: The capacity of whole blood cultures to produce cytokines in response to endotoxin (LPS) was studied in athletes before, 30 min after, 3 h after and 24 h after a half-marathon run. **Methods:** Eight well trained men and 8 well trained women (6 of them in the late luteal phase of their cycle) participated. EDTA blood was incubated with or without LPS for 1 h, and cytokine concentration and gene expression were determined. To quantify LPS-dependent release on a per monocyte basis (LDR), the mean values of the difference (delta) between cytokine concentration in stimulated and unstimulated cultures, normalized to monocyte numbers, were calculated. **Results:** LDR of TNF-α was significantly reduced by exercise with identical kinetic in men and women. TNF-α mRNA expression was slightly down-regulated following exercise *($P<0.05$)*, but significantly so only in women. LDR of IL-6 was also reduced, but with a faster kinetic in women than in men. Similarly, 30 min post-exercise; LDR and spontaneous release of IL-1ra were significantly less in women than men. Concomitantly, IL-1ra mRNA was significantly elevated in unstimulated and in stimulated cultures in men only. IL-10 and IL-10 mRNA were significantly induced 30 min following exercise in absence of any detectable LDR. Women showed significantly lower

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levels than men. LDR and spontaneous release of IL-8 was enhanced in men and TGF-β1 in women. A significant up-regulation was seen in unstimulated IL-8 mRNA for women and LPS-stimulated IL-8 mRNA expression for men following exercise. Conclusion: Altogether, LPS-dependent *ex vivo* cytokine release was strongly influenced by exercise and these changes could only in part be attributed to changes in messenger RNA. Results for IL-1ra, IL-6 and IL-10 pointed to a less pronounced anti-inflammatory response in women as compared with men. Our results also indicate an early production of IL-10 by peripheral blood cells in response to exercise.

Key words: Lipopolysaccharide; Endotoxin; Ex vivo; Cytokine; Sex differences, Menstrual cycle;

Introduction

Several authors have theorized that the magnitude of change in immunity that occurs after each bout of prolonged exhaustive exercise in athletes has more clinical significance than training-induced alterations in resting immunity (21, 32). Indeed, prolonged intensive exercise in athletes is associated with impaired immune function. These changes during early recovery from exercise would appear to weaken the potential immune response to pathogens and have been suggested to provide an "open window" for infection (URTI), representing the most vulnerable time period for an athlete in terms of their susceptibility to contracting an infection (20, 32). Several investigations with animal models have provided important support of the "open window" theory. Davis et al. (6), for example, have shown that in mice alveolar macrophage anti-viral resistance is suppressed 8 h following prolonged strenuous exercise to fatigue, an effect due in part to increase circulating catecholamines. Investigations clearly linking the intensity of the post-exercise immunosuppression to the frequency of infections are however missing.

While numerous studies have investigated individual aspects of immune function after intense endurance exercise, the overall picture of the immunological changes in elite athletes is not clarified in detail. It has been documented that prolonged strenuous exercise not only induces pyrogenesis but also elicits mobilization and augmentation of neutrophil function and also some aspects of monocyte function, whereas it suppresses other parts of cellular immunity leading to increased susceptibility to infections. As mediators and regulators of these phenomena, cytokines released into the circulation, became a natural focus of attention (26, 29, 40). Reviews of several studies that have been performed on the cytokine reaction to strenuous exercise are available (28, 38). For example, increases in different cytokines, like interleukin (IL)-6, IL-8, IL-10 and IL-1 receptor antagonist (IL-1ra) and occasionally traces of TNF-α and IL-1β have been observed after prolonged, exhaustive endurance exercise in plasma or urine of athletes (29, 44), with levels falling back to normal on the following day. Data on exercise-induced changes in plasma cytokines describes the situation in the circulation but

do not necessarily characterize the effect of exercise on the cellular components of the immune system (9). In particular, IL-6 has been shown to be produced and released by contracting muscle cells (30, 33).

On the other hand, the capacity of leukocytes to produce cytokines upon adequate challenge is an interesting question with potentially far reaching consequences for the entire functional capacity of the immune system. It is highly likely to reflect the capacity of an individual to defend itself against intruding microorganisms. The influence of exercise on the cytokine production capacity can be measured by investigating the *in vitro* cytokine response to mitogens, antigens or endotoxin (LPS) in blood cell cultures set up before, and after exercise. LPS is a cell wall component of gram-negative bacteria which strongly activates toll-like receptor (TLR)-4 in mammals, resulting in release of tumour necrosis factor (TNF)-α, IL-1, IL-6 and an array of regulatory factors like IL-1ra, IL-10 and TGF-β (3). In this study we used LPS stimulation of whole blood cultures before, 30 min, 3 h, and 24 after a half-marathon and compared cytokine release to that of unstimulated control cultures. The supernatants of the latter thus represent mainly plasma levels plus eventual spontaneous production during the (short) culture period. This model probably comes closest to the natural environment avoiding artefacts from preparation and allowing natural interactions and also fast, stringent kinetics. Further, in contrast to previous studies we used a relatively short incubation time (1 h), investigated a wide spectrum of cytokines and evaluated the potential differences in cytokine reaction between sexes. Recent studies had shown that exercise induced gene expression differs in women – especially in their luteal phase - as compared to men (27). We hypothesized that in agreement with the literature we would find reduced TNF-α production following the half-marathon, but in addition changes in the capacity to produce anti-inflammatory cytokines, and differences in cytokine responses between sexes.

Methods and Materials

Subjects

Eight well-trained male athletes [34.8 ± 9.4 yr, body mass index (BMI) 23.4 ± 2.2 kg/m^2] and eight well-trained female athletes [38.5 ± 5.7 yr, body mass index (BMI)] 21.9 ± 1.0 kg/m^2] participated in the study. The individuals had been engaged in specific endurance training for at least 2 yr (52.2 km ± 25.5 km/week, running) (Table 1). None of the athletes suffered from acute or chronic diseases or reported intake of medication, including antioxidants and nicotine abuse. Informed written consent was obtained from each subject, and the study was approved by the University Ethics Committee. All were experienced athletes with normal dietary habits. The women included in the study had regular menstrual cycles and did not use oral contraception. We did not aim to select our female subjects from special menstrual cycle phase, but to know in which phase of menstrual cycle they are, the individual questionnaire was used and hormonal status of women was determined by measuring estrogen, progesterone, LH, and FSH using the ADVIA Centaur immunoassay system (Siemens Healthcare Diagnostics, Fer-

nwald, Germany). Interestingly, 6 of 8 female athletes were in the luteal phase of their menstrual cycle, and 2 other female subjects were on contraceptive use.

Preliminary Testing

One week before participating in the main study, the athletes performed an incremental exercise test on a treadmill (Saturn, HP Cosmos, Traunstein, Germany) to determine the running velocity (V_{IAT}) at the individual anaerobic threshold (IAT). Capillary blood for lactate measurement (EBIO, Eppendorf, Hamburg, Germany) was obtained from the earlobe after every stage and heart rate was monitored continuously using a heart rate monitor (Polar Electro, Finland). V_{IAT} was calculated by the method of Dickhuth et al (1991) (8) using a PC-routine.

Exercise program

All the athletes performed an official half marathon run under competition conditions (21.1 km). The run started at 10:00 AM on a cool and humid December day (1°C) and took place on a hilly and demanding terrain.

Blood sampling

Venous blood samples were drawn from the antecubital vein in a sitting position and collected into endotoxin-free K3-EDTA tubes (Vacuette, Greiner bio-one-Frickenhausen, Germany). Samples (a total of 20 ml whole blood) were obtained from each subject at times before (t0), 30 min after (t1), 3 h after (t2) and 24 h (t3) after the exercise run. Leukocyte numbers and differential counts before and after the run were determined using an automated Abbott Ruby Coulter counter.

In vitro stimulation of whole blood with LPS

Sixteen ml whole blood was cultured using a whole-blood culture system as developed in our laboratory. Briefly, 2 × 8-ml tubes containing K3-EDTA were drawn. One blood sample of each athlete was stimulated with lipopolysaccharide

Table 1. Physical characteristics of the subjects

	Men	***Women***
Age (yr)	34.8 ± 9.4	38.5 ± 5.7
Weight (kg)	77.8 ± 9.1	65.5 ± 6.3
Height (cm)	182.2 ± 4.1	168.7 ± 8.2
BMI (kg/m^2)	23.41 ± 2.2	21.9 ± 1
V_{IAT} (km/h)	13.6 ± 0.8	11.8 ± 1.1
Training volume (km/week)	49.3 ± 16.5	46.2 ± 16.6
Average running time (min)	95.5 ± 8	114 ± 12

Values are mean ± std; V_{IAT}, velocity at IAT; BMI, body mass index

(LPS) (Escherichia coli serotype 055:B5; Sigma, St Louis, MO, USA; final concentration 10 ng/ml). The other tube (spontaneous) was incubated after the addition of 8 μl PBS. Immediately after incubation of samples for 1 h at 37°C and slow rotation, both tubes were centrifuged at 1000 g for 10 min to obtain platelet-poor plasma. Aliquots were stored at -70°C until assay. The rest of the blood including cells was used to extract RNA.

Measurement of Blood Inflammatory Protein Markers

Plasma of stimulated and spontaneous (unstimulated) blood samples was analyzed for TNF-α; IL-1β, -1ra, -6, -8, -10, -12p40, -12p70; interferon (IFN)-γ; granulocyte-macrophage colony stimulating factor (GM-CSF) and MCP-1 at baseline (t0), 30 min after (t1), 3 h after (t2), and 24 h after (t3) the strenuous exercise using a multiplex bead-based assay (Human Multiplex Antibody Bead Kits for Millipore) according to the manufacturer's recommendations. The samples were measured using the antibody bead mix in duplicate with a biotinylated detection antibody followed by streptavidin-phycoerythrin. The plate was read using the Luminex XYP platform (Luminex, Austin, TX), and data were collected for 100 beads per cytokine from each well. The raw data (mean fluorescent intensity) were processed on Masterplex Quantitation software (MiraiBio, Alameda, CA) to obtain concentration values.

Measurement of plasma TGF-β1

The concentration of TGF-β1 in plasma of both stimulated and nonstimulated blood cultures was measured using a Quantikine human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (R & D systems, Minneapolis, MN, USA). Briefly, standards, controls and samples (50 μl) were pipetted into the pre-coated wells and any TGF-β1present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme linked polyclonal antibody specific for TGF-β1 was added to the wells to sandwich the TGF-β1 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of TGF-β1 bound in the initial step. The color development was stopped by adding stop solution, and the absorbance at 450 nm was detected via the use of a plate reader. The final concentration of the samples was extrapolated from the standard curve.

Total RNA isolation and cDNA synthesis

LPS-stimulated and unstimulated samples (2.5 ml/tube, 2 tubes/ subject) from male and female athletes were transferred into two PaxGene Blood RNA Tubes (PreAnalytix/Switzerland). Total RNA was isolated using the PaxGene Blood RNA kit (PreAnalytix/Switzerland) according to the manufacturer's protocol, with minor modifications. The concentration of the extracted RNA was measured spectrophotometrically (Nanodrop 1000/Thermo Scientific) and the quality was assessed by a lab-on-a-Chip-System on the Bioanalyzer 2100 (Agilent/Germany) to ensure that samples with intact 18s and 28s ribosomal RNA

peaks and low degradation factor were used for quantitative real-time PCR analysis. Five hundred nanograms (ng) of total RNA were used as a template for cDNA synthesis using the Transcriptor First-Strand cDNA Synthesis kit (Roche/Germany) with random hexamer primers. Reverse Transcription was performed at initial 25°C for 10 min, 50°C for 60 min, and 85°C for 5 min, followed by a quick chilling on ice. The cDNA was stored at -20°C and diluted 1:10 before PCR amplification.

Quantitative real-time PCR

The relative expression analysis for marker-specific mRNA was performed by quantitative real-time PCR (qRT-PCR). The PCR amplifications were detected using the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen/Germany) and the Primers for the cytokines TNF-α, IL-1ra, IL-6, IL-8, IL-0, TGF-β1 and housekeeping genes were designed with QuantPrime, Primer3 or PrimerBlast Software and synthesized by Metabion (Germany) (Table 2). The PCR reactions were per-

Table 2. Primer sequences for qRT-PCR

Target mRNA	NM	Forward primer (5'->3')	Reverse primer (5'->3')	product length [bp]
ACTB	NM 001101.3	TCCCTGGAGAAGAGCTACGA	AGGAAGGAAGGCTGGAAGAG	98
GAPDH	NM 002046.4	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	66
USP34	NM 014709.3	TTGGCTCGATTGGCTACCAGTG	TGGTCCATACCACACAGCTCAG	65
IL-1RN	NM 173842.2	GAAGATGTGCCTGTCCTGTGT	CGCTCAGGTCAGTGATGTTAA	80
IL-6	NM 000600.3	ACCTGAACCTTCCAAAGATGGC	TCACCAGGCAAGTCTCCTCATTG	75
IL-8	NM 000584.3	TCTGCAGCTCTGTGTGAAGGTG	TTCTGTGTTGGCGCAGTGTG	150
IL-10	NM 000572.2	GAACCAAGACCCAGACATC	CATTCTTCACCTGCTCCAC	137
TGF-b1	NM_000660.4	CACCAACTATTGCTTCAGCTCCAC	GAGGTCCTTGCGGAAGTCAATG	76
TNF-a	NM 000594.2	CCAGGCAGTCAGATCATCTTCTCG	ATCTCTCAGCTCCACGCCATTG	142

formed in triplicates on a 384-well plate (Biozym Scientific GmbH, Oldendorf, Germany) and the amplifications were measured on the Light Cycler 480 instrument (Roche/Germany) with following parameters: initial hot start at 95°C for 15min, followed by 45 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 20 s, with SYBR green fluorescence reading. A melting curve analysis was generated and a single melting peak was observed for each sample, validating that only one product was present. PCR efficiency was acquired by 5-fold serial dilutions of a mixture of sample cDNAs and calculated by the equation: $E=10^{(-1/slope)}$. Human GAPDH, β-Actin and USP34 served as reference genes and were selected according to their M-values and used for normalization of the qRT-PCR analysis. The relative expression of each Gene of interest was determined by transferring the Ct values to the REST 2009 Software (developed by M.Pfaffl and Qiagen) in order to calculate the fold changes.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Data are presented as means ± SEM. Student`s t-test was used to compare data from control and treated whole blood at each time point. ANOVA was used to compare haematology data across samples. Cytokine measures and were analyzed using 2 (Control and LPS) × 4 (times of measurement) repeated-measures ANOVA. If $P<0.05$ for the group × time interaction, the change from baseline for the 30 min post-exercise, 3-h post-exercise and 24-h post-exercise values was compared between groups using Student`s t-tests. For these two multiple comparisons across groups, a Bonferroni post-hoc test was used. These same statistical procedures were used to compare the pattern of change in cytokine mRNA and between genders. A value of $P<0.05$ was considered as significant.

Results

Table 1 summarizes individual characteristics of the male and female runners. Groups did not differ significantly in any of the training and fitness parameters measured. The 16 runners can be characterized as elite and highly experienced and committed to half-marathon running. Although 6 of 8 female athletes were clearly in luteal phase, the other 2 subjects which were on contraceptive use showed, interestingly, very similar pattern of kinetics, therefore, we did not exclude any subject from our study and the data was reported for all the female athletes.

Exercise

The runners completed the half-marathon race (21.1 km) in an average running time of 95.5 ± 8 min for men and 114 ± 12 for women. All athletes completed the race.

Table 3. Changes in peripheral blood leukocyte numbers [× 109 cells/l] before, 30 min after, 3 h after and 24 h after exhaustive exercise

	Pre-exercise (t0)	30min after Exe. (t1)	3h after Exe. (t2)	24 h after Exe. (t3)
Men				
Total cells	5.68 ± 0.72	13.29 ± 1.27*	14.01 ± 0.98*	6.32 ± 0.55
Neutrophils	2.84 ± 0.28	11.09 ± 0.02*	11.83 ± 0.09*	3.41 ± 0.01
Lymphocytes	2.08 ± 0.24	1.33 ± 0.12*	1.26 ± 0.07*	1.20 ± 0.02
Monocytes	0.46 ± 0.05	0.70 ± 0.02*	0.83 ± 0.42*	0.54 ± 0.04
Women				
Total cells	6.47 ± 0.67	13.10 ± 1.03*	14.83 ± 0.90*	6.94 ± 0.73
Neutrophils	3.45 ± 0.03	11.07 ± 0.01*	12.51 ± 0.09*	3.90 ± 0.02
Lymphocytes	2.31 ± 0.02	1.34 ± 0.05*	1.43 ± 0.06*	2.20 ± 0.02
Monocytes	0.48 ± 0.04	0.54 ± 0.03*	0.70 ± 0.48*	0.50 ± 0.04

Values are means ± SEM
*Significantly different from pre-exercise values, $p < 0.0001$

Table 4. Cytokine concentration (pg/ml) in stimulated (LPS) and unstimulated (CON) whole blood cultures after exhaustive exercise

		Before Exe. (T0)	30 min after Exe. (T1)	3h after Exe. (T2)	24h after Exe. (T3)	*P* value
Men						
TNF-a	CON	3,90 ± 0,65	3,78 ± 0,62	3,55 ± 0,24	3,02 ± 0,35	NS
	LPS	203,11 ± 30,51*	52,80 ± 9,35*§	245,89 ± 39,1*	126,24 ± 16,93§*	P <0.0001
IL-8	CON	4,27 ± 2,14	7,10 ± 1,84§	7,06 ± 2,66§	3,32 ± 1,06	P <0.0001
	LPS	99,95 ± 13,61*	150,09 ± 20,37§*	276,91 ± 24,02§*	84,92 ± 7,88*	P <0.0001
IL-6	CON	2,21 ± 0,65	13,81 ± 2,14§	3,06 ± 1,59	1,45 ± 0,79	P <0.0001
	LPS	22,19 ± 7,59*	40,82 ± 8,12*§	10,11 ± 1,65*	9,08 ± 1,61*	P <0.0001
INF-γ	CON	11,03 ± 8	7,19 ± 3,65	8,46 ± 5	5,94 ± 2,44	NS
	LPS	6,37 ± 2,64	5,71 ± 2,37	4,13 ± 1,19	3,82 ± 1,12	NS
IL-1ra	CON	4,68 ± 1,85	17,96 ± 4,66§	20,08 ± 10,9§	2,70 ± 0,74	P <0.0001
	LPS	26,7 ± 5,3*	59,16 ± 5,2*§	59,82 ± 12,79*§	13,79 ± 3,32*	P <0.0001
IL-10	CON	0,41 ± 0,24	130,43 ± 38,28§	7,08 ± 2,69§	2,5 ± 0,57§	P <0.0001
	LPS	0,84 ± 0,55	132,89 ± 37,51§	6,65 ± 2,52§	2,68 ± 0,43§	P <0.0001
IL-12 p40	CON	11,27 ± 5,2	10,02 ± 3,6	3,29 ± 1,48	4,97 ± 1,48	NS
	LPS	10,32 ± 4,9	8,75 ± 2,86	5,47 ± 2,17	4,76 ± 1,57	NS
IL-12 p70	CON	9,63 ± 5,09	5,21 ± 3,02	6,70 ± 2,9	4,66 ± 1,8	NS
	LPS	3,66 ± 1,7*	4,60 ± 1,4	2,62 ± 1,21	3,32 ± 0,55	NS
MCP-1	CON	161,34 ± 15,9	287,44 ± 33,30§	187,94 ± 28,19	152,92 ± 18,7	P <0.0001
	LPS	169,11 ± 14,69	319,10 ± 44,52§	189,22 ± 27,95	155,76 ± 12,4	P <0.0001
TGF-β1	CON	8,15 ± 0,93	14,27 ± 1,68§	10,03 ± 1,92	14,07 ± 4,03	P <0.0001
	LPS	11,82 ± 1,16	20,37 ± 2,93§*	19,76 ± 4,41*	14,05 ± 3,24	P <0.0001
GM-CSF	CON	ND	ND	ND	ND	
	LPS	ND	ND	ND	ND	
IL-1β	CON	ND	ND	ND	ND	
	LPS	ND	ND	ND	ND	
Women						
TNF-a	CON	4,23 ± 0,64	3,75 ± 0,29	3,61 ± 0,33	2,17 ± 0,70§	NS
	LPS	178,67 ± 19,27*	49,37 ± 6,97*§	187,85 ± 28,92*	141,98 ± 23,42*	P <0.0001
IL-8	CON	3,95 ± 2,33	8,51 ± 1,34§	4,50 ± 1,09	3,29 ± 1,29	P <0.0001
	LPS	104,72 ± 13,59*	146,04 ± 25,78§*	184,04 ± 27,17*§#	100,51 ± 12,12*	P <0.0001
IL-6	CON	1,54 ±0,54	10,21 ± 2,14§	2,86 ± 0,79	2,47 ± 1,2	P <0.0001
	LPS	20,18 ± 3,18*	18,25 ± 3*#	12,03 ± 2,57*	11,04 ± 3,5*	NS
INF-γ	CON	4,35 ± 2,08	10,42 ± 6,68	8,94 ± 6,09	12,76 ± 7,9	NS
	LPS	6,70 ± 3,46	6,83 ± 4,9	11,65 ± 7,87#	8,63 ± 4,44	NS
IL-1ra	CON	5,79 ± 1,58	10,85 ± 3,6	20,58 ± 6,7§	6,89 ± 2,73	P <0.0001
	LPS	18,22 ± 4,56*	24,38 ± 5,56*#	51,25 ± 12,02*§	21,46 ± 4,34*	P <0.0001
IL-10	CON	0,56 ± 0,26	61,35 ± 14,45§#	3,42 ± 0,47§	1,81 ± 0,71	P <0.0001
	LPS	0,50 ± 0,20	57,87 ± 15,63§#	3,90 ± 0,56§	2,57 ± 0,8§	P <0.0001
IL-12 p40	CON	5,14 ± 2,02#	5,39 ± 1,91	6,81 ± 2,03	3,99 ± 1,8	NS
	LPS	7,38 ± 3,09	5,30 ± 1,95	6,85 ± 2,26	5,31 ± 2,92	NS
IL-12 p70	CON	2,41 ± 1,02#	1,77 ± 0,5	2,22 ± 0,9	3,85 ± 1,79	NS
	LPS	1,79 ± 0,96	1,44 ± 0,44	2,67 ± 1,07	2,49 ± 1,02	NS
MCP-1	CON	175,49 ± 18,12	343,82 ± 54,42§	154,51 ± 15,59	155,1 ± 20,23	P <0.0001
	LPS	190,76 ± 16,91	355,95 ± 50,96§	160,45± 13,13	176,94 ± 20,27	P <0.0001
TGF-β1	CON	14,92 ± 2,47#	18,39 ± 2,47	17,59 ± 3,6	15,18 ± 2,33	P <0.0001
	LPS	18,47 ± 4,28	30,74 ± 3,68§*#	19,61 ± 4,28	17,22 ± 4,6	P <0.0001
GM-CSF	CON	ND	ND	ND	ND	
	LPS	ND	ND	ND	ND	
IL-1β	CON	ND	ND	ND	ND	
	LPS	ND	ND	ND	ND	

Plasma samples (means ± SEM) for the following were measured at baseline (t0), 30 min after (t1), 3h after (t2), and 24 h after (t3) the exhaustive exercise using a multiplex bead-based assay and analyzed by ANOVA for time effect: tumor necrosis factor (TNF)-α; TGF-β1; interleukin (IL)-1β, -1ra, -6, -8, -10, -12 p40, -12 p70; interferon (IFN)-γ; granulocyte-macrophage colony stimulating factor (GM-CSF), monocyte chemotactic protein (MCP)-1. NS represent not significant. ND represent not detected. * Significantly different between LPS and CON values p< 0.0001., § significantly different from pre-exercise values p <0.001., # represents significant difference between genders p <0.001

Leukocyte numbers

The effect of half-marathon running on total leukocyte, granulocyte, lymphocyte and monocyte counts is illustrated in Table 3. Total leukocyte counts were increased significantly 30 min after exercise. Throughout the 3 h recovery period, the total white cell count remained significantly elevated ($P<0.0001$). The prolonged exercise bout induced a pronounced granulocytosis, which was largely responsible for the changes in total white cell count. Changes were significant at 30 min ($P<0.0001$) and remained elevated at 3 h post-exercise ($P<0.0001$). The circulating monocyte count also rose mildly but significantly after exercise at the same time points as granulocytes. Circulating total lymphocyte counts decreased 30 min after exercise and remained attenuated for at least 3 h post-competition ($P<0.0001$). The total leukocyte, granulocyte, monocyte and lymphocyte counts reached pre-exercise levels at 24 h post-exercise (Table 3). No significant gender differences were observed in counts of white blood cells at any time points.

Cytokine concentrations in stimulated and unstimulated whole blood

Results for all markers tested are summarized in Table 4. Mean values for evaluated cytokines are depicted in Figure 1 A-H. LPS-depended release per 1000 monocytes (LDR) was calculated by normalizing the raw data to monocyte numbers and subtracting the values of control cultures from the values of LPS-stimulated cultures. Mean values are presented in Figure 2 A-H. IL-1β and GM-CSF concentrations were undetectable in both unstimulated and stimulated cultures of athletes at either time point.

In unstimulated cultures, the concentrations of IL-10, IL-1ra, IL-6, MCP-1 and IL-8 were elevated significantly following exercise ($P<0.0001$), peaking at 30 min post-run for IL-10, MCP-1, and IL-6, and peaking at 3 h post-run for IL-1ra and IL-8 (Figure 1, Table 4). The concentration of TGF-β1 was increased significantly in men and borderline significantly in women at 30 min post-run. Twenty four hours after the half-marathon, pre-run levels for all cytokines were reached. We could not detect an effect of exercise on plasma concentrations of IFN-γ, IL-12p40 and IL-12p70 at either time point (Table 4).

As expected, LPS induced pronounced alterations in cytokine concentrations in cultured whole blood of athletes (Table 4). Before exercise, *ex vivo* LPS-stimulated production of TNF-α, IL-8, IL-6, and IL-1ra was substantial and highly significant as compared to unstimulated cultures in both sexes ($P<0.0001$) (Figure 1, Table 4). Following exercise there was a significant suppression in LDR of TNF-α, as compared to pre-run values ($P<0.001$)(Figure 2-A), whereas LDR of IL-8, IL-1ra, and TGF-β1 were increased significantly at least in one of the sexes ($P<0.001$)(Figure 2). Interestingly the suppression of TNF-α LDR was still significant 24 h after exercise. LDR of IL-6 was reduced following exercise in both sexes, with more rapid reduction in women ($P<0.001$) (Figure 2-C). There was no LPS-dependent release (LDR) of IL-10 and MCP-1 before and after exhaustive exercise (Figure 2. G-H).

Figure 1. Cytokine concentration in stimulated (+ LPS) and Unstimulated (- LPS) whole blood cultures in male and female athletes after exhaustive exercise. * Significantly different between LPS and CON values P< 0.0001.

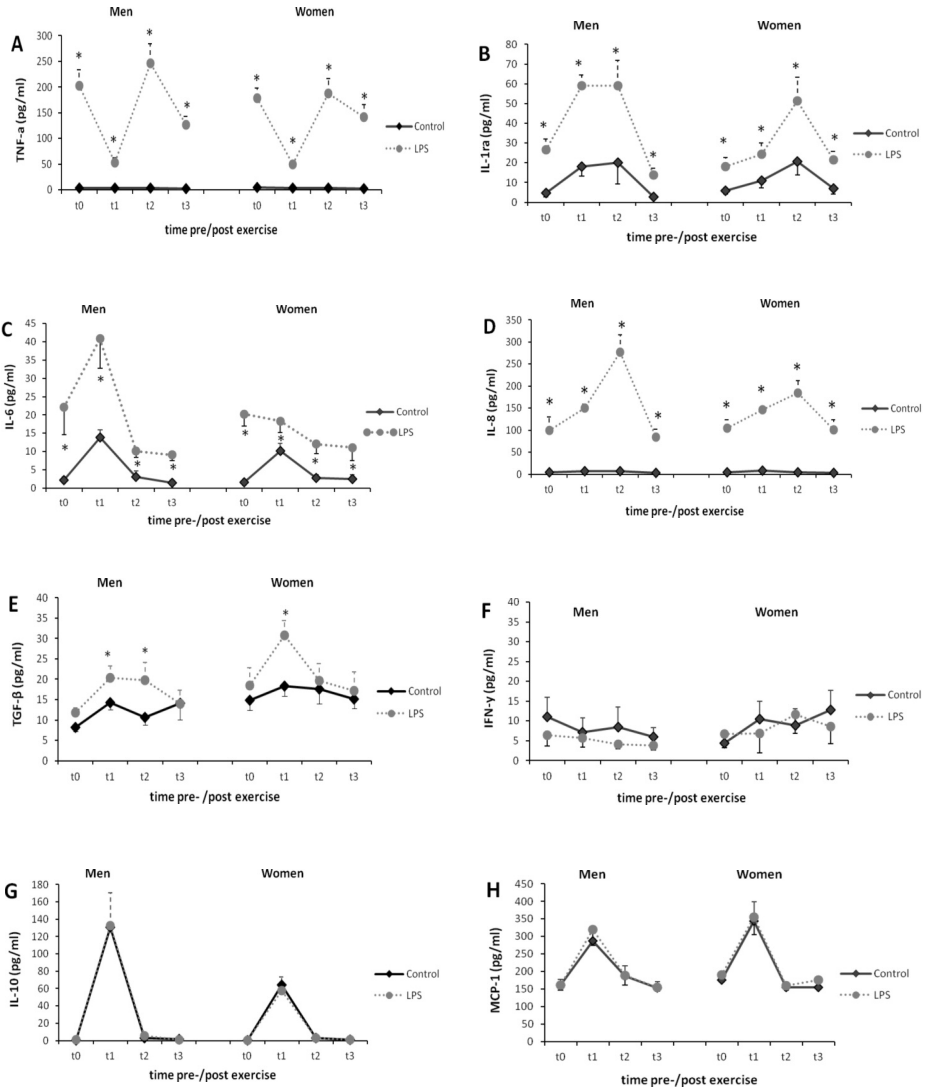
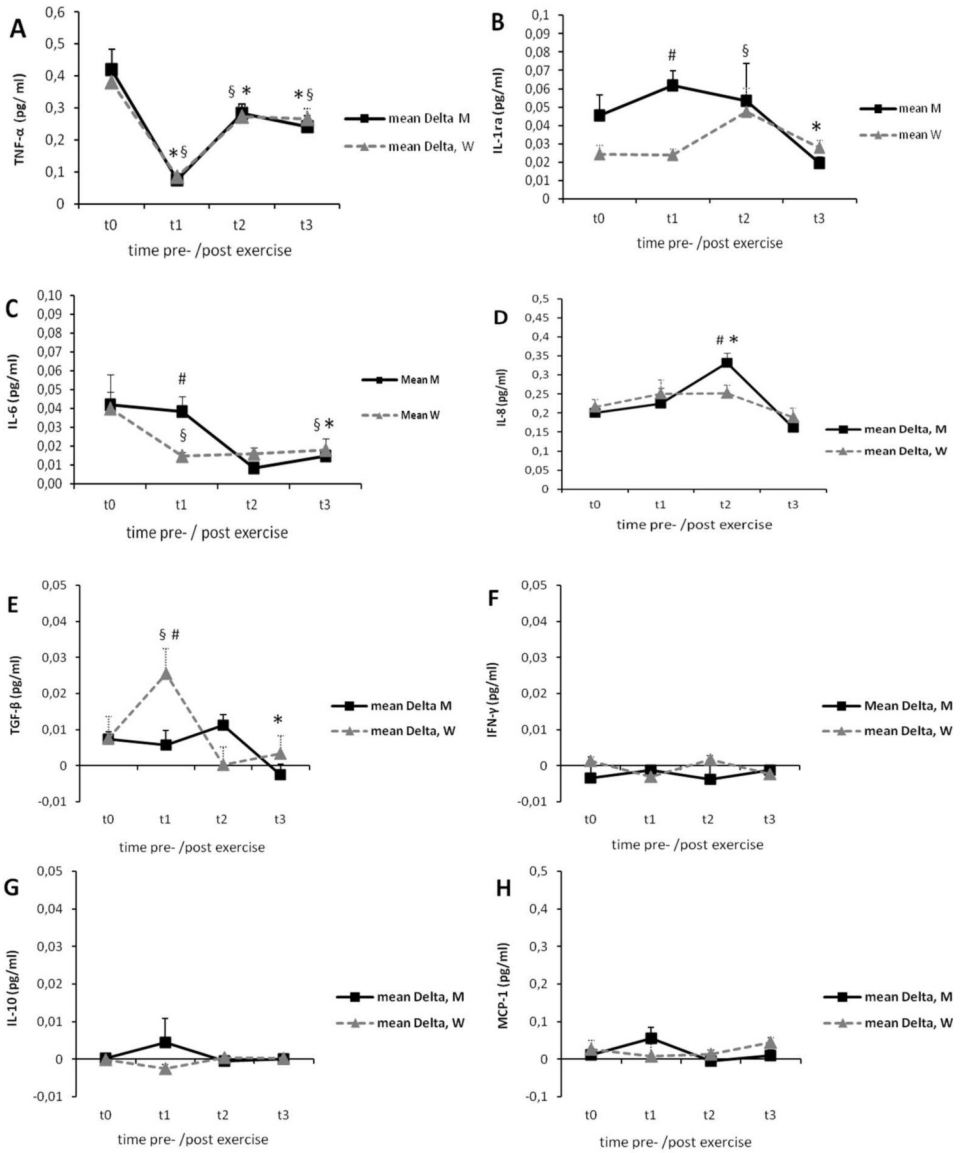


Figure 2. LPS-dependent release per 1000 monocyte (LDR) of cytokines (concentration in stimulated cultures minus concentration in un-stimulated cultures). LPS-dependent release of IFN-γ was normalized to 1000 lymphocytes. Data represent the means ± SE of the values. **M** represents men and **W** represents women. * Significant change from before to after exercise in men, § Significant change from before to after exercise in women, # Significant difference between men and women.



Sex differences in cytokine release

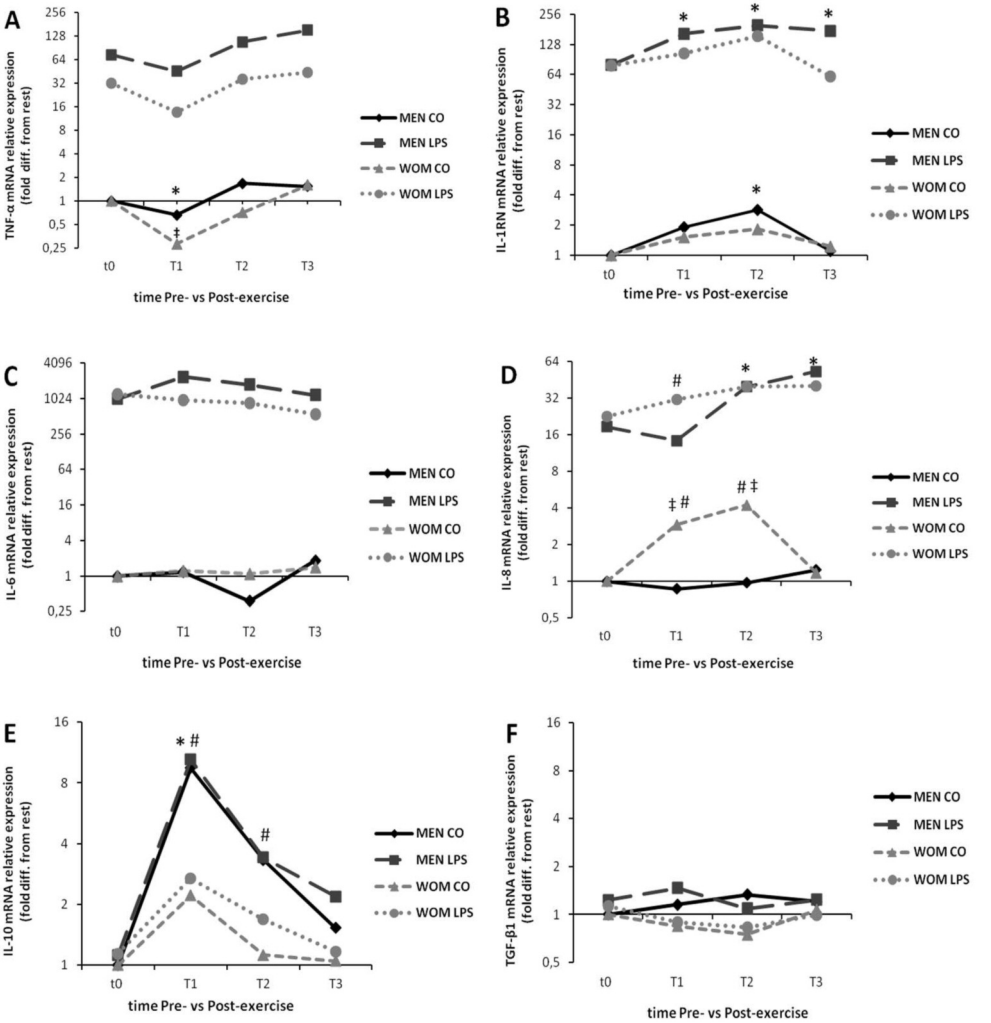
No significant difference was observed between sexes in LDR and unstimulated levels of TNF-α and MCP-1 at either time point (P>0.05) (Figure 2, Table 4). Men showed significantly higher LDR of IL-6 and IL-1ra and also significantly higher unstimulated levels of IL-10 at 30 min post race. As compared to women, men showed significantly higher LDR of IL-8 at 3 h post-run (P<0.001) (Figure 2). In contrast, the LDR of TGF-β1 was greater in women than in men at 30 min post-run (P<0.001) (Figure 2-E). The unstimulated levels of IL-12p40 and IL-12p70 were significantly greater in men and levels of TGF-β1 were greater in women at baseline (P<0.001) (Table 4).

Cytokine mRNA relative expression in stimulated and unstimulated whole blood

As expected, most of the cytokines except IL-10 and TGF-β1 showed a significant elevation of mRNA expression for LPS-stimulated cultures in relation to unstimulated cultures. Cytokine mRNA expression of TNF-α, IL-1ra, IL-6, IL-8, IL-10, and TGF-β1 is depicted in Figure 3 A-F.

A slight down-regulation of TNF-α mRNA expression was seen in both stimulated and unstimulated whole blood 30 min post-exercise in relation to pre-exercise values (t0 =1) for men and women. This was only significant in women (P<0.05), reaching a factor of 0.28-fold and 0.42-fold for unstimulated and LPS-stimulated samples, respectively (Figure 3-A). It is necessary to note that due to cell shifts, there were only 5% monocytes at t1 in contrast to 8% at t0 which amounts to a factor of 0.64 in both sexes. IL-1ra mRNA expression increased (P<0.05) 1.8- to 2.83-fold for unstimulated men`s blood and 2- to 2,48-fold for LPS-stimulated men`s blood and 1.52- to 1.82-fold for unstimulated women's blood and 1.31- to 1.98-fold for LPS-stimulated women`s blood 30 min-24 h post-exercise relative to pre-exercise, with the peak at 3 h post-run for both sexes. LPS-induced IL-1ra mRNA expression remained significantly elevated in men 24 h post-exercise (2.2-fold and P<0.05) (Figure 3-B). There was no significant change in IL-6 mRNA expression in the 24 h following exercise (Figure 3-C). However, in accordance with protein data men and women showed a different trend of LPS-induced IL-6 response in mRNA level. While men showed a slight increase in IL-6 mRNA (2.36-fold), women exhibited a trend to decrease 0.80 to 0.46-fold 30 min-24 h post-exercise in relation to pre-exercise values. This remained suppressed for 24 h post-exercise in women. There was no significant change in unstimulated IL-8 mRNA expression for men following exercise, however LPS-induced mRNA expression increased (P<0.05) 2,13-fold at 3 h post-exercise and remained elevated (2.8-fold P<0.05) for 24 h post-exercise in relation to pre-exercise (Figure 3-D). An increase in unstimulated IL-8 mRNA expression 2.91- to 4.22-fold was seen in women 30 min-3 h post-race, with peak expression at 3 h post-exercise. This was significantly different from men in both time points (2.66-fold for t1 and 4.77-fold for t2)(P<0.05). A tendency toward up-regulation in LPS-induced IL-8 mRNA expression in women at post- exercise compared to pre-exercise was not significant, but there was still a significant up-regulation (1.72-fold) in relation to men at 30 min post-exercise (P<0.05). As mentioned above there was no signifi-

Figure 3. The changes in LPS-stimulated and unstimulated whole blood cytokine mRNA expression at before (t0), 30 min after (t1), 3 h after (t2) and 24 h after exercise (t3) in both sexes. **CO** represents unstimulated control cultures and LPS represents LPS-stimulated cultures. **WOM** represents women. * Represents significant change from before to after exercise in men, ‡ Represents Significant change from before to after exercise in women, # Represents significant difference between men and women.



cant difference between LPS-stimulated and unstimulated IL-10 mRNA expression following exercise for both genders (Figure 3-E). A significant up-regulation in IL-10 gene expression was seen only in men at 30 min post-race compared to pre-race (9.48-fold and 9.29-fold for unstimulated and LPS-stimulated IL-10 gene expression, respectively) ($P<0.05$). Women showed only a trend toward up-regulation following exercise. By 3 h post-exercise there was still a significant difference between sexes in IL-10 mRNA expression ($P<0.001$). TGF-β1 mRNA showed only minimal changes, although there was a trend toward up-regulation in men at t2 ($P<0.05$) (Figure 3-F).

Discussion

The major aim of the study was to further clarify the capability of human blood cells to respond to challenge with endotoxin by producing cytokines, in relation to previous exercise. Cytokines are important mediators governing the immune response, and their regulation or enhancement may yield valuable information pertinent to questions like transient post-exercise immunosuppression, beneficial anti-inflammatory (e.g., anti-atherosclerotic) effects of cytokines, and/or exercise-induced asthma.

Some previous studies including our group's work have followed a similar approach (9, 10, 44). This study is unique in so far as it uses a very short pulse (1 hour) of challenge (LPS), focusing on the early events of stimulation. We argued that this may lead to miss some of the reactions seen in studies with long term stimulation (e.g. 24 h) but may also allow a more differentiated picture for others. We also included cytokines which have not been evaluated until now (TGF-β1) and, importantly, also looked for possible differences in the cytokine responses between sexes. Previous studies from our group using microarray analysis had pointed to a more inflammation prone reaction of female athletes in the luteal phase of their menstrual cycle as compared to women in follicular phase or men immediately after 1 h aerobic exercise (27).

The rationale to prefer whole blood stimulation over cultivation of mononuclear cell fractions has been outlined in the introduction and relates to our aim to analyze the early events as tightly and precisely as possible and to allow cytokine production in an environment which is as close as possible to the natural one. Accordingly, unstimulated whole blood cultures had to be chosen as adequate controls. Supernatants from these unstimulated (control) cultures thus reflect plasma values plus possible unstimulated "spontaneous" production of cytokines during the 1 hour culture period. Normally, unstimulated cultures of healthy subject`s resting blood do not produce measurable cytokines. The lack of plasma values for comparison makes it impossible to determine, if there was any measurable spontaneous production in culture post-exercise. Cytokines appearing in plasma in vivo are, at least in part, produced outside of the blood (e.g, IL-6 and IL-8) (33). Thus, although our approach does not yield information about the exact origin of cytokines in our control cultures, it serves as an unequivocal means to determine endotoxin-induced production of cytokines by blood leukocytes (in

relation to exercise) by using the delta values between stimulated and unstimulated cultures.

To properly quantify the effect of exercise and sexes on LPS-inducible production (LDR) of the cytokines under evaluation, cell numbers needed to be considered in addition to plasma values (which may also reflect production outside of the blood-see above). To deal with this, we calculated the mean values of the delta between cytokine concentration in stimulated and unstimulated cultures, normalized to monocytes (except IFN-γ) as presumed producer cells, although some of the measured cytokines are produced also by other cell types, including T cells (IL-6, IL-10, TNF-α, TGF-β), muscle cells (IL-6, IL-8), and endothelial cells (IL-8). This LPS-dependent release per 1000 monocytes (LDR) of cytokines was compared to corresponding changes in mRNA accumulation in the same cultures. Here, also, the different cell composition needs to be acknowledged. We could not normalize the data, but for interpretation it should be kept in mind, that the percentage of monocytes was lower at 30 min and 3 h post-exercise (~ 5%) than pre-exercise (~ 8%). Messenger RNA of cytokines which are predominantly or exclusively monocyte-derived (e.g. TNF-α) was therefore estimated too low at 30 min and 3 h post-exercise when considered on a per monocyte basis. The pattern of cytokine concentrations obtained in supernatants from our unstimulated cultures is largely in accordance with the pattern of plasma cytokines described in many previous studies using similar settings (22-24, 31, 39, 40).

Tumor necrosis factor-alpha (TNF-α). In accordance with the literature there was no induction of TNF-α through exercise (10, 40). By contrast, LDR of TNF-α was strongly reduced shortly after exercise (t1), which is comparable with the results of other investigators (9, 10, 42, 44). Surprisingly, it was still significantly reduced even at 24 h post-exercise, suggesting a longer lasting effect of exercise. This has not been found previously. It is of interest to note that there was no difference in LPS-induced production of TNF-α between sexes. Real time PCR results showed that LPS-stimulated and unstimulated TNF-α gene expression were both slightly down-regulated 30 min after exercise, but (borderline) significantly so only in women. We further have to realize that the percentage of monocytes was lower in samples drawn post-exercise (~ 5%) than in samples drawn before exercise (~ 8%) (see above).Together, this supports the concept that suppression of LPS-induced TNF-α release post-exercise cannot be sufficiently explained by changes in messenger RNA, but must be related mainly to post-translational modification (e.g., degradation of protein)(19, 42).

Interleukin-1 receptor antagonist. IL-1ra release in unstimulated and LPS-stimulated cultures was significantly increased following exercise, peaking at 3 h post-exercise for both sexes. This is in agreement with the results of Drenth et al (1998), who reported a similar pattern following a 5km run in recreationally trained athletes (men and women) (9). In our study, men produced moderately higher amounts of LPS-induced IL-1ra per monocyte (LDR) at rest (t0) and significantly higher amounts at t1, as compared with women. When comparing the IL-1ra release curves of women and men, the conclusion is warranted that during

the first 3 h after exercise, men produced substantially more IL-1ra in total. Twenty four hours post-exercise, men showed a significantly lower LDR of IL-1ra in relation to pre-exercise values. IL-1ra protein and gene expression showed a similar pattern of kinetics from t0-t2. The significant drop in LPS-induced protein production at t3 in men is, however, not paralleled by gene expression, and may thus be due to post-translational modifications. The up-regulation of IL-1ra gene expression through exhaustive exercise has been reported previously (9, 23, 24, 30, 47). In contrast, the significant difference between sexes in LDR of IL-1ra shortly after exercise has not been described so far. They are, however, compatible with our previous finding that following 1 h aerobic exercise, several anti-inflammatory genes including IL-1ra were down-regulated in women in their luteal phase, but not in women in follicular phase or men (27). Given that 6 of 8 women included in this study were also in the luteal phase of their menstrual cycle, our results confirmed that sex and probably menstrual cycle play a role in early phase of the immune response to exercise. Others have demonstrated that luteal phase has inflammatory bias compared to follicular phase (4, 46). Lynch EA et al (1994) showed greater amount of IL-1ra production in follicular phase as compared with luteal phase (18). Changes in phenotype and secretary activity of some leukocytes to a more pro-inflammatory, pro-migratory profile during luteal phase have also been reported (16,36).

Interleukin-6. IL-6 concentrations in our unstimulated cultures perfectly reflect the usual rise in plasma IL-6 during and sometime after endurance exercise which has been documented many times since discovered in the early 1990s (26, 28). Later, it was shown that IL-6 mRNA was not significantly induced in peripheral blood by exercise, and that the observed plasma levels of IL-6 are probably produced in muscle and play an important role in the energy supply chain (34). In the present study we also found no IL-6 mRNA induction by exhaustive exercise, which is consistent with the finding of previous studies (19, 23, 24). Apart from energy metabolism IL-6 plays a major role in the immunological network, and most of its pleiotropic effects are anti-inflammatory or restorative (35). LPS-induced IL-6 – a model for immunologically induced IL-6 – was only mildly reduced after prolonged-exhaustive exercise as opposed to the massive suppression of TNF-α and IFN-γ which are both clearly pro-inflammatory cytokines (9, 44). In our study, sex differences in the effect of exercise on LPS-induced IL-6 release became visible, with women showing a faster kinetics in suppression of IL-6 LDR. Although we found no significant exercise-dependent changes in LPS-induced IL-6 messenger RNA, the observed sex difference in the protein pattern was confirmed in trend by mRNA: while men even showed an increase of LPS-induced IL-6 mRNA after exercise, women showed a mild decrease. Differential regulation between sexes of IL-6 induction and release after exercise is a new finding but not entirely unexpected, since previous studies using microarrays had pointed to a less prominent anti-inflammatory response to exercise in women in the luteal phase (see above). General consensus for spontaneously produced IL-6 demonstrates that female sex hormones, especially estrogen, decrease plasma IL-6 concentration. Decreased plasma IL-6 in luteal phase compared with follicular has been reported by Angstwurm et al (1). Schwarz et al (2000) reported a lower level of LPS-stimulated IL-6 in healthy women during luteal phase, as compared

with women in follicular phase and healthy men (41). The observed pattern of IL-6 resembles the behaviour of IL-1ra.

IL-10. Of the various cytokines measured in this study, IL-10 was most strongly influenced by exercise. In unstimulated control cultures the concentration of IL-10 showed a sharp, significant peak at t1 which was accompanied by a concomitant peak of IL-10 mRNA at the same time point. These are in agreement with the findings of previous studies (23, 24). However, LPS stimulation did not cause any increase in IL-10 levels, nor did it change IL-10 mRNA levels significantly. Obviously, the 1 h incubation with LPS was not long enough to influence IL-10 concentrations in our cultures. According to the literature, the alteration of IL-10 in LPS-stimulated cultures is secondary to the release of pro-inflammatory cytokines and begins only 3-5 h after exposure to the LPS (15). It is of interest to note that exercise-induced IL-10 levels and mRNA expression were considerably and significantly higher in men as compared with women at 30 min post-exercise. We see these results in parallel with the results for IL-1ra and IL-6. Independent of these sex-related differences, mRNA elevation seems high enough to possibly enable a substantial contribution of peripheral leukocytes to the appearance of IL-10 in plasma after exercise. In this light, the lack of any effect of 1 h incubation with LPS may add a new facet to our understanding of IL-10 modulation by exercise, suggesting that induction of IL-10 by exercise alone may work through entirely different pathways than LPS-stimulated induction. The exact pathways remain however unknown.

Interleukin-8 and TGF-β1. The production and expression of IL-8 and TGF-β1 may be discussed together. TGF-β1 is a pleiotropic multifunctional cytokine and has a broad spectrum of effects, with prominent anti-inflammatory facets (43, 45). TGF-β1, for example, can block NK cell proliferation and cytotoxicity as well as inhibit induction of IL-12 and NK cell IFN-γ production (2). It does, however, also have clearly pro-inflammatory effects (45) and these are largely overlapping with effects of IL-8: both are chemotactic for granulocytes and boost their phagocytic and bactericidal functions (17). Improvement of granulocyte functions by exercise has been described (31, 37). In the present study, unstimulated levels of IL-8 rose significantly following exercise but remained in a relatively low range, which is in agreement with previous reports (22, 24, 25, 40). It has been suggested that this may represent a spillover from IL-8 production in muscle (33). Interestingly, in our study, this post-exercise rise in unstimulated levels of IL-8 was accompanied by a significant up-regulation of IL-8 mRNA in women only while in men there was even a mild decrease.

We do not know if this relates to the fact that most of the women were in luteal phase of their menstrual cycle. We also do not know if it signifies that there was IL-8 production in peripheral blood cells in female athletes or if there was post transcriptional protein suppression. In agreement with the results of Degerstrøm (7), there was a significant exercise related increase in IL-8 LDR. This was, however, more pronounced and significant in men only (3 h post-exercise). IL-8 mRNA in LPS stimulated cultures showed an exercise related up-regulation in both sexes however it was only significant in men. At 24 h post-exercise, mRNA

remained up-regulated while protein was decreased to pre-exercise levels, suggesting post-translation modifications.

Unstimulated levels of TGF-β1 were elevated at 30 min post-exercise in both sexes, but became significant only in men. Mild elevation of TGF-β1 by exercise in circulation and tissues has been reported previously (5, 12, 13), and like IL-8, may result from spillover in skeletal muscle or tendons (13, 14). In contrast to IL-8, TGF-β1 release was only mildly increased by LPS stimulation in the present study, and exercise caused a significant increase in TGF-β1 LDR at 30 min post-exercise in women only. This was not paralleled by messenger RNA changes which remained minimal at all times in both sexes. Thus, in the early hours after exercise, both sexes showed a peak in LPS-induced release of one of the granulocytotropic cytokines (IL-8 at 3 h post-exercise for men and TGF-β1 at 30 min post-exercise for women), which could be related to the known improvement of functionality of granulocytes by exercise. Of course, any clinical significance of our observations is not easy to prove and would require confirmation in future studies. Still it cannot be excluded either, and may mean that the antibacterial response in both sexes is augmented by exercise albeit by different means. In any case, booster effects of exercise on LPS-inducible IL-8 or TGF-β1 release were gone 24 h post-exercise, even if, in males, IL-8 message remained up-regulated.

Other cytokines. Like IL-10, MCP-1 showed significant induction by exercise in unstimulated control cultures in both sexes. This is in agreement with the findings of Garcia et al (11), who reported increased circulating MCP-1 concentrations following one session of cycling (1 h at ~70% of VO_2max). There was no LPS effect on MCP-1 protein level in stimulated cultures, suggesting that 1 h incubation is not long enough to induce MCP-1. For technical reasons, we could not determine MCP-1 mRNA in our samples. We also couldn't find any change in IFN-γ and IL-12p70 levels. In contrast to Suzuki et al (40) and Peake et al (31) we could not even find changes in IL-12p40 in our control supernatants. IL-12p40 is antagonistic to IL-12p70, and therefore to IFN-γ and other type 1 cytokines. The reason for this discrepancy remains unclear so far. Possibly it might be related to the different exercise challenges (marathon vs. half-marathon) (39,40).

In summary, the cytokine response to the bacterial stimulus LPS was dramatically changed in samples drawn 30 min and 3 h post-exercise. When calculated on a per monocyte basis, LPS-dependent release (LDR) of TNF-α was significantly reduced by exercise with the same kinetic for men and women. LDR of IL-6 was likewise reduced, but with a faster kinetic in women. Both sexes presented a sharp peak of unstimulated levels of IL-10 at 30 min post-exercise accompanied by upregulation of IL-10 mRNA. IL-10 and IL-10 mRNA were both significantly higher in men than women. Due to the short incubation time, LPS stimulation was not associated with any additional release of IL-10. These results also indicate an early production of IL-10 by peripheral blood cells in response to exercise. LDR of IL-8 was enhanced in men and TGF-β1 LDR in women. Thirty minutes after exercise women showed significantly less LDR of IL-1ra than men. Altogether, changes in cytokine release could only in part be attributed to changes in mRNA. Results for IL-1ra, IL-6 and IL-10 pointed to a less pronounced anti-inflammatory response in women as compared with men.

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Urinary excretion of cytokines versus their plasma levels after endurance exercise

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ABSTRACT

It has been consistently shown that circulating levels of interleukin (IL)-6, IL-8, IL-1 receptor antagonist (IL-1ra) and IL-10 increase remarkably following endurance exercise longer than 2 h such as marathon and triathlon races. However, no studies have compared changes in the plasma and urinary levels of these cytokines after endurance exercise, including the recovery period. In the present study, we investigated kinetic changes in the urinary excretion of cytokines following endurance exercise up to 3 h after exercise to evaluate the magnitude of change in comparison to the plasma levels and to explore the possible biological significance and the mechanisms of cytokine dynamics following exercise. Fourteen male athletes participated in a duathlon race consisting of 5 km of running, 40 km of cycling, and 5 km of running. Venous blood and urine samples were collected before, immediately after, 1.5 h and 3 h after the race. Plasma and urine were analyzed using enzyme-linked immunosorbent assays (ELISA). Plasma concentrations of IL-1β, IL-1ra, IL-6, IL-8, IL-10 and monocyte chemotactic protein (MCP)-1 increased significantly after the race, whereas tumour necrosis factor (TNF)-α, IL-2, IL-4, IL-12 and interferon (IFN)-γ did not change significantly. Urinary concentrations of IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN-γ and MCP-1 increased significantly after the race. When the urine concentrations were adjusted by creatinine concentration, urine volume and sampling time, the increases of IL-2, IL-4, IL-8, IL-10, IFN-γ and MCP-1 were evident and these were notably present in urine of the stressed athletes suffering from renal tubular epithelial damage. The present study provides new evidence that the kinetics and magnitude of changes in urinary cytokine concentrations differ from plasma cytokine concentrations following endurance exercise, especially, in the recovery

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period several hours after exercise, and that the damaged kidney might be responsible at least in part for the kinetics of some cytokines. Urinary cytokines may be sensitive biomarkers of the impact of exhaustive exercise workload on renal damage and inflammation in the recovery period after endurance exercise.

Key words: exertion, anti-inflammation, chemokine, urinalysis, acute kidney injury (AKI)

INTRODUCTION

Cytokines are potent intercellular signalling molecules that usually act within the local tissues in an autocrine or paracrine manner. However, systemic spillover of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 from damaged tissues can occur in response to a variety of serious insults such as severe trauma, burns, hemorrhagic shock, sepsis and ischaemia-reperfusion injuries, which are integrated as a systemic inflammatory response syndrome (SIRS) and multiple organ failure syndrome characterized by hypercytokinaemia (1, 19, 46). For example, in a sepsis model, TNF-α is the first cytokine released systemically, and peaks within 2 h after the onset of sepsis, followed shortly thereafter by peaks of IL-1, and then IL-6 within 4 h after the disease onset (1, 37). These pro-inflammatory cytokines induce pyrogenesis and promote subsequent acute inflammatory responses such as neutrophilia, lymphocytopenia by inducing IL-8, interferon (IFN)-γ and monocyte chemotactic protein (MCP)-1 (19, 37). In contrast to these pro-inflammatory cytokines, anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1ra), IL-4 and IL-10 may prevent inflammatory tissue damage but cause immunosuppressive states of the body (10, 16, 46, 52).

The plasma concentrations of the classical pro-inflammatory cytokines, TNF-α and IL-1β, in general, do not increase immediately after exercise (4, 9, 14, 24, 33-35, 45-49). In contrast, IL-6 is the first cytokine present in the circulation during exercise (9, 14, 17, 24-26, 28, 37, 39, 46). The level of circulating IL-6 increases up to 100-fold, depending on the intensity and duration of endurance exercise, and declines in the post-exercise period (28-30, 32-35, 45-49). Contracting skeletal muscle is the main source of IL-6 in the circulation. In response to exercise, an increase in the IL-6 mRNA content in the contracting skeletal muscle is detectable after 30 min of exercise, and up to 100-fold increases of the IL-6 mRNA content occur at the end of the exercise bout (17, 25, 33-35). In addition, it is demonstrated that both intramuscular IL-6 mRNA expression and protein release are enhanced when intramuscular glycogen is low, indicating that IL-6 is involved in energy metabolism during endurance exercise (17, 33-35, 48).

Concerning immunomodulatory cytokines that are potent functional activators of cellular immunity (29, 30, 36, 37, 38), there are no reports showing significant increases in plasma IFN-γ concentration after exercise, whereas there are several reports demonstrating a decrease in the plasma concentration of IL-2 after exercise (11, 28-30, 39, 53). Interestingly, many studies have shown that the capacity of blood leucocytes to produce IL-2 and IFN-γ decreases after endurance exercise (28-30, 39, 53). The findings that pro-inflammatory and immunomodulatory

cytokines only increase to a small extent, or are even down-regulated after exercise, could well be attributed to the actions of anti-inflammatory cytokines such as IL-1ra, IL-4 and IL-10. Indeed, we previously observed more than 200-fold increase of plasma IL-1ra after a marathon race (45) and delayed secretion of IL-4 several hours after short-duration maximal-intensity exercise (46). Furthermore, we also observed renal excretion of several cytokines, which may be one of the underlying mechanisms that plasma cytokines either remain unchanged or exhibit relatively small, delayed increments following exhaustive exercise.

Although many researchers have consistently shown that circulating levels of IL-6, IL-8, IL-1ra and IL-10 increase remarkably following endurance exercise longer than 2 h such as marathon and triathlon races (4, 14, 24, 33-37, 45-49), less is known concerning changes in urinary cytokine excretion after endurance exercise and during the recovery period (29, 30, 46, 47, 53). The present study investigated kinetic changes in the urinary levels of cytokines following endurance exercise lasting several hours to evaluate the magnitude of change in comparison to the plasma concentrations of the same cytokines. It is known that deterioration of glomerular filtration rate and oliguria are induced following endurance exercise (50), and that endurance exercise causes rhabdomyolysis (5, 31, 41), produces reactive oxygen species (ROS), and contributes to the onset of acute renal failure through reduced renal blood flow and damage of tubular cells (15). In the previous studies, after renal ischaemia-reperfusion, IL-2, IL-8, IL-10, MCP-1 and IFN-γ appeared in the kidney (7, 8, 12, 21, 42, 51). Accordingly, in this study, we evaluate acute kidney injury (AKI) following endurance exercise in relations with the changes of plasma and urine levels of various cytokines. Our hypothesis was that plasma levels of cytokines depend on urinary excretion and the excreted cytokines into urine might provide important information on renal damage and inflammation after endurance exercise.

METHODS

Subjects

Fourteen male triathletes (age 28.7 ± 7.9 (mean ± SD) yr and body mass 63.2 ± 6.0 kg) participating in the 19th Kikunotsuyu duathlon race, held on March 16th 2008, volunteered for this study. The protocol was approved in advance by the institutional ethics committee of Waseda University. None of the athletes had been ill in the month prior to the race, which was confirmed from medical questionnaire.

Race conditions

All participants agreed to avoid the use of vitamin/mineral supplements, herbs and medications from the day prior to the race until the end of experimental period (3 h post-exercise). They ate the same breakfast containing 574 kcal with 22.1 g protein, 13.7 g fat, and 88.8 g carbohydrate at 08:30. They emptied their bladders before 08:30 and just before the race to eliminate any effect of remaining urine. The race consisted of 5 km of running, 40 km of cycling, and 5 km of running, and began at 14:00. The weather was fair, with the ambient temperature at 24.6 degrees Celsius (°C). They did not exercise for approximately 18 h before

the pre-race blood and urine sampling. With the subjects resting quietly, the pre-race blood and urine samples (Pre) were collected at 10:30. They ate the same lunch containing 211 kcal with 9.3 g protein, 2.4 g fat, and 38.6 g carbohydrate at 11:00. The post-race blood and urine samples were collected immediately (0 h), 1.5 h (1.5 h) and 3 h (3 h) after the race. All participants drank the same quantity of fluid during exercise. They each drank 600 ml of fluid before the race, 1400 ml of fluid during the race and 1500 ml of fluid until 3 h after the race, respectively.

Blood and urine sampling

Approximately 7 ml of blood was drawn by a standard venipuncture technique from the antecubital vein using vacutainers containing no additive or disodium EDTA as an anticoagulant to obtain serum and plasma samples, respectively. Collected blood samples containing no additives were allowed to clot at room temperature for one hour before centrifugation at 1000 *g* for 10 min for serum preparation, whereas blood samples containing disodium EDTA were centrifuged immediately for plasma preparation. Plasma was stored at –80 °C until the day of analysis. Serum concentrations of creatinine (Cr) and albumin (ALB) were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo), and urine osmolality was determined using an auto-osmometer (OSMOSTAT, Kyoto Daiichi Kagaku, Kyoto).

Urine samples were collected directly in graduated cylinders and the volume was measured and then approximately 8 ml of urine was stored at 4 °C without centrifuge until analysis of sediments, respectively. Remaining urine samples were centrifuged immediately at 1000 *g* for 10 min to remove sediments, and the supernatants were stored at –80 °C until the day of analysis. Urinary concentrations of Cr, ALB and *β-N*-acetyl-D-glucosaminidase (NAG) activity were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo). White blood cells and red blood cells in urinary sediments were counted by flow cytometer (UF-1000i, Sysmex, Kobe), and other cells and casts in urinary sediments were analyzed by microscope (x400 high power field: HPF).

Assays for cytokines

Cytokine concentrations were measured in EDTA-plasma and urine samples with enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions. Concentrations of IL-1β, IL-6, IL-12 and TNF-α were measured with Quantikine high sensitivity (HS) kits (R&D Systems, Inc., Minneapolis, MN). IL-1ra and MCP-1 were measured with Quantikine kits (R&D Systems, Inc.). IL-2, IL-4, IL-8, IL-10 and IFN-γ were measured with OptEIA kits (Beckton Dickinson Biosciences, San Diego, CA). For all assays, the absorbance was measured spectrophotometrically on a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA), and the concentration of each cytokine was calculated by comparison with a calculation curve established in the same measurement.

Data analyses

Data are presented as means ± SD. Statistical validation was made using Friedman's test. If significance was detected, the Scheffe method was used for multiple comparisons. Statistical significance was evaluated at $p < 0.05$.

RESULTS

Plasma and urine cytokines

As shown in Table 1, the plasma concentrations of TNF-α did not change significantly following the race. Plasma IL-1β concentration increased significantly at 1.5 h after the race (16-fold) and remained elevated at 3 h after the race (5-fold) compared with the pre-exercise values. Plasma IL-1ra concentration showed more substantial changes significantly above pre-exercise values at immediately after the race (100-fold) and at 1.5 h post-exercise (169-fold), and at 3 h post-exercise (66-fold). The plasma concentrations of IL-6, IL-8, IL-10 and MCP-1 also increased significantly at immediately after the race, but decreased thereafter. The plasma concentrations of IL-2, IL-4, IL-12 and IFN-γ did not change significantly following exercise.

Urine volume and urinary creatinine concentration changed markedly following exercise as shown in the bottom of Table 1. Therefore, the urinary concentrations of cytokines are reported as raw data, those corrected for creatinine concentration, the gross amount (raw concentration × urine volume), and the gross amount per minute (urinary excretion rate). Urinary TNF-α did not increase significantly. Urinary IL-1ra, IL-6, IL-12 and MCP-1 were significantly higher after the race compared with pre-race values, but these differences were no longer significant when corrected for creatinine. Urinary levels of IL-2 (59-fold), IL-4 (348-fold), IL-8 (20-fold), IL-10 (54-fold) and IFN-γ (18-fold) were significantly elevated at 3 h post-exercise compared with pre-exercise values.

Urinary sediments

As shown in Table 2, renal tubular epithelial cells and renal tubular epithelial casts were observed in the urinary sediments of 7 subjects, among the fastest 8 subjects for the race time. Granular casts were also observed in the urinary sediments of 6 of these 7 subjects. White blood cells were more dominant than red blood cells in most subjects, and 13 of the 14 subjects (1~3 counts/ every field) recovered at 3 hours after the race, but only one subject (No.6) did not recover (11~15 counts/ every field). Based on these results, we analyzed between two subgroups that were divided according to existence (damaged group, n=7) or non-existence (minor-damaged group, n=7) of renal tubular epithelial cells in the urinary sediments as follows.

Parameters of renal functions

As shown in Figure 1, in the damaged group, serum Cr concentration increased significantly at 0 h post-exercise (0.54mg/dL; 1.66-fold) and at 1.5 h after the race (0.36mg/dL; 1.44-fold) compared with the pre-exercise values, whereas serum Cr concentration increased significantly at 0 h post-exercise (0.32mg/dL; 1.41-fold) as compared to pre-exercise values in the minor-damaged group. In the minor-damaged group, urinary excretion volume per minute (excretion volume rate) exhibited no significant change. Excretion volume rate in the damaged group and creatinine clearance (Ccr) in both groups decreased after the race compared with the pre-exercise values. Ccr and excretion volume rate in both groups did increase from 0 h, but still remained below the pre-exercise levels at 3 h after exercise (damaged group only: Ccr $p<0.05$). There was a significant decrease in

Table 1. Changes in plasma and urinary cytokines following the duathlon race.

	Unit	Pre	0 h	1.5 h	3 h	Fried-man test	Scheffe test
TNF-α-P	pg/mL	0.31 ± 0.45	0.10 ± 0.04	0.26 ± 0.28	0.92 ± 1.69	-	-
TNF-α-U	pg/mL	2.1 ± 2.1	2.6 ± 2.0	4.2 ± 6.0	2.3 ± 2.9	-	-
	pg/mgCr	1.8 ± 1.6	2.0 ± 1.8	1.8 ± 2.0	2.8 ± 3.8	-	-
	pg	442.4 ± 450.7	227.0 ± 250.9	251.9 ± 302.8	404.5 ± 580.2	-	-
	pg/min	3.7 ± 3.8	1.3 ±1.3	2.8 ± 3.4	4.5 ± 6.5	-	-
IL-1ß-P	pg/mL	0.06 ± 0.12	0.43 ± 1.21	0.96 ± 2.03	0.29 ± 0.38	**	Pre-1.5 h* Pre-3 h**
IL-1ß- U	pg/mL	0.97 ± 0.74	6.32 ± 9.43	10.82 ± 27.73	1.66 ± 2.76	**	0 h-3 h*
	pg/mgCr	0.90 ± 0.71	4.53 ± 5.88	4.15 ± 8.42	1.78 ± 2.84	*	0 h-3 h*
	pg	199.9 ± 180.9	267.0 ± 283.9	480.6 ± 772.7	269.2 ± 468.5	-	-
	pg/min	1.7 ± 1.5	2.2 ± 2.5	5.3 ± 8.6	3.0 ± 5.2	-	-
IL-1ra-P	pg/mL	50.5 ± 47.0	5058.7 ± 8270.7	8223.7 ± 8692.5	3333.9 ± 4540.9	**	Pre-0 h** Pre-1.5 h** Pre-3 h*
IL-1ra-U	pg/mL	189.8 ± 222.8	1658.2 ± 2313.5	7751.2 ± 13390.6	2990.1 ± 8537.6	*	Pre-1.5 h*
	pg/mgCr	160.9 ± 186.1	1182.5 ± 2507.6	3175.5 ± 6314.9	1991.1 ± 4471.9	-	-
	pg	28828 ± 24904	107482 ± 236347	476039 ± 1105060	282657 ± 597769	-	-
	pg/min	240.2 ± 207.5	865.9 ± 1992.0	5289.3 ± 12278.4	3140.6 ± 6641.9	-	-
IL-2-P	pg/mL	0.14 ± 0.11	0.26 ± 0.29	0.28 ± 0.35	0.23 ± 0.31	-	-
IL-2-U	pg/mL	6.3 ± 13.5	21.7 ± 48.2	305.3 ± 383.1	373.8 ± 382.1	*	-
	pg/mgCr	4.3 ± 7.0	7.2 ± 10.0	117.2 ± 165.5	195.6 ± 197.4	**	Pre-3 h** 0 h-3 h*
	pg	947.6 ± 1369.8	1005.0 ± 1410.9	18316.2 ± 26638.7	25476.4 ± 28349.0	*	0 h-3 h*
	pg/min	7.9 ± 11.4	5.1 ± 5.8	203.5 ± 296.0	283.1 ± 315.0	**	Pre-3 h* 0 h-3 h*
IL-4-P	pg/mL	0.25 ± 0.05	0.36 ± 0.14	0.30 ± 0.10	0.25 ± 0.08	-	-
IL-4-U	pg/mL	118.0 ± 403.2	1260.6 ± 2890.1	34928.9 ± 61276.3	41073.7 ± 61409.3	*	Pre-3 h*
	pg/mgCr	109.3 ± 384.3	329.1 ± 603.4	10529.2 ± 18532.6	20119.0 ± 30392.5	**	Pre-3 h* 0 h-3 h*
	pg	29472 ± 104996	35335 ± 64621	1786140 ± 3634239	2272571 ± 3488557	**	Pre-3 h* 0 h-3 h**
	pg/min	245.6 ± 875.0	262.2 ± 454.5	19846.0 ± 40380.4	25250.8 ± 38761.7	**	Pre-3 h* 0 h-3 h**

IL-6-P	pg/mL	0.41 ± 0.40	11.84 ± 8.43	4.42 ± 3.67	1.61 ± 1.52	**	Pre-0 h** Pre-1.5 h** 0 h-3 h**
IL-6-U	pg/mL	0.20 ± 0.15	0.36 ± 0.29	0.58 ± 0.45	1.58 ± 3.38	**	Pre-0 h* Pre-1.5 h**
	pg/mgCr	0.17 ± 0.07	0.23 ± 0.16	0.28 ± 0.20	0.79 ± 1.59	-	-
	pg	39.9 ± 19.3	22.2 ± 18.0	41.2 ± 31.5	75.3 ± 95.5	**	Pre-0 h* 0 h-3 h**
	pg/min	0.33 ± 0.16	0.14 ± 0.09	0.46 ± 0.35	0.84 ± 1.06	**	Pre-0 h* 0 h-1.5 h** 0 h-3 h**
IL-8-P	pg/mL	16.6 ± 10.4	44.9 ± 20.2	32.8 ± 12.6	22.1 ± 11.2	**	Pre-0 h** Pre-1.5 h** 0 h-3 h**
IL-8-U	pg/mL	9.0 ± 8.9	31.8 ± 28.7	153.2 ± 158.4	184.6 ± 145.2	**	Pre-1.5 h** Pre-3 h*
	pg/mgCr	7.7 ± 6.6	18.8 ± 15.2	60.9 ± 70.5	113.2 ± 70.8	**	Pre-3 h** 0 h-3 h*
	pg	1698 ± 1658	1649 ± 1364	9325 ± 11202	15150 ± 10602	**	Pre-3 h** 0 h-3 h**
	pg/min	14.2 ± 13.8	11.7 ± 11.1	103.6 ± 124.5	168.3 ± 117.8	**	Pre-3 h** 0 h-1.5 h* 0 h-3 h**
IL-10-P	pg/mL	3.1 ± 8.2	16.1 ± 31.4	11.5 ± 38.4	10.7 ± 37.0	**	Pre-0 h** 0 h-3 h**
IL-10-U	pg/mL	6.7 ± 8.5	34.1 ± 48.0	311.7 ± 376.5	364.2 ± 337.9	**	Pre-3 h*
	pg/mgCr	5.7 ± 7.5	14.1 ± 20.4	115.6 ± 147.3	203.3 ± 170.4	**	Pre-3 h** 0 h-3 h**
	pg	1352 ± 1959	1808 ± 2414	18318 ± 25117	26594 ± 23686	**	Pre-3 h** 0 h-3 h**
	pg/min	11.3 ± 16.3	13.7 ± 20.2	203.5 ± 279.1	295.5 ± 263.2	**	Pre-3 h** 0 h-3 h**
IL-12-P	pg/mL	0.20 ± 0.08	0.30 ± 0.17	0.25 ± 0.13	0.21 ± 0.08	-	-
IL-12-U	pg/mL	1.5 ± 1.1	4.4 ± 4.3	3.0 ± 3.0	1.4 ± 1.1	**	Pre-0 h* 0 h-3 h*
	pg/mgCr	1.4 ± 1.3	2.9 ± 3.4	1.4 ± 1.3	2.3 ± 3.7	-	-
	pg	298.1 ± 257.1	182.9 ± 112.9	198.9 ± 188.2	303.5 ± 429.1	-	-
	pg/min	2.5 ± 2.1	1.4 ± 1.0	2.2 ± 2.1	3.4 ± 4.8	-	-

IFN-γ-P	pg/mL	0.04 ± 0.71	0.15 ± 0.29	0.07 ± 0.08	0.08 ± 0.11	-	-
IFN-γ-U	pg/mL	11.8 ± 24.0	45.7 ± 78.2	179.0 ± 189.5	212.6 ± 165.4	**	Pre-1.5 h** Pre-3 h* 0 h-1.5 h*
	pg/mgCr	8.4 ± 12.1	17.0 ± 23.1	73.6 ± 85.4	129.5 ± 86.5	**	Pre-1.5h* Pre-3 h** 0 h-3 h**
	pg	1828 ± 2397	2125 ± 2590	11201 ± 13173	17481 ± 13265	**	Pre-3 h* 0 h-1.5 h** 0 h-3 h**
	pg/min	15.2 ± 20.0	12.0 ± 13.5	124.5 ± 146.4	194.2 ± 147.4	**	Pre-3 h* 0 h-1.5 h** 0 h-3 h**
MCP-1-P	pg/mL	2.8 ± 2.6	7.9 ± 3.5	6.3 ± 2.9	4.0 ± 2 .5	**	Pre-0 h** Pre-1.5 h** 0 h-3 h**
MCP-1-U	pg/mL	1.3 ± 0.9	8.7 ± 24.8	5.1 ± 8.4	8.4 ± 16.3	**	Pre-1.5 h*
	pg/mgCr	1.1 ± 0.7	2.6 ± 4.7	1.7 ± 1.3	4.5 ± 6.8	-	-
	pg	260.9 ± 179.1	206.3 ± 329.6	240.4 ± 157.4	661.5 ± 1064.4	**	0 h-3 h*
	pg/min	2.2 ± 1.5	1.5 ± 2.4	2.7 ± 1.8	7.4 ± 11.8	**	0 h-3 h*
Urine volume	mL	220.7 ± 107.2	57.5 ± 46.4	83.1 ± 44.1	191.7 ± 201.3	**	Pre-0 h**
Cr-U	g/L	1.1 ± 0.3	2.0 ± 1.4	2.4 ± 1.5	1.3 ± 0.8	**	Pre-1.5 h*
Osmotic Pressure-S	mOsm/L	296.1 ± 5.1	299.8 ± 8.1	288.6 ± 5.3	288.3 ± 5.4	**	Pre-1.5 h* Pre-3 h* 0 h-1.5 h** 0 h-3 h**
Osmotic Pressure-U	mOsm/L	719.6 ± 193.4	482.4 ± 258.7	630.6 ± 225.5	527.9 ± 337.0	*	Pre-0 h*

Values: means ± SD (n=14). Statistics: $^{**}p < 0.01$, $^{*}p < 0.05$.
-P: plasma. -U: urine. Cr: creatinine was used for the adjustment of urine concentration. The gross amount of urine (pg) was calculated by the raw concentration (pg/mL)×urine volume (mL). Urinary excretion rate was calculated by the raw concentration (pg/mL)×urine volume (mL) / one minute (min). Abbreviations: interleukin (IL)-1 receptor antagonist (IL-1ra), tumour necrosis factor (TNF), interferon (IFN), monocyte chemotactic protein (MCP).

Ccr at 0 h following exercise in the damaged group ($p<0.05$). Urinary excretion rate of ALB increased significantly at 0 h (30.3-fold) after the race in the minor-damaged group, and until 1.5 h (43.4-fold) after exercise in the damaged group, but were then reduced. The change in urinary excretion rate of NAG activity in the minor-damaged group was not significant. In the damaged group, urinary excretion rate of NAG activity was not significantly different from pre-exercise at 0 h, however, levels then increased significantly compared with 0 h post-exercise. Urinary excretion rate of NAG activity did not return to pre-exercise levels at 3 h

Table 2. Cells and casts in the urinary sediments following the duathlon race.

A: renal tubular epithelial cell

race time (min)	subject No.	Pre	0 h	1.5 h	3 h
99.6	1		+	+	
101.1	2			+	+
103.0	3		+		
108.7	4				
109.4	5			+	
110.0	6			+	+
111.8	7		+		
113.0	8			+	
118.2	9				
124.1	10				
124.8	11				
138.7	12				
152.3	13				
167.4	14				

B: renal tubular epithelial cast

Pre	0 h	1.5 h	3 h
	+	+	
		+	+
	+		
		+	
		+	+
	+		
		+	

C: granular cast

Pre	0 h	1.5 h	3 h
	1-3/s.f.	1-3/e.f.	
		1-3/e.f.	1-3/s.f.
	1-3/s.f.		
		1-3/s.f.	
		1-3/s.f.	
		1-3/e.f.	
	1-3/s.f.		

D: white blood cell

subject No.	Pre	0 h	1.5 h	3 h
1	1-3/s.f.	1-3/e.f.	7-10/e.f.	1-3/e.f.
2	1-3/s.f.		7-10/e.f.	1-3/e.f.
3	1-3/s.f.	7-10/e.f.	4-6/e.f.	1-3/e.f.
4	1-3/s.f.	7-10/e.f.	1-3/s.f.	1-3/s.f.
5	1-3/e.f.	11-15/e.f.	16-20/e.f.	1-3/e.f.
6	1-3/s.f.	1-3/e.f.	11-15/e.f.	11-15/e.f.
7	1-3/s.f.	7-10/e.f.	1-3/e.f.	1-3/s.f.
8	1-3/s.f.		7-10/e.f.	1-3/e.f.
9	1-3/s.f.	16-20/e.f.	7-10/e.f.	1-3/e.f.
10	1-3/s.f.	16-20/e.f.	4-6/e.f.	1-3/s.f.
11	1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/e.f.
12	1-3/s.f.		1-3/e.f.	1-3/e.f.
13	1-3/s.f.	1-3/e.f.	1-3/s.f.	1-3/s.f.
14	1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/s.f.

E: red blood cell

Pre	0 h	1.5 h	3 h
1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/s.f.
1-3/s.f.		1-3/e.f.	1-3/e.f.
1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/e.f.
1-3/s.f.	1-3/e.f.	1-3/s.f.	1-3/s.f.
1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/s.f.
1-3/s.f.	1-3/s.f.	1-3/e.f.	1-3/s.f.
1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/e.f.
1-3/s.f.		1-3/e.f.	1-3/e.f.
1-3/s.f.	1-3/s.f.	1-3/e.f.	1-3/s.f.
1-3/s.f.	1-3/e.f.	1-3/s.f.	1-3/s.f.
1-3/s.f.	1-3/s.f.	1-3/e.f.	1-3/s.f.
1-3/s.f.		1-3/e.f.	1-3/s.f.
1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/s.f.
1-3/s.f.	1-3/e.f.	1-3/s.f.	1-3/s.f.

A-renal tubular epithelial cell
B-renal tubular epithelial cast
C-granular cast: Data are 1-3 counts by several field (/s. f.), 1-3 counts by every field (/e. f.)
D-white blood cell: Data are 1-3 counts by several field (/s. f.), 1-3, 4-6, 7-10, 11-15 and 16-20 counts by every field (/e. f.)
E: red blood cell: Data are 1-3 counts by several field (/s. f.), 1-3 counts by every field (/e. f.)
Urinary sediments were analyzed by microscope (x400 high powerd field)

after exercise in the damaged group. Thus, the degree of renal disorder of Ccr, urinary excretion rates of ALB and urinary excretion rate of NAG activity in the damaged group were higher than the minor-damaged group.

Plasma and urine cytokines in relation with renal damage

In both groups, plasma concentrations of IL-1ra, IL-6, IL-8 and MCP-1 increased significantly after the race compared with the pre-exercise values. Plasma IL-10 concentration increased significantly at 0 h after exercise compared with the pre-exercise value in the damaged group only. However, urinary excretion rates of IL-8, IL-10 and IFN-γ increased significantly, and urinary excretion rates of IL-2

Table 3. Criteria of AKI

RIFLE

	Cr/GFR Criteria	Urine Output (UO) Criteria
Risk	Increased Cr ×1.5 or GFR decreases ＞25%	UO ＜0.5ml/kg/hr ×6hr
Injury	Increased Cr ×2 or GFR decreases ＞50%	UO ＜0.5ml/kg/hr ×12hr
Failure	Increased Cr ×3 or GFR decreases ＞75% or Cr ≧4mg/dl (with acute rise of ≧0.5mg/dl)	UO ＜0.3ml/kg/hr ×24hr or anuria ×12hr
Loss	Persistent ARF = Complete loss of renal function for ＞4weeks	
ESRD	End Stage Renal Disease	

AKIN

	Cr Criteria	Urine Output (UO) Criteria
Stage1	Increased Cr ×1.5 or ≧0.3mg/dl	UO ＜0.5ml/kg/hr ×6hr
Stage2	Increased Cr ×2	UO ＜0.5ml/kg/hr ×12hr
Stage3	Increased Cr ×3 or Cr ≧4mg/dl (with acute rise of ≧0.5mg/dl)	UO ＜0.3ml/kg/hr ×24hr or anuria ×12hr

Modified by Cruz et al. Critical care, 13:211.2009 (6)

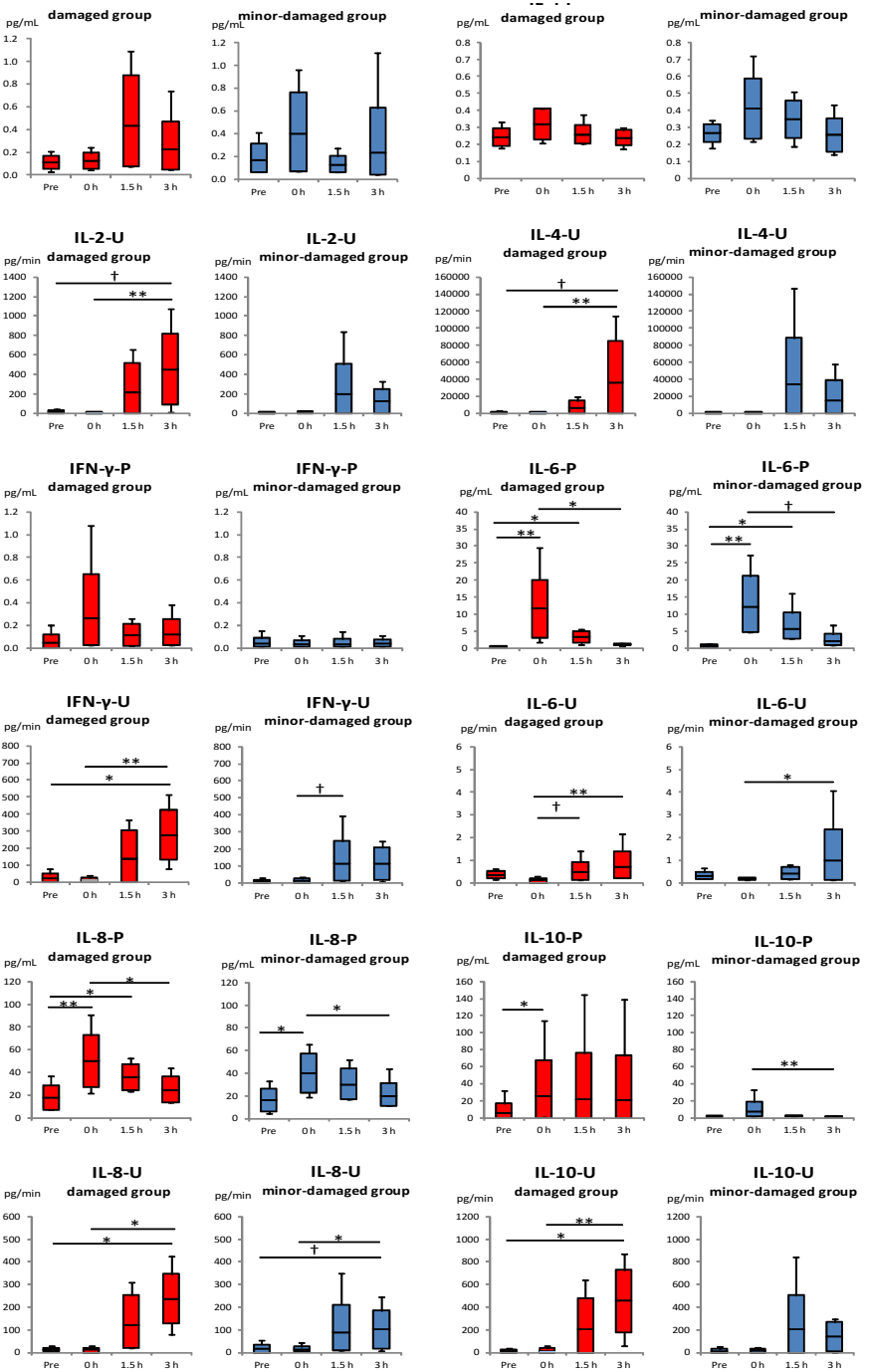
Abbreviations: Risk, Injury, Failure, Loss, End Stage Kidney Disease (RIFLE), acute kidney injury network (AKIN), creatinine (Cr), glomerular filtration rate (GFR), acute renal failure (ARF).

and IL-4 tended to increase after the race compared with the pre-exercise values in the damaged group. There was a trend for urinary excretion rate of IL-8 to increase following exercise in the minor-damaged group. In both groups, urinary excretion rates of IL-6 were significantly elevated at 3 h after the race as compared to 0 h-post exercise values. In the damaged group, urinary excretion rates of IL-2, IL-4 and MCP-1 increased significantly at 3 h after the race compared with 0 h-post exercise. On the other hand, urinary excretion rates of TNF-α, IL-1β, IL-1ra and IL-12 did not change significantly after the race in both groups (Figure 1, Table 4).

Table 4. Changes of urine volume and cytokines following the duathlon race.

	Renal tubular epithelial cell	Unit	Pre	0 h	1.5 h	3 h	Fried-man test	Scheffe test
Urine volume	(+) n=7	mL/min	1.8 ± 0.84	0.31 ± 0.22	0.79 ± 0.36	1.5 ± 0.95	**	Pre-0 h** 0 h-3 h†
	(-) n=7		1.9 ± 1.0	0.62 ± 0.41	1.1 ± 0.59	2.9 ± 3.0	†	Pre-0 h†
TNF-α-P	(+)	pg/mL	0.40 ± 0.61	0.11 ± 0.03	0.22 ± 0.22	1.6 ± 2.3	-	-
	(-)		0.23 ± 0.25	0.10 ± 0.05	0.30 ± 0.34	0.28 ± 0.29	-	-
TNF-α-U	(+)	pg/min	3.7 ± 4.4	0.61 ± 0.53	2.7 ± 2.3	5.6 ± 8.4	-	-
	(-)		3.7 ± 3.3	2.0 ± 1.4	2.9 ± 4.4	3.4 ± 4.0	-	-
IL-1ß-P	(+)	pg/mL	0.09 ± 0.15	0.70 ± 1.7	1.4 ± 2.8	0.23 ± 0.24	-	-
	(-)		0.03 ± 0.08	0.16 ± 0.38	0.49 ± 0.83	0.35 ± 0.50	**	Pre-0 h† Pre-1.5 h† Pre -3 h*
IL-1ß-U	(+)	pg/min	2.1 ± 2.0	1.2 ± 1.3	8.6 ± 11.2	4.8 ± 7.0	-	-
	(-)		1.2 ± 0.74	3.2 ± 3.0	1.8 ± 2.4	1.2 ± 1.2	*	-
IL-1ra-P	(+)	pg/mL	54.7 ± 52.1	3545 ± 7386	9391 ± 8791	3846 ± 3996	**	Pre-1.5 h** Pre -3 h†
	(-)		46.3 ± 45.1	6572 ± 9398	7057 ± 9124	2822 ± 5300	**	Pre-0 h** Pre -1.5 h**
IL-1ra-U	(+)	pg/min	186.8 ± 163.5	175.4 ± 360.4	8328 ± 17263	2500 ± 4362	†	-
	(-)		293.7 ± 244.8	1556 ± 2712	2251 ± 2663	3781 ± 8695	-	-
L-12-P	(+)	pg/mL	0.21 ± 0.07	0.30 ± 0.12	0.24 ± 0.09	0.22 ± 0.07	-	-
	(-)		0.20 ± 0.09	0.29 ± 0.21	0.27 ± 0.17	0.20 ± 0.09	-	-
IL-12-U	(+)	pg/min	2.6 ± 2.9	1.0 ± 1.2	2.6 ± 1.8	3.2 ± 4.3	-	-
	(-)		2.4 ± 1.3	1.9 ± 0.67	1.8 ± 2.5	3.5 ± 5.6	-	-

Values: means ± SD (n=7). Statistics: **p < 0.01, *p < 0.05, †p < 0.1.
-P: Data are plasma concentration. -S: Data are serum concentration.
-U: Data are the gross amount in the volume of urinary excretion per one minute.
pre-exercise (Pre), immediately post-exercise (0 h), 1.5 hour post-exercise (1.5 h) and 3 hour post-exercise (3 h) are sampling points.
(+): damaged group; renal tubular epithelial cells existed in the urinary sediments.
(-): minor-damaged group; renal tubular epithelial cells did not exist in the urinary sediments.
Abbreviations: tumour necrosis factor (TNF), interleukin (IL), IL-1 receptor antagonist (IL-1ra).


damaged group
minor-damaged group
damaged group
minor-damaged group
IL-2-U
damaged group
IL-2-U
minor-damaged group
IL-4-U
damaged group
IL-4-U
minor-damaged group
IFN-γ-P
damaged group
IFN-γ-P
minor-damaged group
IL-6-P
damaged group
IL-6-P
minor-damaged group
IFN-γ-U
dameged group
IFN-γ-U
minor-damaged group
IL-6-U
dagaged group
IL-6-U
minor-damaged group
IL-8-P
damaged group
IL-8-P
minor-damaged group
IL-10-P
damaged group
IL-10-P
minor-damaged group
IL-8-U
damaged group
IL-8-U
minor-damaged group
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damaged group
IL-10-U
minor-damaged group
pg/mL
pg/min
Pre
0 h
1.5 h
3 h

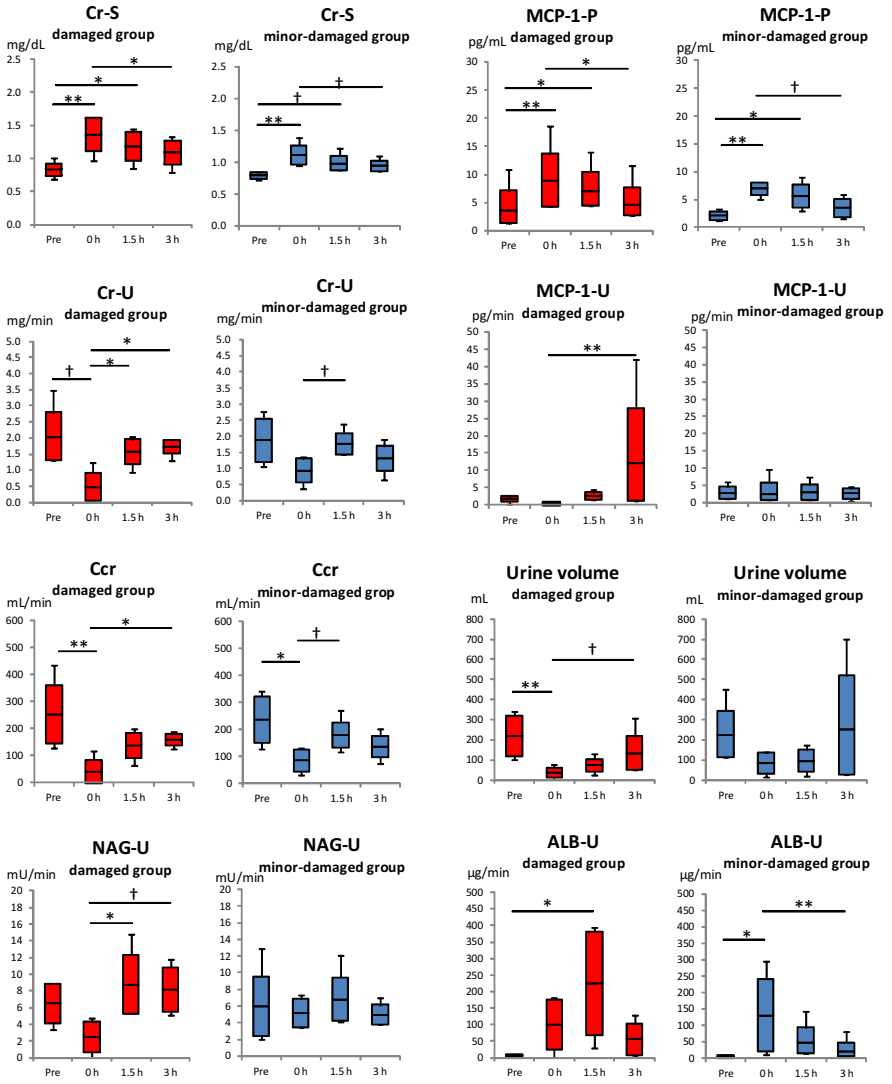

Figure 1. Changes of cytokines and parameters of renal damage following the duathlon race. pre-exercise (Pre), immediately post-exercise (0 h), 1.5 hour post-exercise (1.5 h) and 3 hour post-exercise (3 h) are sampling points.
damaged group: renal tubular epithelial cells existed in the urinary sediments.
minor-damaged group: renal tubular epithelial cells did not exist in the urinary sediments.
Abbreviations: creatinine (Cr), creatinine clearance (Ccr), albumin (ALB), *β-N*-acetyl-D-glucosaminidase (NAG) activity, interleukin (IL), interferon (IFN), monocyte chemotactic protein (MCP)-1.
Values: means ± SD (n=7). Statistics: *$p < 0.05$, **$p < 0.01$, †$p < 0.1$.
Box plot: minimum values means SD means means SD maximum values.
-P: Data are plasma concentration.
-S: Data are serum concentration.
-U: Data are the gross amount in the volume of urinary excretion per one minute (urinary excretion rate).

DISCUSSION

Studies investigating changes in the urinary levels of cytokines several hours after exhaustive endurance exercise have not yet been reported. In the present study, we present new evidence that changes in the urinary excretion of some cytokines were much greater than plasma cytokine changes, and might provide sensitive biomarkers of responses to exercise. IL-8 and IL-10 increased in both plasma and urine after exercise. In contrast, IL-1β, IL-1ra, IL-6 and MCP-1 mainly increased in plasma, whereas IL-2, IL-4 and IFN-γ only increased in urine after exercise. These results suggest that following exercise IL-1β, IL-1ra and MCP-1 were cleared slowly from the circulation with the plasma levels reflecting the systemic release, whereas IL-2, IL-4 and IFN-γ were rapidly excreted from the circulation into urine, without any increase in the systemic circulation, or the kidney might be the source of these cytokines as discussed later.

TNF-α has been considered as a primary mediator of the SIRS response, but the plasma and urine concentrations of TNF-α did not change significantly in the present study. On the other hand, the plasma concentration of IL-6 increased significantly by more than 30-fold immediately after the race, which is consistent with previous findings (4, 9, 14, 28-30, 32-34, 37, 46). However, the urine concentration of IL-6 did not change markedly—especially when corrected for urinary creatinine clearance. Although the plasma concentration of IL-1β increased significantly 1.5 h after the race, this response occurred later than the IL-6 response, and the concentration of IL-1β was relatively lower than IL-1ra, indicating that the cascade of cytokine production during exercise differs from the classical pro-inflammatory cytokine cascade that occurs during acute inflammation such as sepsis (1, 10, 19, 33, 37, 46). Chemokines are also considered important in the pro-inflammatory process. IL-8 is a potent neutrophil chemotactic and activation protein, whereas MCP-1 promotes monocyte activation and extravasation into inflammatory tissues (22). The plasma concentrations of IL-8 and MCP-1 increased significantly immediately after the race in the present study, which is also consistent with previous studies (24-27, 32-35, 44-49). Although exhaustive exercise enhances the capacity of neutrophils to produce reactive oxygen species (27, 43, 44), which is also one of the main pathogenic mechanisms of multiple organ failure in SIRS (1), these chemokines may, at least in part, mediate such exercise-induced pathogenesis as muscle inflammatory damage, exertional rhabdomyolysis and heat-related multiple organ failure (27, 44-46).

In contrast to these pro-inflammatory cytokines, we demonstrated that plasma IL-1ra concentration increased by more than 170 times in the present study. We have previously shown an increase of IL-1ra by 214-fold after a marathon race (45). IL-1ra is a natural antagonistic cytokine that competes with IL-1 for receptor binding without inducing signal transduction. Regardless of whether IL-1β is released in a small quantity after exercise in a delayed-onset manner, the higher concentration of IL-1ra appears to block the IL-1 bioactivity in advance—at least in the circulation. In the present study, IL-10 increased not only in plasma (5-fold) but also in urine by more than 50-fold. IL-10 causes immunosuppression associated with various forms of trauma by attenuating IL-1β, TNF-α, IFN-γ, IL-6 and IL-8 and enhancing IL-1ra production (16, 33, 52). There was no increase in plasma IL-4, but we observed a significant increase in urinary concentration of

IL-4 after exercise in the present study. In support of the present findings, we have previously shown no changes in plasma IL-4 concentration following a marathon (45). However, we have reported an increase in urinary IL-4 concentrations 2 h after short-duration, maximal exercise (46). The 350-fold increase in urinary IL-4 concentration in the present study is, to our knowledge, the most dramatic change reported among existing studies on exercise and cytokines. IL-4 has been shown to down-regulate pro-inflammatory cytokines and up-regulate IL-1ra production (52). In view of the large changes and biological characteristics of these anti-inflammatory cytokines, it is possible that they play a role in immunosuppression and increased susceptibility to infection after endurance exercise (3, 23, 39). IL-4 is also a strong inducer of type-2 cytokines such as IL-6 and IL-10, and its involvement in patients with atopic disposition and past history of exercise-induced allergy and anaphylaxis seems worthy of further investigation (13, 40).

We did not collect blood samples during exercise, so it is uncertain whether IL-2 was elevated and active within the bloodstream during exercise. IL-2 may bind to soluble receptors, and/or act locally within lymphoid tissues (36), which could explain why it did not appear in plasma following exercise (11, 46). We observed that plasma concentration of IFN-γ was low, whereas urinary excretion of both IL-2 and IFN-γ was large following exhaustive exercise in this study. Although one study reported the urinary excretion of IFN-γ after 20-km running (38), other groups have not reported any change (11, 29, 53). Several factors might explain these inconsistencies. Firstly, we used a different manufacturer's ELISA kit from the previous studies that may capture a different epitope or have enhanced sensitivity. Secondly, the exercise conditions and time course of sampling points especially for the recovery period in the present study were different from previous studies. Lastly, other investigators have not standardized fluid intake or corrected for glomerular filtration by expressing cytokine concentrations relative to urinary creatinine content. Although we observed, somewhat unexpectedly, that the post-exercise urinary concentrations of IL-2, IL-4, IL-8, IL-10 and IFN-γ were several orders of magnitude higher compared with plasma, this might be because we provided fluid replacement during exercise to prevent dehydration and augment urine output with enhanced renal clearance and reduced re-absorption, or the kidney in itself might be the main source of the cytokine production. In any case, the present data demonstrate that IL-2, IL-4 and IFN-γ were produced in the body following exhaustive endurance exercise.

In this study, according to the values of serum Cr in the AKI diagnosis criteria such as “Risk, Injury, Failure, Loss, End Stage Kidney Disease (ESKD): RIFLE criteria” (2) and “acute kidney injury network: AKIN” (18, 20) (Table 3), AKI following endurance exercise showed “Risk” or “Stage1”at 0 h and 1.5 h after the race in the damaged group and at 0 h after exercise in the minor-damaged group. Moreover, the degree of renal disorder of Ccr, urinary excretion rate of ALB and urinary excretion rate of NAG activity in the damaged group were higher than the minor-damaged group. In the present study, the relations among the changes of plasma and urine levels of various cytokines and AKI were investigated. In both groups, plasma concentrations of IL-2, IL-4 and IFN-γ did not change significantly after the race as compared with the pre-exercise values whereas urinary excretion of IFN-γ increased significantly and urinary excretion

of IL-2 and IL-4 tended to increase after the race compared with the pre-exercise values in the damaged group only. Therefore, it might be possible that IL-2, IL-4 and IFN-γ were produced in the kidney that caused AKI following endurance exercise. Moreover, urinary excretion of IL-8 and IL-10 also increased significantly after the race as compared with the pre-exercise values in the damaged group only, but plasma concentrations of IL-8 and IL-10 also increased significantly post-exercise when compared with the pre-exercise in both groups of IL-8 and in the damaged group of IL-10. In the damaged group only, urinary excretion rates of MCP-1 increased significantly at 3 h after the race as compared with 0 h-post exercise values. However, urinary excretion of MCP-1 was increased until 3 h-post exercise. Furthermore, urinary excretion of IL-8, IL-10 and MCP-1 in the damaged group was elevated markedly more than the ones in the minor-damaged group. Accordingly, it might be possible that IL-8, IL-10 and MCP-1 were produced in the circulation and/or kidney, which caused AKI following endurance exercise.

The present data suggest that although some cytokines may not appear in the circulation during exercise, they do appear in urine after several hours following endurance exercise, which indicates their production and/or clearance from the bloodstream in the kidney. These findings might help to develop reliable biomarkers of immunity and inflammation for the assessment of endurance exercise workload as well as the pathological mechanisms of strenuous exercise. Further research is needed to determine the mechanisms of the production and clearance of the cytokines in the kidney from the circulation following prolonged endurance exercise. Although the wider physiological and pathological implications are still not clearly understood, such cytokine kinetics may partly explain the exercise-induced suppression of cell-mediated immunity (3, 23, 34, 36, 39), organ damage and inflammation (4, 14, 29, 32-34, 37, 43-49), and increased allergic reactions (13, 40, 46). The relationships among these changes, their clinical significance and the possibility of intervention must be clarified in future research.

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Cytokine kinetics in nasal mucosa and sera: new insights in understanding upper-airway disease of marathon runners

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ABSTRACT

Recently, many authors have proposed that mechanisms such as inflammation and/or allergies could be partly responsible for cases of upper respiratory tract illnesses that affect athletes after exhaustive exercise. Here we studied the kinetics of cytokines in the serum and nasal mucosa of athletes after a marathon. We were able to demonstrate an increase in serum levels of all interleukins studied immediately after the marathon in athletes that present or not with upper airways symptoms followed by a return to basal levels 72 hours after the race, as described in the literature. Interleukin (IL)-10 behaviour differed in the group of asymptomatic athletes. Measurement of this cytokine in protein extract of nasal mucosal cells showed increase 72 hours after the marathon. Levels of this cytokine in sera were increased at rest in athletes that did not present symptoms. These fin-

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dings suggest that the maintenance of a non-inflammatory environment in the mucosal airways is an active process that requires participation of the systemic and mucosal immune systems. We propose that the understanding of the upper airway disease of the athlete involves the study of mucosal and systemic immune systems.

Keywords: upper respiratory tract infections, cytokines, secretory immunoglobulin A, exhaustive exercise, mucosal immune system.

INTRODUCTION

Peters and Bateman (17) were the first authors to report an increased prevalence of respiratory tract infection in athletes after competitions or periods of intensive training. It has been suggested that a relationship exists between training load and susceptibility to infection (9, 15). Among the hypotheses put forward to explain these findings, it is worth mentioning the "open window" and "J curve" theories, which suggest that infection may be caused by systemic immunosuppression. However, these theories are still under considerable debate (16) and fail to explain why systemic immunosuppression in these athletes usually causes only upper respiratory tract infections (URTIs) without any other manifestations.

Spence and others (19), examining athletes with symptoms of URTIs, reported that inflammatory allergic reactions associated with vasomotor phenomena and the inhalation of cold and dry air could be observed in most cases, and that confirmed infection by pathogen identification could be detected in a smaller number of cases. Bermon (3) also suggested that the mechanism that causes manifestations of symptoms of upper respiratory tract illness symptoms (e.g. sore throat, runny nose) in the respiratory tract can be associated with either infections or inflammation/allergies. Cox (6) reported similar results, showing that allergy and asthma were responsible for at least 30% of non-infectious cases. Thus, the question of whether sore throats are actually caused by infections or are a reflection of other inflammatory stimuli continues to be the subject of debate (20).

The study of local factors involved in upper respiratory tract illnesses essentially focuses on determining the concentration of secretory immunoglobulin A (SIgA) in athletes' saliva, which has been reported to decrease after prolonged endurance events. Exercise has an important effect on salivary SIgA levels, which vary according to the level of the athlete's training. The absolute concentration of SIgA has been reported to fall after prolonged strenuous exercise. Thus, there appears to be a relationship between repeated high-intensity training or competitions and altered production of mucosal immunoglobulins and, therefore, the local immune response (20).

However, the role played by the immune system in this regulatory response is not yet fully understood (8). For this reason, the study of immune system cells involved in mechanisms that protect the upper respiratory tract, as well as their products, such as immunoglobulins and cytokines, is of fundamental importance

in understanding the mechanisms responsible for breaking the homeostasis, which manifest as infection, clinical inflammation or allergy.

In spite of the importance of cytokines in the regulation of the immune response, their role in respiratory tract infection in athletes has yet to be clarified. As shown by Cox (7), athletes prone to respiratory illness had low absolute plasma concentrations of IL-10, IL-1ra and IL-8 when at rest, and high absolute plasma concentrations of IL-6 after exercise, suggesting a possible association between impaired inflammatory regulation and respiratory illness. However, the studies cited above only investigated levels of cytokines in peripheral blood samples. Despite the differences between systemic and mucosal immune responses, local factors in the upper airway, after exhaustive exercise, have not been studied to date, except for the SIgA response.

The aim of this study was to evaluate the immune response elicited by exhaustive exercise in different compartments, namely, the local (upper airway mucosa) and systemic (serum) compartments, by comparing athletes that presented or not with symptoms of upper airway disease after completing a marathon.

MATERIAL AND METHODS

Subjects and study design

The study population consisted of 22 recreational male marathon runners with a mean age of 41.4±9.4 years living in the city of São Paulo. All the athletes were aware of the possible risks involved in the study, having given their agreement to the study protocol and signed a consent form, both of which were approved by the UNIFESP-EPM Ethics Committee. This study meets the ethical standards defined by Harris and Atkinson (10).

The volunteers were evaluated before the race by clinical examination and those with upper respiratory symptoms were excluded. Athletes were instructed to report any manifestations of respiratory illness symptoms in the two weeks following their participation in the race. The individuals recruited to the study kept to their usual training/physical exercise schedules throughout the study (mean of 100 km/week and 12 hours/week) to prepare for the race. The last training session was performed 24 hours before samples were collected. We recommended that all marathon runners ingested 500 mL of water before the marathon. The same drink was offered by the marathon organisation team during the race and consumed according to each runner's needs. One week after the race, the volunteers were divided into two groups based on the athletes' reports: asymptomatic individuals (n=15) and symptomatic individuals (n=7). Symptoms began within five to seven days after the marathon and lasted for three to five days. The main complaints reported by the athletes were coryza (nasal congestion) and an itchy and runny nose. All athletes who reported upper-airway symptoms were examined by a doctor.

Collection of the samples
Samples of serum, saliva and cells from the nasal mucosa, which were chosen as being representative of the upper airways, were collected at three different times: at rest, immediately after and 72 hours after the marathon.

One millilitre of saliva sample from each volunteer was collected directly into sterile 15-mL Falcon® tubes without prior stimulation. A total of 250 µL of each sample was transferred to 1.5-mL Eppendorf tubes and kept frozen at -80°C until SIgA concentration was measured. No buffers or preservatives were added. Each sample was centrifuged at 3000 rpm for 5 minutes, and the supernatant was used to measure SIgA concentration. Samples containing blood were discarded and replaced with newly collected samples.

Samples of nasal cells (representative of the upper airways) were collected from the nasal cavities with a cytobrush (Kolplast LTDA, São Paulo, Brazil), and the total protein extract was obtained as previously described (11). A total of 200 µL of supernatant was collected and frozen at -80ºC for subsequent analysis of intracellular cytokines.

Cell collection for cytogram
At rest and after 72 hours, nasal cells were collected to determine the percentage of neutrophils in the sample. These data were used together with the report of the athletes and blind clinical evaluation performed by an otolaryngologist to determine the inflammatory status of their upper airway.

Determination of salivary immunoglobulin A (SIgA) concentration
Salivary IgA concentration was determined by ELISA using the Salivary Secretory IgA Indirect Enzyme Immunoassay kit (Salimetrics™) according to the manufacturer's instructions. Standards, positive and negative controls were prepared as described by the manufacturer.

Determination of cytokine concentrations in serum and nasal cell extract
The concentration of the cytokines IL-6 and IL-10 in serum and in the nasal cell extract previously stored at -80°C was measured using the Luminex bead-based system for human cytokines with the LINCOplex kit for simultaneous multi-analyte detection (Linco Research, Inc. St Charles Missouri, IL) following the manufacturer's instructions. The cytokine concentrations obtained in the nasal cell extract were normalised by the Bradford method (5), using the ratio of total protein (in milligrams) to cytokine concentrations in each sample.

Statistical analysis
Secretory immunoglobulin A (SIgA) and cytokine concentrations are shown as means and standard deviation (SD). Data analysis was performed using a Two-Way ANOVA test followed by a Fisher's LSD post hoc test to compare if the cytokine or SIgA concentrations and three different times of sample collection were significant between each group of marathon runners. The Mann-Whitney test was used to determine whether the relation between neutrophils infiltration

percentage PRE and 72 hours after (POST) marathon [(POST/PRE)*100] were significant. The significance level was set at 5% ($p < 0.05$).

RESULTS

Performance and maintenance of hydration during the marathon race

The athletes performed the marathon in a mean time of 4 hours (h) and 31 minutes (min) (SD ± 29 min). No statistically significant differences in times were observed between the groups [asymptomatic group = 4h 36min (SD ± 31min) and symptomatic group = 4h 25min (SD ± 29min)]. Immediately after the marathon, the haematocrit percentage did not alter [asymptomatic group = 44.7±4.1 (baseline) and 43.4±3.9 (immediately after); symptomatic group = 44.9±5.2 (baseline) and 45.1±5.8 (immediately after)], indicating that the athletes were adequately hydrated during the race.

Differences in salivary SIgA levels observed before and after the marathon race

Analysis of salivary SIgA levels in all the athletes immediately after the marathon showed a significant reduction compared with baseline levels ($p<0.005$), returning to the levels at rest, 72 hours after the marathon (Table 1). When we evaluated the concentration of salivary SIgA levels at rest, comparing asymptomatic with symptomatic athletes, there was no significant difference between the groups (symptomatic = 215±35 mg/L and asymptomatic = 243±47 mg/L, Figure 1). In both groups, SIgA levels were reduced in relation to levels at rest immediately after the marathon (symptomatic = 168±31 mg/L and asymptomatic = 153±18 mg/L, $p=0.001$). No difference was observed between the groups (Figure 1). Seventy-two hours after the race, salivary SIgA levels in both groups had returned to their respective baseline values (symptomatic = 223±47 mg/L and asymptomatic = 237±12 mg/L), with no statistical difference between the groups (Figure 1).

Cellular analysis of nasal mucosa

The cytological study of cells obtained from the nasal cavities at rest and 72 hours after the marathon showed that upper-airway complaints were compatible with alteration in the nasal cellularity. While in the symptomatic group there was an increase from 1.1±1.6 to 21.7±14.6% in the percentage of neutrophils infiltration, in the asymptomatic group the percentage of neutrophils after the marathon (0.7±1.2) was similar to that

Table 1. Salivary secretory immunoglobulin A (SIgA) levels (mg/L) of all marathon runners on three different occasions: at rest (baseline), immediately after and 72 hours after running a marathon. The results are shown as means and standard deviation.

Salivary SIgA (mg/L)	Marathon runners (n=22)
Baseline (at rest)	274±24
Immediately after the marathon	168±19*
72 hours after the marathon	266±28

* $p<0.005$ (immediately after X baseline and 72 hours after the run)

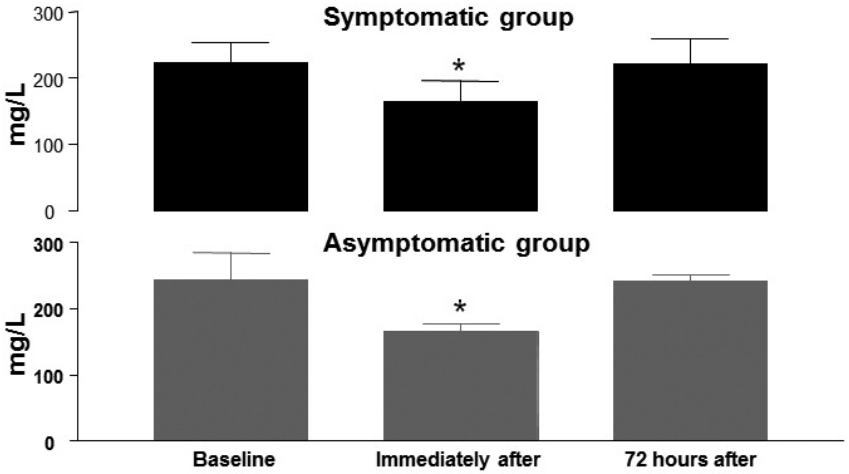

Figure 1. Secretory immunoglobulin A levels (mg/L) in saliva of marathon runners in the asymptomatic group and symptomatic group were evaluated on three different occasions: at rest (baseline), immediately after and 72 h after running a marathon. The values ware expressed as mean and standard deviation with a significant difference from baseline ($p<0.05$) indicated with an asterisk (*).

observed at rest (1.2±1.9). The statistical analysis of difference between the ratio [(Post/Pre)*100] of neutrophils infiltration percentage in both groups showed that percentage of neutrophils in symptomatic group was significantly higher than asymptomatic group ($p<0.0001$). These findings reinforced both the information reported by the athletes and the blind clinical examination performed by an otolaryngologist.

Alteration in cytokine levels before and after the marathon race in the sera and upper-airway mucosa.

According to the results obtained in the Two-way ANOVA analysis, we obtained a significant effect of time and time-symptoms interaction for IL-6 and IL-10 cytokines. Based on this observation, was performed the Post-hoc comparisons between groups. Baseline analysis of serum IL-6 concentrations (Figure 2A) did not show differences between the groups (symptomatic = 8.2±20.8 pg/mL and asymptomatic = 14.9±32.3 pg/mL). However, the evaluation of IL-10 levels (Figure 2B) showed that in the asymptomatic group (17.4±34.0 pg/mL), the concentration was statistically higher ($p<0.03$) than the symptomatic group (0.76±0.1 pg/mL) at rest. Immediately after the marathon, the IL-6 (symptomatic = 40.9±30.9 pg/mL and asymptomatic = 39.2±26.5 pg/mL, Figure 2A) and IL-10 (symptomatic = 57.2±32.7 pg/mL and asymptomatic = 30.7±25.3 pg/mL, Figure 2B) levels were significantly increased in both groups ($p<0.01$), when compared to basal concentrations. Seventy-two hours after the marathon, IL-6 (symptomatic = 10.6±20.4 pg/mL and asymptomatic = 16.9±34.3 pg/mL, Figure 2A) and IL-10 (symptomatic = 1.1±0.9 pg/mL and asymptomatic = 4.5±11.4 pg/mL, Figure 2B)

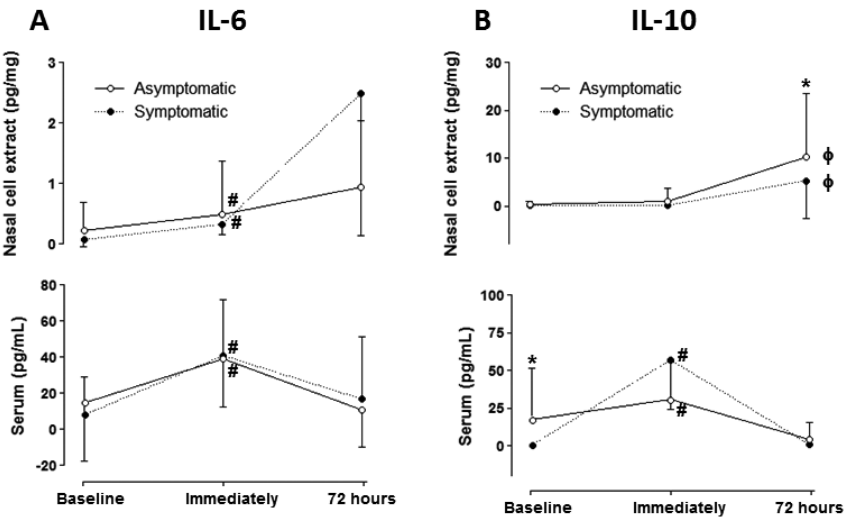

Figure 2. Concentrations of cytokine IL-6 (A) and IL-10 (B) in serum (pg/mL) and extracts from nasal cells (pg/mg total protein) from marathon runners in asymptomatic and symptomatic groups on three different occasions: at rest (baseline), immediately after and 72 hours after running a marathon. The significance level was $p<0.05$ and * denotes differences between the values obtained for the groups at the same moment; # denotes differences in values obtained at baseline moment and immediately after the marathon; φ denotes differences in values obtained at immediately and 72 hours after the marathon

levels returned to basal values in both groups. In relation to cytokine concentrations in nasal cell extracts, previously normalised by the Bradford method to pg/mg, we observed differences between the groups, as shown in Figure 2A and Figure 2B. Analysis of IL-6 concentrations at rest (Figure 2A) did not show a difference between the groups (symptomatic = 0.07±0.11 pg/mg and asymptomatic = 0.22±0.47 pg/mg). Immediately after the marathon, the IL-6 levels (Figure 2A) increased significantly compared with levels at rest in both groups (symptomatic = 0.33±0.17 pg/mg, $p<0.005$ and asymptomatic = 0.49±0.89 pg/mg, $p<0.01$) and remained elevated 72 hours after the marathon (Figure 2A, symptomatic = 2.49±2.35 pg/mg, $p<0.001$ and asymptomatic = 0.94±1.1 pg/mg, $p<0.001$). The IL-10 levels (Figure 2B) in both groups were significantly increased 72 hours after the marathon (symptomatic = 5.33±8.00 pg/mg, $p<0.05$ and asymptomatic = 10.26±13.31 pg/mg, $p<0.001$) in relation to levels at rest (symptomatic = 0.22±0.30 pg/mg and asymptomatic = 0.28±0.69 pg/mg) and immediately after the marathon (symptomatic = 0.15±0.15 pg/mg and asymptomatic = 1.00±2.68 pg/mg). Furthermore, the elevation of IL-10 levels observed 72 hours after the marathon in the asymptomatic group was statistically higher ($p<0.05$) than the symptomatic group (Figure 2B).

DISCUSSION

In this study we were able to observe significant differences in the kinetics of IL-10 in the sera and mucosa of symptomatic and asymptomatic groups of athletes after a marathon. Our results on serum IL-6 behaviour are in accordance with the literature, and the study of the kinetics in the nasal mucosa showed an increase of IL-6 concentration immediately after the marathon that persisted until 72 hours but without statistical differences between the groups.

The serum cytokines studied behaved according to previous reports in the literature; however the kinetics of IL-10, both in the serum and nasal mucosa extract suggests new insights in the comprehension of the upper-airway disease of the athlete.

An interesting finding was that sera IL-10 was significantly increased in the asymptomatic group even before the marathon, suggesting that these athletes were prone to activate anti-inflammatory mechanisms to cope with the inflammatory response expected due to the exhaustive exercise and hyperventilation that occurs during the marathon, as consequences of mucosal dryness and excessive exposure to air pollutants. This group of athletes probably developed this adaptive behaviour during their training period.

The results obtained in the mucosa were not remarkable except for the behaviour of IL-10, whose levels were significantly increased in the extract of nasal mucosa obtained from the group that did not present symptoms compared to the symptomatic group. This finding suggests that asymptomatic individuals must actively induce a local immune response directed to inhibit the inflammatory response. Although levels of IL-10 were increased in both groups 72 hours after the marathon, the values obtained for the asymptomatic group were significantly higher, so that we can infer that levels of cytokines in the extract of nasal mucosa are not simply a reflection of the changes serum cytokine concentrations, which is a finding that, as we will see below, has great importance.

"Mucosal immunity has the function of maintaining homeostasis on exposed mucosal surfaces. It is noteworthy that this system is anatomically and functionally distinct from its blood-borne counterpart of the systemic (or peripheral) immune system and is strategically located at the portals by which most microorganisms enter the body. The development of this specific branch of the immune system alongside and separate from the peripheral immune system may have been necessitated by the size of the mucosal surfaces, which cover an area of ~400 m^2 in the adult human, as well as the large number of exogenous antigens to which these surfaces are exposed"(4). In light of these facts we must take into account that the study of mucosal responses cannot be correlated only on results obtained from the systemic immune response, although both branches of the immune system are directed towards the same goals, in some instances they can act in different ways.

The upper-airway mucosa is continuously in contact with different types of antigenic substances and so it is of extreme importance to have tight control of the

mechanisms that can modulate the response to these antigens. This can be done by inhibition of pro-inflammatory cytokines or by increased secretion of IL-10 by epithelial cell-conditioned dendritic cells. Moreover, epithelial cell-conditioned T lymphocytes showed increased differentiation towards IL-10-producing type 1 T-regulatory (Tr1) cells, suggesting that epithelial cells induce non-inflammatory microenvironments that regulate the local immune response (12). Awasthi et al. (1) discuss the importance of T regulatory cells in maintaining the balance of immune homeostasis and the potential of Tr1 cells in regulate T-cell function by demonstrating that dendritic cells modified by Treg cells induced the generation of IL-10-producing Tr1 cells.

Many other studies in the literature have highlighted the importance of IL-10 in blocking the airways´ inflammatory responses (13, 14, 18, 21) so, the elevated levels of IL-10 that we found in the asymptomatic group can be a factor of protection to avoid mucosal inflammation.

Bazzoni et al (2), in a review about the interaction between IL-10 and the innate immune system, showed that neutrophils, a key cellular target for IL-10, act in response to the cytokine and its actions on macrophages and tissue chemoattractants, modulating their responsiveness and their capacity to produce and release cytokines, thus inducing an anti-inflammatory response. In light of these facts, the percentage of neutrophils obtained from nasal mucosa of athletes at rest and after the race assumed great importance, as we found in the symptomatic group a significant increase of cells after the marathon that was not seen in the asymptomatic individuals, a mucosal environment that suggests a blockade of the inflammatory response. Although IL-10 acts on many others types of immune cells, we chose to study neutrophils because we are dealing with an acute response.

Regarding to salivary SIgA we found decreased concentrations in both groups. According to Walsh et al (20) falls in SIgA levels can occur during intensive periods of training, and these authors reinforce the idea that there is reasonable evidence to indicate that a reduced concentration of saliva SIgA is associated with an increased risk of URTI. In agreement on the fact that reduction of the levels of SIgA is a key event in triggering upper respiratory symptoms, we believe that cytokines in serum and especially those that are locally produced play important role to determine whether the athlete will or not became symptomatic.

Based on our findings on the behaviour of IL-10 in the serum and nasal mucosa of asymptomatic and symptomatic groups of athletes, we suggest that the research on upper-airway disease of athletes must, as often as possible, include the study of local factors, and that after exhaustive exercises associated with hyperventilation and usually with inhalation of substances that can cause an inflammatory response of the upper airway mucosa, the maintenance of a non-inflammatory status is an actively induced process in which both, systemic and locally released IL-10 has a key action.

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Effects of regular exercise on neutrophil functions, oxidative stress parameters and antibody responses against 4-hydroxy-2-nonenal adducts in middle aged humans

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Abstract

Regular exercise has recognized health benefits, partly because it reportedly lowers the levels of the oxidation products of proteins and DNA at rest, in contrast with the effect of acute exercise. However, when we compared oxidative response markers in active middle-aged subjects with those in sedentary ones, the level of urinary 8-OHdG was higher in active subjects. Because neutrophils are the first line of defense against a variety of infectious diseases, we then compared the cell density, functions and apoptosis of neutrophils in active subjects with those in sedentary ones. The cell density of neutrophils and phagocytosis of opsonized zymosan by neutrophils were higher in active subjects, being similar with the reported effects of acute exercise. To determine any beneficial effects of oxidative stress in active subjects, we then compared the levels of antibodies against 4-hydroxy-2-nonenal adducts in active subjects with those in sedentary ones, because 4-hydroxy-2-nonenal is one of the most common bioactive aldehyde products of oxidative stress, and because the IgM class of antibodies against oxidized low-density lipoprotein is associated with atheroprotective properties. The level of the IgM but not the IgG class of antibodies against 4-hydroxy-2-nonenal adducts was higher in active subjects. Overall, this study revealed that our active

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middle-aged subjects showed both oxidative responses and a higher IgM response to reactive carbonyl derivatives, possibly providing a basis for a health benefit by exercise in our active subjects.

Key words: regular exercise, oxidative response, anti-oxidative response, antibodies against 4-hydroxy-2-nonenal adducts.

Introduction

Regular exercise is beneficial for health. Although the underlying mechanisms for the positive effects of regular exercise have not been fully elucidated, there is the widely accepted hypothesis that each bout of exercise causes oxidative stress that eventually induces an anti-oxidative response, thereby augmenting the capacity of our body to exert the anti-oxidative response (16). If this mechanism holds true, then repeated bouts of exercise, i.e. regular exercise, may decrease the oxidation products of proteins and DNA at rest (16). This is perhaps why regular exercise or a physically active lifestyle reduces the risk of the development of coronary artery disease and ameliorates the symptoms in patients with established cardiovascular disease (21). It may also partly account for the association between regular exercise and reduced risk of other diseases, including type 2 diabetes, cancer and senile dementia (6, 21). It should be noted that a major cause of these diseases is oxidative stress.

The oxidation products can be measured non-invasively based on the levels of reactive carbonyl derivatives (RCD) of serum proteins (22) and urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) (14). Serum proteins are secreted from cells and react with reactive oxygen species (ROS) outside the cells, whereas 8-OHdG is derived from the intracellular oxidation products of DNA. Therefore, the levels of carbonylated serum proteins and 8-OHdG are affected by extracellular and intracellular ROS, respectively, although they are not mutually exclusive.

Regular exercise may also be beneficial in reducing common cold symptom duration and severity, possibly by means of the products of white blood cells including ROS derived from neutrophils. Neutrophils are a major constituent of white blood cells, and are one of the main producers of ROS in the body. Consequently there is the possibility that the cell density of neutrophils and/or the capability to produce ROS may be related to the beneficial effects of regular exercise. In addition, if regular exercise induces an anti-oxidative response in our body, neutrophil apoptosis may decrease, thereby possibly increasing our capacity to combat against pathogens.

Recently, RCD, such as formaldehyde-generated RCD, were implicated as the active moieties that drive adaptive immune responses (12). Therefore, there is the possibility that regular exercise causes an adaptive immune response through RCD. Clinical and experimental studies have suggested that the IgG class of antibodies against oxidized low-density lipoprotein (OxLDL) is associated with pro-atherogenic properties, whereas the IgM class of antibodies against OxLDL is associated with atheroprotective properties (10). This raises the possibility that regular exercise may cause a higher IgM response to RCD, thereby decreasing the incidence of atherosclerosis.

In this study, we recruited middle-aged male games players (soccer) and sedentary males of similar age, and examined the effects of regular exercise on the oxidation products of proteins and DNA at rest and on apoptosis of neutrophils, the cell density and ROS production of neutrophils. We also examined the possibility that regular exercise may cause a higher IgM response to RCD such as 4-hydroxy-2-nonenal (HNE) adducts.

Materials and methods

Experimental design

This experiment was designed to examine the relationship between regular exercise and health, and was approved by the bioethical committee of Toho University School of Medicine. The experiments were carried out in three different periods, (1) from 28 May 2005 to 2 July 2005, (2) from 30 October 2005 to 4 December 2005, and (3) from 30 September 2006 to 18 November 2006. We recruited 37 male volunteers involved in regular exercise (soccer) (55.3±7.7 years-old; 41-67 years-old) and 15 male volunteers who were not regularly active (53.4±6.9 years-old; 44-65 years-old) for this experiment. All the active volunteers had played soccer for 2 to 3 hours every Sunday for more than 10 years. Three to four of the volunteers joined the experiment at 10 am on each Saturday when they had not exercised on that day. We obtained informed consent from all the volunteers under the condition that personal information would be number coded and stored anonymously. Then, 25 ml of peripheral blood was taken from each volunteer by a nurse to obtain serum and a neutrophil-rich population. Urinary samples (5 ml each) were also obtained from each volunteer in the morning. Serum and urine samples were aliquoted and stored at -80°C until analysis. Each volunteer then performed a supervised VO_2max test based on the Astrand-Rhyming submaximal cycle ergometer test, on an Aerobike 75XLII (Combi Wellness Co. Ltd., Tokyo). VO_2max in active subjects was higher than that in sedentary ones ($p<0.01$) (Fig. 1), as expected.

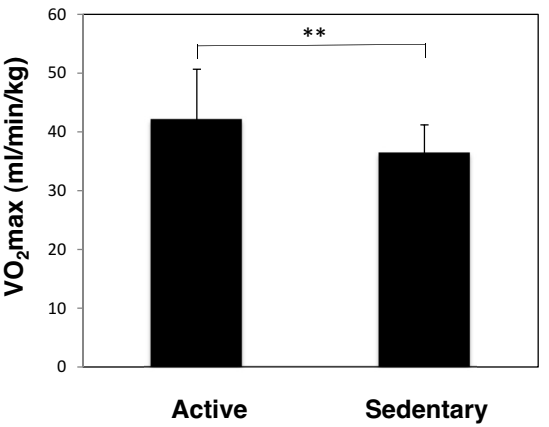

Fig. 1. VO_2max.
The VO_2max data are expressed as means ± standard error. The difference is statistically significant ($p<0.01$, **).

Measurement of urinary 8-OHdG

Urinary 8-OHdG was determined using a high sensitivity ELISA kit (NOF, Tokyo). The obtained values were corrected for urinary creatinine concentration.

Measurement of carbonylated serum albumin

Carbonylated serum albumin was measured by derivatization of carbonyls

with dinitrophenylation, followed by Western blot analysis according to the method previously described (13). The amount of carbonylated serum albumin was normalized to serum albumin concentration, which was determined by Western blot analysis with anti-human serum albumin antibodies.

Preparation of a neutrophil-rich population
Ten ml of peripheral blood was mixed with an equal volume of 3% dextran in saline, followed by incubation for 30 min at room temperature. The supernatant was then centrifuged, and the red cells lysed with distilled water for 30 to 60 s. Neutrophils were then separated from lymphocytes by Ficoll density gradient centrifugation, the purity being 54±17% (n=45) based on FSC vs. SSC profiles on flow cytometric analysis using a FACS Calibur (Becton Dickinson, CA).

Phagocytosis of zymosan by a neutrophil-rich population
Zymosan (*Saccharomyces cerevisiae;* Sigma) was labeled with FITC and then washed several times with PBS. The zymosan was then opsonized with fetal calf serum (3). A neutrophil-rich population was incubated with either opsonized or unopsonized FITC-zymosan (15 mg/ml) at the cell density of 1.25 x 10^6 cells/ml for 30 min at 37°C, followed by flow cytometric analysis. Neutrophils were identified by means of FSC vs. SSC profiles.

Superoxide anion production by a neutrophil-rich population
Two hundred μl of a neutrophil-rich population at the density of 1.25 x 10^6 cells/ml was left in a tube for 3 min at room temperature. Three min after the addition of 25 μM lucigenin, the cells were stimulated with PMA (0.81 μM), followed by measurement of chemiluminescence with a luminometer.

Induction and analysis of apoptosis (9)
A neutrophil-rich population was incubated for 24 h at the cell density of 5 x 10^5 cell/ml in RPMI1640 medium containing 7% fetal calf serum. The cells were then washed and stained with FITC-Annexin V (Bender MedSystems, Vienna, Austria) and/or propidium iodide (PI) for 10 min at room temperature. Cells were then analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, CA). For flow cytometric analysis of apoptosis, neutrophils were identified by means of FSC vs. SSC profiles. Annexin V single-positive cells are defined as early apoptotic ones, and Annexin V and PI double-positive cells as late apoptotic ones.

Measurement of antibodies against HNE adducts in serum
Antibodies against HNE adducts were measured according to the method published previously (8). Briefly, 50 μl of 5 μg/ml bovine serum albumin in 0.05 M carbonate buffer (pH 9.6) was added to each well of a 96-well U-bottomed vinyl plate (BD Falcon), followed by incubation for 2 h at room temperature. After washing with PBS, 50 μl of 200 μM HNE in PBS or a vehicle control was added to each well, followed by incubation for 4 h at room temperature. After washing with PBS containing 0.05% Tween 20 (wash buffer), PBS containing 3% skim milk was added, followed by incubation for 2 h at room temperature. After washing with wash buffer, samples or rabbit anti-HNE adducts antibodies (Academy Biomedicals) as a positive control were added, followed by incubation for 2 h at

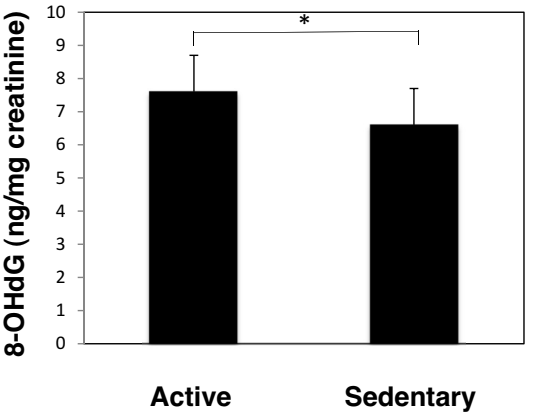

Fig. 2. Urinary 8-OHdG.
Urine samples were obtained from each subject, and the levels of urinary 8-OHdG were measured by means of a specific ELISA. The data were subjected to correction as to creatinine concentration, and expressed as means ± standard error. The difference is statistically significant ($p<0.05$, *).

room temperature. After washing, each well was incubated with biotinylated secondary antibodies (anti-rabbit IgG antibodies (American Qualex), anti-human IgM antibodies (Southern Biotech), anti-human IgG antibodies (Southern Biotech), or anti-human IgA antibodies (VECTOR)), streptavidin-HRP (R&D), and the substrate, ABTS (Sigma).

Statistics
The data were analyzed by means of Student's *t* test. *p* values of less than 0.05 were considered significant.

Results

Effects of regular exercise on urinary 8-OHdG and carbonylated serum albumin.
Urinary 8-OHdG and carbonylated serum albumin were chosen as markers of oxidative responses. As shown in Fig. 2, the level of urinary 8-OHdG in active subjects was higher than that in sedentary ones ($p<0.05$). Carbonylated serum albumin was then detected by means of derivatization of carbonyls through dinitrophenylation (13) (Fig. 3A, B, and C). The levels of carbonylated serum albumin, however, were not significantly different between active and sedentary subjects (Fig. 3D).

Effects of regular exercise on the cell density and functions of neutrophils
We then determined the neutrophil cell density in the peripheral blood of each subject, because acute exercise is known to release neutrophils from bone marrow into the periphery (19), and because regular exercise lowers the neutrophil density (1). The neutrophil cell density in active subjects was higher than that in sedentary ones ($p<0.05$) (Fig. 4).

We then determined the functions of neutrophils, namely phagocytosis and superoxide anion production. The superoxide anion can be quantitated by means of luminol- or lucigenin-enhanced chemiluminescence, the latter being superior to the former due to the high selectivity as to the superoxide anion produced outside of cells (2). We therefore employed lucigenin-enhanced chemiluminescence for determination of superoxide anion. Although zymosan was phagocytosed by neutrophils, opsonization with fetal calf serum enhanced phagocytosis (6.3% vs. 28%) (Fig. 5A, B, and C). Phagocytosis of opsonized zymosan by neutrophils of active subjects was higher than that by neutrophils of sedentary ones ($p<0.05$)

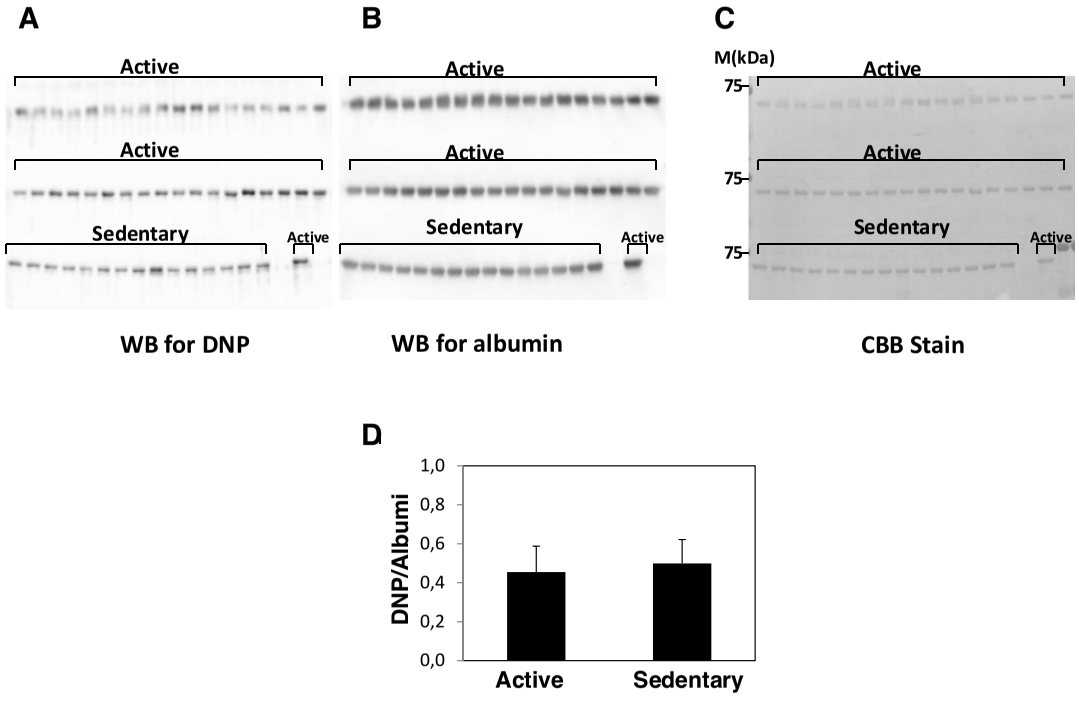

Fig. 3. Carbonylated serum albumin.
(A, B, C) Serum was obtained from the venous blood of each subject, and the level of carbonylated serum albumin was measured by derivatization of carbonyls with dinitrophenylation, followed by Western blot analysis according to the method previously described (A). The amount of carbonylated serum albumin was normalized with regards to the amount of serum albumin (B). The results of CBB staining are also shown in (C). (D) The data are expressed as means ± standard error. The difference is not statistically significant.

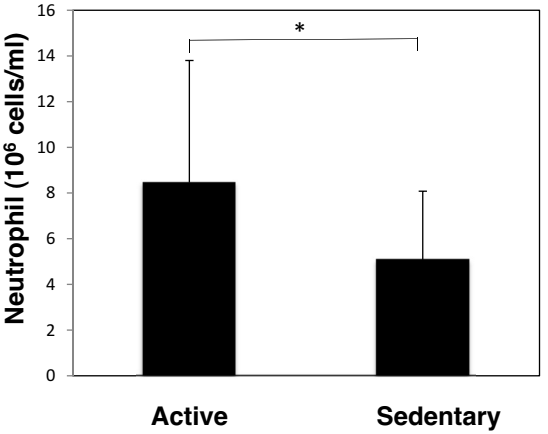

Fig. 4. Neutrophil cell density.
The cell density of a neutrophil-rich population was determined and corrected to the percentage of neutrophils, which was determined by FSC vs. SSC profiles on flow cytometric analysis. The data are expressed as means ± standard error. The difference is statistically significant ($p<0.05$, *).

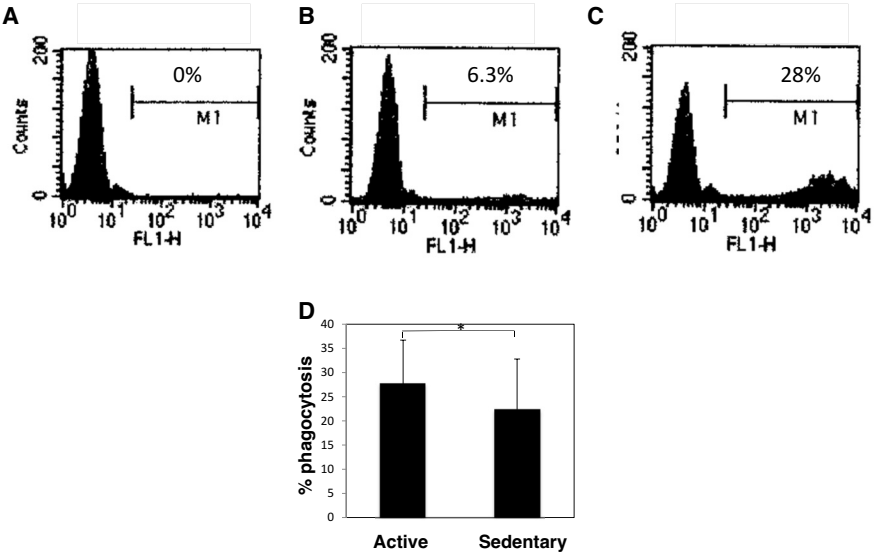

Fig. 5. Phagocytosis of opsonized zymosan by neutrophils.
(A, B, C) A neutrophil-rich population was obtained, and incubated alone (A), or with FITC-labeled unopsonized (B) or opsonized zymosan (C). (D) The data are expressed as means ± standard error. The difference is statistically significant ($p<0.05$, *).

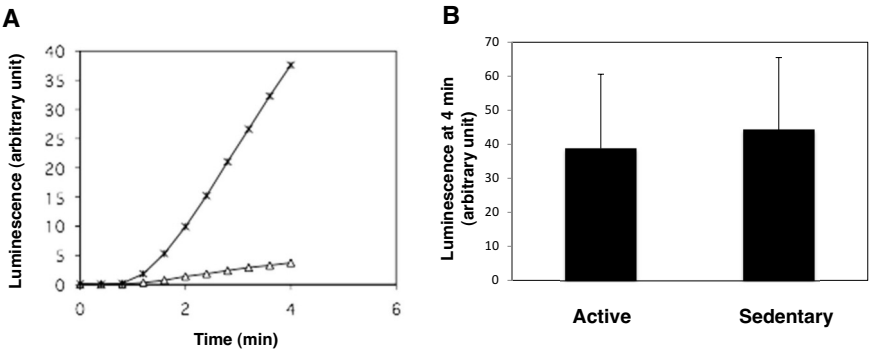

Fig. 6. PMA-induced production of the superoxide anion by neutrophils. (A) A neutrophil-rich population was obtained and lucigenin was added to it. They were then incubated alone (triangles) or with PMA (crosses), followed by measurement of chemiluminescence with a luminometer. (B) The data are expressed as means ± standard error. The difference is not statistically significant.

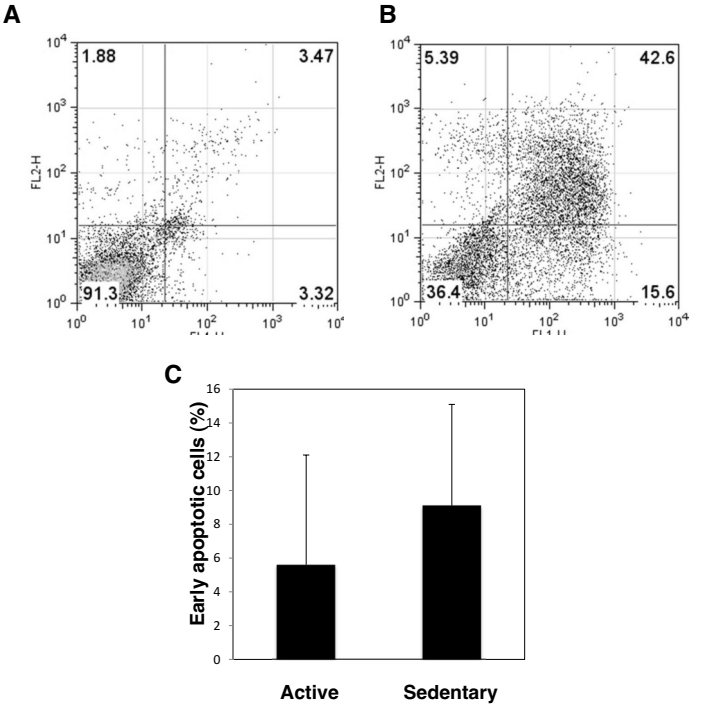

Fig. 7. Apoptosis of neutrophils after culturing.
(A, B) A neutrophil-rich population was obtained, and apoptosis was immediately analyzed (A) or analyzed after 24-h culture (B). FITC fluorescence is shown on the abscissa, and PI fluorescence on the ordinate. (C) The data are expressed as means ± standard error. The difference is not statistically significant ($p<0.1$).

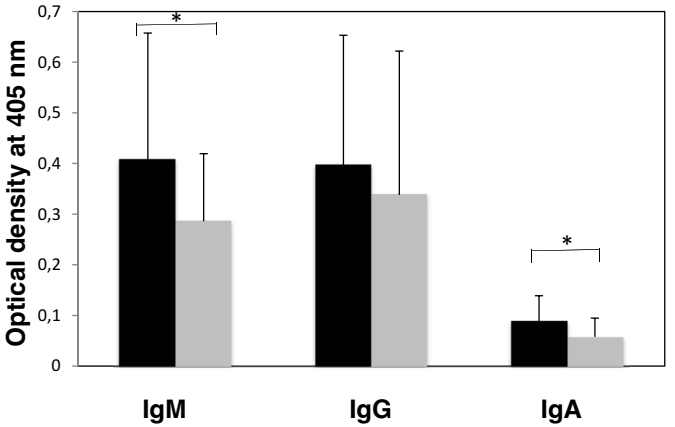

Fig. 8. The IgM, IgG, and IgA classes of anti-HNE adduct antibodies in serum. The data are expressed as means ± standard error (black for active subjects, gray for sedentary subjects). The differences in IgM and IgA are statistically significant ($p<0.05$, *), whereas that in IgG is not statistically significant.

(Fig. 5D). When neutrophils are stimulated with PMA, they produce the superoxide anion after a short time lag (Fig. 6A). However, superoxide anion production was not significantly different between active and sedentary subjects (Fig. 6B).

Neutrophils undergo apoptosis upon culturing, and the process can be quantitated with a flow cytometer. Before culture, neutrophils showed marginal levels of apoptosis, those of early and late apoptosis being 3.3 and 3.5%, respectively (Fig. 7A), whereas, after 24h-culture, they showed significant levels of apoptosis, those of early and late apoptosis being 15.6 and 42.6%, respectively (Fig. 7B). Early apoptosis in neutrophils of active subjects was lower than that in neutrophils of sedentary ones, although the difference was statistically not significant ($p<0.1$) (Fig. 7C). Late apoptosis was also not different significantly between active and sedentary subjects (data not shown, $p>0.1$).

Effects of regular exercise on the levels of antibodies against HNE adducts

HNE is one of the most common bioactive aldehyde products of oxidative stress (5), and it was reported that model antigens modified by HNE or other aldehydes resulted in antigen-specific Th2-type antibody responses under adjuvant-free conditions (12). We therefore determined the levels of antibodies against HNE adducts (IgM, IgG, and IgA) in serum after the serum had been diluted appropriately. Fig. 8 shows the results for one-hundredth diluted samples. The levels of IgM and IgA in active subjects were higher than those in sedentary ones ($p<0.05$). Although the levels of IgM, IgG and IgA should not be compared with each other due to the lack of individual standards, the IgA response would be weaker than the IgM and IgG ones, judging from the results of one-tenth and one-thousandth diluted samples (data not shown).

Discussion

In this study, we found significant differences in the level of urinary 8-OHdG, the cell density of circulating neutrophils and the phagocytosis of opsonized zymosan by neutrophils, which were all higher in active subjects. Neutrophil apoptosis after culturing, on the other hand, was lower in active subjects, although statistically not significant. The level of the IgM but not the IgG class of antibodies against HNE adducts was significantly higher in active subjects.

Since the urinary volume affects the concentration of urinary 8-OHdG, the urinary 8-OHdG data were corrected for creatinine concentration. Although we expected that regular exercise would decrease the oxidation products of protein and DNA at rest, the corrected urinary 8-OHdG levels were higher in active subjects compared with those in the sedentary males. In contrast, the level of carbonylated serum albumin did not differ between active and sedentary subjects. One of the plausible reasons for the different responses to oxidative stress between DNA and the serum protein would be the location of ROS production that causes the modifications, i.e. extracellular vs. intracellular; this is an area worthy of further study.

Neutrophil cell density was higher in active subjects, contrary to our expectation. In one study involving highly trained cyclists who were cycling distances of 120 km per week, the neutrophil cell density at rest was significantly lower than that in sedentary subjects, although in these cyclists acute exercise caused an increase in the neutrophil cell density to a similar extent as sedentary subjects (1). One of the plausible reasons for the discrepancy between our study and that of Blannin et al. is that the participants in the latter study were much younger and were also more highly trained than our active subjects, as judged from the VO_2max values. The other possible reason is that the carry-over effect from the last bout of exercise would persist in the active subjects even after a week. On the contrary, acute exercise results in first, rapid and profound neutrophilia, followed by a second, delayed increase in the blood neutrophil count a few hours later, the magnitude of which is related to both the intensity and duration of the exercise (15, 17). The initial increase is likely due to demargination caused by shear stress and catecholamines, whereas the later increase may be due to cortisol-induced release of neutrophils from the bone marrow (11).

Neutrophil phagocytosis of opsonized zymosan was higher in active subjects, contrary to our expectation. In contrast, PMA-induced superoxide anion production did not differ between active and sedentary subjects. Neutrophil phagocytosis and oxidative burst activity are increased by an acute bout of exercise (15, 17, 19) or by moderate exercise (18), whereas they are decreased in highly trained subjects (1). Of note, Blannin et al (1) used formazan deposits as an indicator of phagocytosis, which are actually an indicator of intracellular ROS production, whereas we employed FITC-labeled opsonized zymozan for analysis of phagocytic activity.

Neutrophil apoptosis was delayed in active subjects, although statistically not significant. This was in good agreement with a recent publication (20), in which the authors suggested that regular exercise induces an anti-oxidative response of neutrophils.

All the above data except for neutrophil apoptosis raise the possibility that an increase in ROS that can increase HNE adduct generation may induce an anti-HNE adduct antibody response of the Th2 type. This possibility may be supported by an increase in the IgA class of anti-HNE adduct antibodies, because Th2 cells promote preferential B cell production of IgA in mice (4) and presumably in humans (7). On the other hand, an increase in the IgM class of anti-HNE adduct antibodies may suggest one beneficial outcome of an increase in ROS in the active subjects in this study. This is because the IgG class of antibodies against oxidized low-density lipoprotein (OxLDL) is associated with pro-atherogenic properties, whereas the IgM class of antibodies against OxLDL is associated with atheroprotective properties (10). The level of urinary 8-OHdG appeared to be more closely related to the IgM class of anti-HNE adduct antibodies than the other classes of anti-HNE adduct antibodies, although the difference was statistically not significant. The mechanism underlying the increases in the IgM and IgA classes of anti-HNE adduct antibodies in active subjects should be explored in the future by means of experimental animals.

To summarize, this study demonstrated that active middle-aged males showed both oxidative responses and a higher IgM response to HNE adducts, possibly through increases in the levels of ROS, compared with sedentary males of similar age. Thus this study provides a basis for a health benefit by exercise in active individuals.

Acknowledgements

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Eccentric exercise-induced delayed-onset muscle soreness and changes in markers of muscle damage and inflammation

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[4] Mebiol Inc., Japan.

ABSTRACT

The purpose of this study was to determine the relationships among delayed-onset muscle soreness (DOMS), muscle damage and inflammatory responses to eccentric exercise and investigate the underlying mechanisms. Nine healthy males performed one-leg calf-raise exercise with their right leg on a force plate. They performed 10 sets of 40 repetitions of exercise at 0.5 Hz by the load corresponding to the half of their body weight, with a rest for 3 min between sets. DOMS was evaluated by a visual analogue scale (VAS). Blood and urine samples were collected before and 2, 4, 24, 48, 72 and 96 h post-exercise. Blood samples were analyzed for leucocyte differential counts and neutrophil functions (migratory activity and oxidative burst activity). We also determined a serum marker of muscle damage, myoglobin (Mb), and plasma and urinary prostaglandin E_2 as an algesic substance. As for the inflammatory mediators, plasma and urine were analyzed for cytokines (interleukin (IL)-1β, IL-1 receptor antagonist, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, tumour necrosis factor-α, interferon-γ, monocyte chemotactic protein-1, granulocyte colony-stimulating factor, macrophage colony-stimulating factor, and granulocyte macrophage colony-stimulating factor), leucocyte activation markers (calprotectin and myeloperoxidase), and neutrophil chemotactic factor complement 5a. All subjects reported muscle soreness on subsequent days and VAS peaked at 72 h after exercise. Serum Mb concentration significantly increased ($p<0.05$) at 72 h after exercise as compared with the pre-exercise values which was correlated with the increases in VAS at 72 h ($r=0.73$, $p<0.05$). Circulating neutrophil count and migratory activity increased significantly ($p<0.01$, and $p<0.05$, respectively) at 4 h after exercise, whereas there

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were no significant changes in the other plasma and urinary inflammatory mediators. These results suggest that neutrophils can be mobilized into the circulation and migrate to the muscle tissue several hours after the eccentric exercise. There were also positive correlations between the exercise-induced increases in neutrophil migratory activity at 4 h and the increases in Mb at 48 h ($r=0.67$, $p<0.05$). These findings suggest that neutrophil mobilization and migration after exercise may be involved in the muscle damage and inflammatory processes.

Key words: delayed-onset muscle soreness, eccentric exercise, exercise-induced muscle damage, inflammatory mediators, neutrophils

INTRODUCTION

Unaccustomed exercise with eccentric muscle contraction and exhaustive exercise cause muscle damage, inflammation, leakage of muscle proteins into the circulation and soreness on and several days after, which is called delayed-onset muscle soreness (DOMS) (14, 21, 24, 25). Although a large number of researchers have investigated the effects of exercise on muscle damage and DOMS in humans, there were various results in inflammatory responses (13, 17, 30), which may depend on exercise mode, intensity, and duration, as well as an individual's sex and age.

Exercise disrupts skeletal muscle ultrastructurally, resulting in leucocyte infiltration and release of myocellular proteins such as myoglobin (Mb) into the circulation (3, 24, 28). Systemically, marked neutrophilia with a left shift (6, 23), and enhanced capacity of neutrophils to produce reactive oxygen species (ROS) have been documented after endurance exercise (12, 23). Peripheral leucocyte count has also been shown to increase several hours after eccentric exercise (4). Histological examination of muscle biopsy in humans demonstrates leucocyte accumulation in muscle tissues (10, 29). After muscle and connective tissue damage following exercise, neutrophils are rapidly mobilized into the circulation, and soon migrate and infiltrate into the damaged tissue and produce ROS. If neutrophil functions, especially ROS production are over activated, tissue damage may occur. Within 24 h, neutrophils are replaced by macrophages which are active inflammatory cells that produce several pro-inflammatory cytokines, and promote removal of debris and remodeling of muscle tissue (9, 29). Indeed, several researchers investigated the inflammatory responses to eccentric exercise in humans (15, 19, 32). Peake *et al.* reported that changes in circulating leucocyte count after eccentric exercise are dependent on the muscle groups, or the amount of muscle mass recruited during eccentric exercise (16). Concerning changes in leucocyte receptor expression and oxidative burst activity, there were no clear effects of different types of eccentric exercise, but they might be due to differences in the type of eccentric exercise (16).

Cytokines are proteins which regulate immune and inflammatory responses. They are classified into pro-inflammatory cytokines which promote inflammation, anti-inflammatory cytokines which inhibit inflammation, immunomodulatory cytokines which control inflammation, multifunctional cytokines, chemokines

and colony-stimulating factors. Some of these substances are induced remarkably in plasma and urine following exhaustive endurance exercise (26). Other substances, involved in inflammatory responses, are neutrophil chemotactic factor complement 5a (C5a) and prostaglandin E_2 (PGE_2). Although numerous studies have investigated the effects of eccentric exercise on exercise-induced muscle damage, changes in plasma cytokines and neutrophil activation, there are several contradictory findings, partly due to 1) differences in the mode and intensity of exercise, 2) a limited range of variables measured, and 3) unreliable methods of neutrophil activity measurement.

Migration of neutrophils to the tissue microenvironment is the first step to evoke local inflammation. The production of ROS, not only of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), but also of MPO-dependent HOCl production of neutrophils, can be measured by luminol-dependent chemiluminescence (LmCL) (7, 23, 24, 27). Various neutrophil functions, such as migration and ROS production, could work as a dual-edged sword on both sides of host defence and tissue injury (23, 27). Thus, the balance between beneficial and harmful effects of neutrophil functions should be properly assessed. The use of hydrogel made it possible to mimic the *in vivo* microenvironment of neutrophil infiltration into tissues and LmCL can be detected through the transmissive gel (27). In this method, neutrophil migratory activity and ROS producing activity can be measured without the alteration of neutrophil functions by separating process in the conventional methods (7).

The aims of this study were at first to clarify relationships among muscle soreness, muscle damage markers, circulating leucocyte dynamics and changes in inflammatory mediators in blood and urine of which we tried to detect any possible changes. Especially, we applied a newly-developed measurement system of neutrophil migratory activity and ROS-producing activity by use of *ex vivo* hydrogel methodology with extracellular matrix to the investigation of the mechanisms of muscle damage (27).

METHODS

Subjects

Nine untrained healthy males participated in this study. Their mean (±SD) characteristics were as follows: age (24.8±1.3 yrs), body mass (62.3±6.3 kg), and height (1.72±0.05 m). At the time of the study, the subjects had not been involved in any hard exercise or resistance training for at least two weeks before the exercise bout, and were not taking any supplements, or participating in recovery strategies such as massage, stretching, or cryotherapy. The subjects were instructed to maintain their usual daily schedule during the experiment. The study protocol was approved by the ethics committee of Waseda University, Japan, and the subjects provided their informed consent.

Experimental design

Subjects performed a calf-raise exercise, including repetitive eccentric muscle contractions with their right leg on a force plate. The range of motion of the ankle

joint during the exercise was regulated from -20° (dorsiflexion position) to 15° (planter flexion position) using a goniometer (SG 110/A, Biometrics, Newport, UK) with its ends attached onto the skin over the tibia and calcaneus. They performed 10 sets of 40 repetitions of exercise at 0.5 Hz by the load corresponding to half of their body weight, with a rest for 3 min between sets. DOMS was rated using a visual analogue scale (VAS) that had a 100-mm line with "no pain" on one end and "extremely sore" on the other. Blood and urine samples were collected before and 2, 4, 24, 48, 72 and 96 h after the exercise.

Blood and urine sampling and analyses

Approximately 12 ml of blood samples were drawn by a standard venipuncture technique from the antecubital vein. Blood samples were collected into serum separation tubes and vacutainers containing heparin and EDTA. A portion of whole blood was used to measure haemoglobin, haematocrit and complete blood cell counts using an automatic blood cell counter (PocH100i, Sysmex, Kobe, Japan). The serum separation tubes were left to clot at room temperature for 30 min, and the vacutainers containing EDTA for plasma separation were immediately centrifuged at 1000×G for 10 min. Serum and plasma samples were then removed and stored at –80°C for later analysis. Serum concentrations of creatinine (Cr) and Mb were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan). Urine samples were centrifuged immediately at 1000×G for 10 min to remove sediments, and the supernatants were stored at –80°C for later analyses. Urinary concentrations of Cr were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the plasma and urine concentrations of interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra), IL-6, IL-12p70, tumour necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF) (R&D Systems; Minneapolis, MN, USA), IL-2, IL-4, IL-8, IL-10, IL-12p40, interferon-γ (IFN-γ), and C5a (Becton Dickinson Biosciences; San Diego, CA, USA), and calprotectin and myeloperoxidase (HyCult Biotechnology; Uden, the Netherland). ELISA measurements were performed according to the instructions for each ELISA kit using a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA, USA). Plasma and urine concentrations of PGE_2 (ENZO Life Sciences Inc; Farminglale, NY, USA) were measured using a chemiluminescent microplate reader (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany).

Preparation of peptide-bound temperature-responsive polymer (G-TRP)

Collagen peptide (24 g; SCP-5000; Nitta Gelatin Co., Osaka, Japan) was dissolved in 96 ml of distilled water at 37°C and followed by reaction with 3.26 g of N-acryloylsuccinimide (Kokusan Kagaku, Tokyo, Japan) for 4 days at 37°C to obtain polymerizable collagen peptide. N-isopropylacrylamide (108.5 g; Kojin, Tokyo, Japan) and n-butylmethacrylate (4.26 g; Wako Chemical, Osaka, Japan) were dissolved in 600 ml of ethanol and then 123 g of the above aqueous solution of polymerizable collagen peptide was added. Under nitrogen atmosphere, 1 ml of N,N,N',N'-tetramethylethylenediamine (Wako Chemical, Osaka, Japan) and 10 ml of 10 wt% ammonium persulfate (Wako Chemical, Osaka, Japan) aqueous

solution were added to the mixed solution, and then reacted for 5 h at 4°C, maintaining the nitrogen atmosphere. After the reaction, 30 l of cold (4°C) distilled water were added and the mixture was concentrated to 3 l using an ultrafiltration membrane (molecular weight cut off 100,000) at 4°C. This dilution and concentration process was repeated 5 times in order to remove impurities and low molecular species. Lyophilization and sterilization of the final concentrated solution gave 105 g of peptide-bound temperature-responsive polymer (G-TRP).

Preparation of scaffold-thermoreversible gelation polymer (S-TGP) gel
Under a clean-air laminar hood workbench, 0.5 g of G-TRP and 0.5 g of TGP (Mebiol gel; Mebiol Inc, Kanagawa, Japan) were dissolved in 16.7 ml of Hank's balanced salt solution (HBSS) at 4ºC for overnight, yielding a viscous transparent S-TGP gel uniform liquid without any bubbles for use in the experiments (27). Mebiol gel is a pure synthesized biocompatible copolymer composed of thermoresponsive polymer blocks and hydrophilic polymer blocks, characterized by its temperature-dependent dynamic viscoelastic properties and used as a biocompatible scaffold for three-dimensional culture without any toxicity (22). S-TGP gel is a peptide-bound thermoreversible gel formed by mixing G-TRP with the Mebiol gel. It liquefies at low temperature, turns to gel immediately upon warming, and returns to liquid state again when cooled.

Neutrophil functions
Peripheral blood samples were drawn from subjects using 2 ml Na-heparin tubes (Venoject II, Terumo Co., Tokyo, Japan). The blood samples were mixed with 2.5 mM luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Aldrich, MO, USA) at a ratio of 1:1. Then, 150 µl luminol-blood samples were layered on 50 µl S-TGP gel prepared in a tube at 37°C, and was promptly measured by LmCL (relative light unit: RLU) using a luminometer (Gene Light 55; Microtec Co., Ltd, Funabashi, Japan). The samples were incubated at 37°C, and the production of ROS from neutrophils was monitored in a kinetic mode for 60 min. After measurement of LmCL at 60 min, luminol-blood samples were removed and the tubes with 50 µl S-TGP gel in which neutrophils migrated were washed three times with PBS warmed at 37°C. Then, the tubes with gel were cooled on ice, and 50 µl Turk solution (Wako, Osaka, Japan) were added and mixed well. The liquid obtained in this way were set on the C-Chip (Disposable haemocytometer, Neubauer improved, DHC-No.1, Digital Bio, Seoul), and the migratory cell number was counted under the microscope. Migrated neutrophil number was calculated by 20 times multiplication of the counted cell number.

Statistical analysis
Data were analyzed using two-way analysis of variance. When significant time effects were evident, multiple comparisons were analyzed with Bonferroni adjustment. Associations between data were analyzed with Pearson's correlation coefficient (r). Statistical significance was set at $p<0.05$, and data were presented as means ± standard deviations (SD).

RESULTS

Delayed-onset muscle soreness and muscle damage markers

Muscle soreness developed on subsequent days after one-leg calf-raise exercise. It increased significantly 48 h and peaked around 72 h after exercise compared with the pre-exercise values ($p<0.01$) (Figure 1). Muscle soreness after exercise

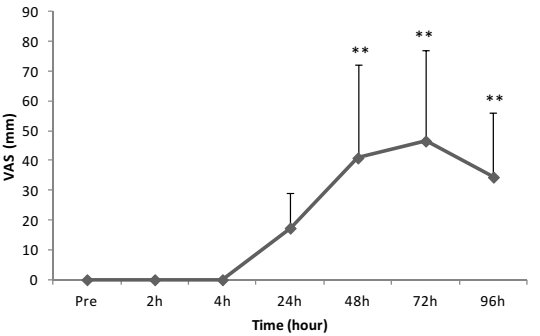

Figure 1. Time course of changes in delayed-onset muscle soreness (DOMS) following the calf-raise exercise as determined by a visual analogue scale (VAS).
Values: means±SD (n=9). Statistics: Two-way ANOVA. Post-hoc test: Bonferroni adjustment. **p<0.01: vs Pre.

decreased at 96 h from 72 h, but remained elevated compared with the pre-exercise values ($p<0.01$) (Figure 1). Concerning blood markers of muscle damage, Mb concentration significantly increased at 72 h after exercise ($p<0.05$) as compared with the pre-exercise values (Figure 2).

Differential leucocyte count

Peripheral leucocyte counts significantly increased at 4 h after exercise, due to the increase in neutrophils ($p<0.01$), and they returned to the pre-exercise values at

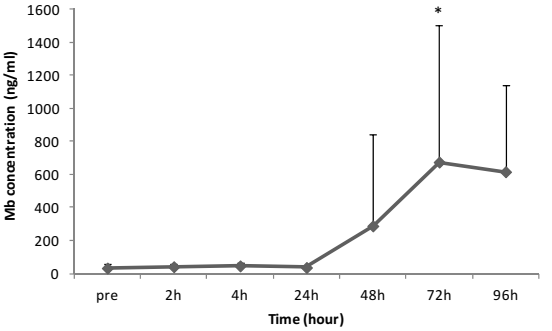

Figure 2. Changes in myoglobin (Mb) concentration following the calf-raise exercise. Values: means±SD (n=9). Statistics: Two-way ANOVA. Post-hoc test: Bonferroni adjustment. *p<0.05: vs Pre.

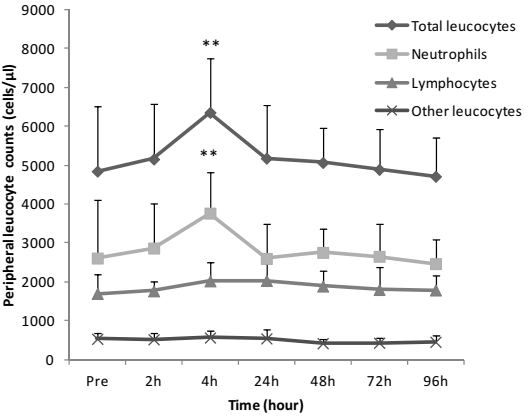

Figure 3. Changes in peripheral leucocyte counts following the calf-raise exercise. Values: means±SD (n=9). Statistics: Two-way ANOVA. Post-hoc test: Bonferroni adjustment. **p<0.01: vs Pre.

24 h after the exercise. Lymphocytes and other leucocytes showed no changes (Figure 3).

Neutrophil functions

Neutrophil migratory activity increased at 4 h after exercise ($p<0.05$) and ROS producing activity showed a trend to significantly increase ($p=0.07$). They both returned to the pre-exercise value at 24 h after exercise (Figure 4).

Inflammatory substances

Plasma and urinary concentrations of inflammatory substances such as pro-inflammatory cytokines, immunomodulatory cytokines, chemokines, anti-inflam-

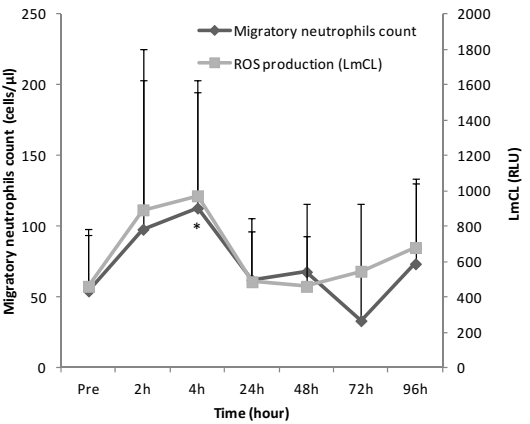

Figure 4. Changes in neutrophil migratory activity and producing activity of reactive oxygen species (ROS) as determined by Luminol-dependent chemiluminescence (LmCL) following the calf-raise exercise. Values: means±SD (n=9). Statistics: Two-way ANOVA. Post-hoc test: Bonferroni adjustment. LmCL p=0.07: vs Pre, migratory neutrophil counts *p<0.05: vs Pre.

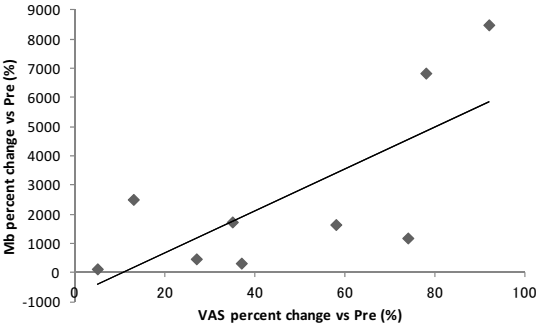

Figure 5. Associations between muscle soreness as determined by visual analogue scale (VAS) at 72 h and value of myoglobin (Mb) at 72 h. Values: percent changes of peak values vs Pre (n=9). Statistics: Pearson's correlation coefficient. VAS (Pre-72 h) vs Mb (Pre-72 h): r=0.73, p<0.05

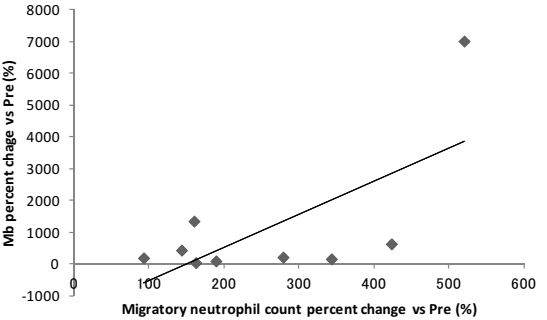

Figure 6. Associations between migratory neutrophil count at 4 h and myoglobin concentration at 48 h. Values: percent changes vs Pre (n=9). Statistics: Pearson's correlation coefficient. Migratory nuetrophil count (Pre-4 h) vs Mb (Pre-48 h) : r=0.67, p<0.05

matory cytokines, colony-stimulating factors, leucocyte activation markers, neutrophil chemotactic factor C5a and algesic substance PGE_2 showed no significant changes (Table 1).

Relationships between muscle soreness and muscle damage markers

We investigated the correlations between the peak VAS value of muscle soreness, and peak value of muscle damage marker, neutrophil dynamics and inflammatory mediators (all percent changes). There was a positive correlation between the percent changes of VAS at 72 h and Mb concentration at 72 h (r=0.73, p<0.05) (Figure 5). There was also a positive correlation between the percent changes of migrated neutrophil count at 4 h and Mb concentration at 48 h (r=0.67, p<0.05) (Figure 6).

Table 1. Changes in plasma and urinary inflammatory substances following the calf-raise exercise

	Unit	Pre	2h	4h	24h	48h	72h	96h
pro-inflammatory cytokines								
IL-1β-P	pg/ml	0.68 ± 0.11	0.57 ± 0.50	0.57 ± 0.39	0.72 ± 0.41	0.61 ± 0.35	0.61 ± 0.38	0.57 ± 0.38
IL-1β-U	pg/min	0.10 ± 0.09	0.58 ± 0.79	0.15 ± 0.12	0.30 ± 0.35	0.13 ± 0.07	0.13 ± 0.12	0.12 ± 0.04
TNF-α-P	pg/ml	0.46 ± 0.23	0.53 ± 0.26	0.64 ± 0.42	0.75 ± 0.35	0.44 ± 0.36	0.53 ± 0.12	0.51 ± 0.20
TNF-α-U	pg/min	0.77 ± 0.84	0.84 ± 1.05	1.33 ± 1.86	0.61 ± 0.69	1.04 ± 1.76	0.47 ± 0.67	0.27 ± 0.28
immunomodulatory cytokines								
IL-2-P	pg/ml	0.93 ± 0.17	0.83 ± 0.08	0.82 ± 0.07	0.78 ± 0.05	0.90 ± 0.30	0.84 ± 0.20	0.88 ± 0.20
IL-2-U	pg/min	0.79 ± 0.84	0.74 ± 0.43	0.40 ± 0.19	0.51 ± 0.18	0.67 ± 0.48	0.54 ± 0.57	0.30 ± 0.08
IL-12p70-P	pg/ml	0.59 ± 0.32	0.28 ± 0.27	0.59 ± 0.35	0.72 ± 0.45	0.80 ± 0.41	0.50 ± 0.23	0.55 ± 0.39
IL-12p70-U	pg/min	0.33 ± 0.24	0.34 ± 0.21	0.22 ± 0.09	0.31 ± 0.14	0.33 ± 0.23	0.56 ± 0.76	0.15 ± 0.07
IFN-γ-P	pg/ml	0.59 ± 0.16	0.60 ± 0.30	0.65 ± 0.32	0.63 ± 0.16	0.52 ± 0.16	0.54 ± 0.12	0.58 ± 0.17
IFN-γ-U	pg/min	0.79 ± 1.01	0.81 ± 0.65	0.30 ± 0.09	0.45 ± 0.16	0.42 ± 0.21	0.14 ± 0.08	0.23 ± 0.07
multifunctional cytokines								
IL-6-P	pg/ml	0.27 ± 0.16	0.38 ± 0.19	0.29 ± 0.18	0.22 ± 0.12	0.26 ± 0.12	0.30 ± 0.11	0.39 ± 0.23
IL-6-U	pg/min	0.14 ± 0.13	0.32 ± 0.15	0.15 ± 0.11	0.18 ± 0.10	0.16 ± 0.08	0.16 ± 0.14	0.29 ± 0.35
anti-inflammatory cytokines								
IL-1ra-P	pg/ml	35.6 ± 5.2	33.1 ± 5.5	34.4 ± 4.8	33.1 ± 8.4	29.3 ± 5.2	29.6 ± 2.6	32.3 ± 5.2
IL-1ra-U	pg/min	231.8 ± 173.2	401.2 ± 233.7	327.5 ± 230.7	837.6 ± 97.1	354.9 ± 192.6	204.0 ± 20.2	278.2 ± 155.0
IL-4-P	pg/ml	0.66 ± 0.35	0.70 ± 0.49	0.88 ± 0.56	1.00 ± 0.98	0.78 ± 0.66	0.76 ± 0.50	0.59 ± 0.31
IL-4-U	pg/min	0.60 ± 0.46	1.12 ± 0.72	0.95 ± 0.60	0.84 ± 0.31	0.65 ± 0.32	0.70 ± 0.69	0.77 ± 0.40
IL-10-P	pg/ml	0.71 ± 0.45	0.75 ± 0.52	0.50 ± 0.29	0.45 ± 0.21	0.57 ± 0.23	0.47 ± 0.31	0.66 ± 0.68
IL-10-U	pg/min	0.40 ± 0.44	0.56 ± 0.55	0.48 ± 0.50	0.56 ± 0.40	0.84 ± 1.11	0.46 ± 0.58	0.44 ± 0.42
IL-12p40-P	pg/ml	10.7 ± 17.8	13.2 ± 24.4	13.2 ± 24.7	10.7 ± 18.1	8.8 ± 13.7	7.7 ± 11.6	8.5 ± 13.2
IL-12p40-U	pg/min	0.81 ± 0.91	0.82 ± 0.64	0.56 ± 0.51	0.76 ± 0.83	1.06 ± 1.19	0.45 ± 0.48	0.33 ± 0.21
chemokines								
IL-8-P	pg/ml	4.5 ± 2.7	4.2 ± 2.4	4.4 ± 1.5	3.7 ± 0.9	3.7 ± 0.9	5.3 ± 4.3	4.3 ± 1.6
IL-8-U	pg/min	0.59 ± 0.42	1.20 ± 0.54	0.76 ± 0.43	1.3 ± 1.5	0.87 ± 0.69	0.65 ± 0.45	0.95 ± 0.80
MCP-1-P	pg/ml	95.4 ± 19.0	90.5 ± 13.8	94.3 ± 19.4	101.5 ± 19.1	89.8 ± 13.1	102.3 ± 13.2	96.8 ± 10.6
MCP-1-U	pg/min	87.5 ± 82.6	258.5 ± 197.9	199.4 ± 122.4	235.3 ± 146.3	189.8 ± 146.2	76.1 ± 29.2	128.3 ± 47.3
colony-stimulating factors								
G-CSF-P	pg/ml	1.8 ± 0.9	2.2 ± 1.4	2.0 ± 1.4	1.9 ± 1.0	2.3 ± 1.7	1.9 ± 1.3	2.0 ± 1.4
G-CSF-U	pg/min	0.19 ± 0.12	0.24 ± 0.13	0.27 ± 0.17	0.27 ± 0.17	0.48 ± 0.54	0.24 ± 0.24	0.16 ± 0.05
M-CSF-P	pg/ml	19.3 ± 2.5	23.1 ± 7.8	22.6 ± 5.4	21.8 ± 7.6	22.5 ± 6.3	17.0 ± 6.2	20.1 ± 3.0
M-CSF-U	pg/min	25.7 ± 23.1	130.8 ± 108.9	121.8 ± 84.9	281.4 ± 310.7	230.6 ± 298.1	40.4 ± 29.1	218.6 ± 280.9
GM-CSF-P	pg/ml	0.27 ± 0.10	0.21 ± 0.37	0.20 ± 0.06	0.28 ± 0.13	0.24 ± 0.09	0.17 ± 0.02	0.22 ± 0.09
GM-CSF-U	pg/min	0.41 ± 0.40	0.66 ± 0.61	0.54 ± 0.38	0.42 ± 0.29	0.69 ± 0.48	0.48 ± 0.50	0.41 ± 0.29
leucocyte activation markers								
calprotectin-P	ng/ml	15.7 ± 12.9	9.9 ± 6.8	10.5 ± 9.7	9.2 ± 7.0	11.7 ± 8.0	12.1 ± 6.3	12.3 ± 8.9
calprotectin-U	ng/min	0.15 ± 0.13	0.33 ± 0.19	0.27 ± 0.23	0.57 ± 0.68	0.45 ± 0.42	0.24 ± 0.23	0.20 ± 0.14
MPO-P	ng/ml	12.7 ± 7.8	12.6 ± 6.9	15.2 ± 9.8	13.2 ± 10.0	13.7 ± 8.1	12.2 ± 7.7	12.2 ± 5.9
MPO-U	ng/min	0.06 ± 0.05	0.10 ± 0.08	0.08 ± 0.06	0.10 ± 0.09	0.12 ± 0.13	0.08 ± 0.08	0.08 ± 0.07
neutrophil chemotactic factors								
C5a-P	ng/ml	3.8 ± 4.3	3.6 ± 3.6	3.2 ± 3.2	3.3 ± 3.5	3.4 ± 3.8	3.5 ± 4.0	3.5 ± 3.8
C5a-U	ng/min	0.03 ± 0.01	0.06 ± 0.02	0.09 ± 0.11	0.09 ± 0.11	0.08 ± 0.10	0.04 ± 0.02	0.06 ± 0.03
algesic substances								
PGE_2-P	pg/ml	64.1 ± 11.7	69.7 ± 12.6	79.0 ± 15.8	60.4 ± 18.0	58.9 ± 24.4	59.9 ± 7.7	58.9 ± 16.9
PGE_2-U	pg/min	1075 ± 886.4	1271 ± 732.9	1372 ± 1044	1354 ± 906.9	991.0 ± 643.2	758.3 ± 630.8	777.1 ± 535.1

Values: means±SD. Statistics: Two-way ANOVA was not significant.
P: Plasma
U: Urine. Data are the gross amount in the volume of urinary excretion per one minute.
Abbreviations: IL: interleukin, TNF: tumour necrosis factor, IFN: interferon, IL-1ra: IL- 1 receptor antagonist, MPO: myeloperoxidase, MCP-1: monocyte chemotactic protein-1, G-CSF: granulocyte colony-stimulating factor, GM-CSF: granulocyte macrophage colony-stimulating factor, M-CSF: macrophage colony-stimulating factor, PGE_2: prostaglandin E_2.

DISCUSSION

The aims of this study were at first to investigate the associations among DOMS caused by calf-raise exercise, muscle damage marker in peripheral blood, and inflammatory mediators. Muscle soreness appeared at 24 h after exercise and subsequently developed from 48 h to 72 h ($p<0.01$), and decreased at 96 h though still high above compared with pre-exercise ($p<0.01$). Generally, DOMS appears from several hours or one day after exercise and peaks at two or three days after exercise (1, 14, 16). This study confirmed that calf-raise exercise with eccentric contractions caused DOMS. As a marker of muscle damage, serum Mb concentration significantly increased at 72 h after exercise compared with pre-exercise. Numerous studies have reported on muscle damage and muscle soreness, however, they did not always occur simultaneously according to the degree of muscle damage and muscle soreness (4, 5, 13, 14). In this study, we observed the similar time course and Mb was closely correlated with muscle soreness at 72 h after exercise ($r=0.73$, $p<0.05$). These results confirm that the present calf-raise exercise caused sufficient muscle damage for the purpose of the present study.

Several studies report that circulating leucocyte and neutrophil count increase within several hours after eccentric exercise, but that is dependent on the intensity, duration, and type of exercise and muscle mass (16, 20). Saxton *et al.* reported that circulating neutrophil count increased 1.48 fold (Pre:2.9±0.4, 4h:4.3±0.5) at 4 h after low systemic stress (repeated eccentric muscle action) and caused 1.76 fold (Pre:2.9±0.3, 4h:5.1±0.4) increase in neutrophils at 4 h after high systemic stress (bench-stepping) (20). Gleeson *et al.* reported the circulating leucocyte number decreased two and three days after a bench-stepping exercise, although it increased immediately after and still more increased at 1 to 4 h (5). We demonstrated that total leucocyte count increased by 31 % ($p<0.01$) and circulating neutrophil count by 44 % ($p<0.05$) above pre-exercise level, respectively. Neutrophilia returned to pre-exercise values at 24 h after exercise. Neutrophil migratory activity was also significantly increased ($p<0.05$) at 4 h after exercise. There have been several studies that investigated the neutrophil migration into the muscle after the muscle-damaging exercise (0-6 h) (2, 3, 8, 9, 11, 33), but only Fielding *et al.* demonstrated neutrophil accumulation in the muscle (3). This could be partly attributable to the fact that biopsy samples can depict quite limited parts of the muscle tissue. Paulsen *et al.* investigated leucocyte accumulation into the exercised-muscle using radiolabeled leucocytes by scintigraphy (15). Although they measured high radioactivity in the exercised-muscle, large individual differences were observed. We demonstrated a positive correlation between the increase in neutrophil migratory activity at 4 h and the increase in Mb concentration at 48 h ($r=0.67$, $p<0.05$). This result shows the possibility that neutrophil is involved in the muscle damage and the inflammatory processes.

ROS production from neutrophils migrated into the hydrogel likened to muscle tissue was assessed with LmCL. There was a trend for this to be increased ($p=0.07$) at 4 h after exercise. Pizza *et al.* reported that neutrophil O_2^- production was increased ($p<0.05$) at 4 h following one-arm eccentric exercise (18). Suzuki

et al. also observed increased neutrophil count with a left shift, enhanced spontaneous mobility and ROS production after 90-min bicycling, suggesting that mobilized neutrophils from the bone marrow reserve have higher activity (23). They also reported that the increases in Mb values at post 3 h were correlated closely with the raise in neutrophil count at post and LmCL response at post and 1 h post 90-min bicycling, suggesting that neutrophil mobilization and activation might affect muscle damage (24). The result in this study was similar, but we could demonstrate the enhanced neutrophil migration by use of newly-developed *ex vivo* methodology in imitation of tissue damage.

Although DOMS, muscle damage marker, circulating neutrophil counts and their functions changed significantly following the eccentric exercise, plasma and urinary inflammatory mediators which we investigated were not changed significantly. Uchida *et al.* reported increasing serum PGE_2 following bench press exercise despite no changes in plasma IL-1β, IL-6 or TNF-α (30). Although we also investigated plasma and urinary PGE_2 concentrations, they did not change. Serum PGE_2 may be produced by leucocytes during the time left to removal of the fibrin clot and blood cells where neutrophilia occurs after exercise that might affect the higher values. These results demonstrated that the one-leg calf-raise exercise caused local inflammation and that neutrophil mobilization and migration were the most affected variables among the wide range of tested inflammatory mediators. However, further research is needed to clarify the mediators that mobilize neutrophils into the circulation and substances which are produced by migrated neutrophils and promote inflammation.

In conclusion, one-leg calf-raise exercise caused DOMS, muscle damage, increases in circulating neutrophil number and migratory activity without changes in the other inflammatory mediators. Neutrophils may be involved in the early stage of muscle damage and the inflammatory processes after eccentric exercise.

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Influence of vitamin D status on respiratory infection incidence and immune function during 4 months of winter training in endurance sport athletes

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Abstract

The purpose of this study was to examine the influence of vitamin D status on mucosal and systemic immunity and the incidence, severity and duration of upper respiratory tract illness (URTI) episodes in endurance athletes during a 16-week winter training period. Blood was collected from 225 subjects at the start of the study and plasma was analysed for total 25-hydroxy vitamin D (25(OH)D) and cathelicidin concentration. Blood was also collected at the end of the study and analysed for 25(OH)D and antigen-stimulated cytokine production. Unstimulated saliva samples were obtained at the start and at 4-week intervals during the study period. Saliva samples were analysed for salivary antimicrobial peptides and proteins (AMPs). Weekly training and daily illness logs were kept. At the start and end of the study 38% and 55%, respectively, of the athlete cohort had inadequate (plasma 25(OH)D 30-50 nmol/L) or deficient (plasma 25(OH)D < 30 nmol/L) vitamin D status. There was a significantly higher proportion of subjects who presented with symptoms of URTI in the vitamin D deficient status group (initial plasma 25(OH)D < 30 nmol/L) during the study period than in the optimal vitamin D group (> 120 nmol/L) and the total number of URTI symptom days and the median symptom-severity score in the vitamin D deficient group was signifi-

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cantly higher than in the other groups. The plasma cathelicidin concentration positively correlated with the plasma 25(OH)D concentration and the saliva secretory immunoglobulin A (SIgA) secretion rate in the optimal vitamin D status group was significantly higher than in the other groups. Low vitamin D status was associated with lower pro-inflammatory cytokine production by monocytes and lymphocytes. Low vitamin D status could be an important determinant of URTI risk in endurance athletes and mucosal as well as systemic immunity may be modified via vitamin D-dependent mechanisms.

Key words: exercise training, cholecalciferol, ergocalciferol, saliva antimicrobial proteins, common cold

Introduction

It has only recently been recognised that vitamin D plays an important role in up-regulating immunity [16]. Vitamin D is a key link between Toll-like receptor (TLR) activation and antimicrobial responses in innate immunity. Vitamin D has a vital role in up-regulating the expression of antimicrobial peptides and proteins (AMPs), such as cathelicidin and β-defensin [23]. These AMPs have a broad range of activities against microorganisms and may be involved in the direct inactivation of viruses [16]. They are produced by epithelial cells and macrophages and in the lungs are secreted into the biofilm covering the inner surface of the airways, thereby creating a barrier that is chemically lethal to microbes. The biologically active form of vitamin D (1,25 dihydroxy cholecalciferol) activates the genes for cathelicidin synthesis and enhances the effectiveness of monocytes and macrophages in killing microbes by enhancing the oxidative burst potential of these phagocytic cells [28]. Furthermore, vitamin D has been shown to be essential in activating and controlling the T-cell antigen receptor and thus enhancing the recognition of antigens by T lymphocytes [30]. Vitamin D may also influence cytokine production during periods of infection [3].

Several recent studies have found a negative association between vitamin D status and respiratory illness incidence in young and elderly adults [1,9,20]. The incidence of respiratory illnesses is generally higher in athletes [10] and low vitamin D status could be a contributing factor as vitamin D insufficiency has been reported to be common in athletes [21] especially if exposure to natural sunlight is limited (e.g. when training in the winter months or when training mostly indoors).

During a 4-month winter training period we conducted a study on a large cohort of endurance athletes who completed daily URTI symptom diaries and reported weekly training loads using validated questionnaires. We also collected blood samples from these athletes at the start and end of the study and saliva at the start and at 4-weekly intervals. Our aims were to determine the influence of vitamin D status on mucosal immunity, antigen-stimulated cytokine production and the incidence, severity and duration of upper respiratory tract illness (URTI) episodes in endurance athletes during a winter training period.

Methods

Subjects

Two hundred and sixty seven subjects who were engaged in regular sports training (predominantly endurance-based activities such as running, cycling, swimming, triathlon, team games and racquet sports) volunteered to participate in the study. Subjects ranged from recreationally active to Olympic triathletes and their self-reported training loads averaged 10 h/week. Subjects were required to complete a comprehensive health-screening questionnaire prior to starting the study and had not taken any regular medication or antibiotics in the 3 months prior to the study. All subjects were fully informed about the rationale for the study and of all experimental procedures to be undertaken. Subjects provided written consent to participate in the study, which had earlier received the approval of Loughborough University ethical advisory committee. Subjects were enrolled after having fulfilled all inclusion criteria, and presenting none of the exclusion criteria (determined by both questionnaire and interview). Subjects could be included if they were currently healthy, had been involved in endurance training for at least 2 years, engaged in at least 3 sessions and at least 3 h of total moderate/high-intensity training time per week and were between 18-40 years of age. Subjects representing one or more of the following criteria were excluded from participation: smoking or use of any medication, suffering from or had a history of cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal, haematological or psychiatric illness. A total of 267 healthy individuals (83 females and 184 males) were recruited as subjects from Loughborough University, UK (latitude 53°N) during November 2011 with the mean age of the study cohort at recruitment being 21 ± 3 years (mean ± SD). For the first visit to the laboratory, subjects arrived in the morning at 08:30-10:30 following an overnight fast of approximately 12 h and their body mass and height were recorded. Information about the study was given to them and they then signed an informed consent form. Subjects then sat quietly for 10 min and completed a health-screening questionnaire and inclusion/exclusion criteria questionnaire before providing an unstimulated saliva sample by passive dribble into a pre-weighed sterile collection tube for a timed period (usually 2 min; longer was allowed if the volume of saliva collected after 2 min was insufficient). After centrifugation for 2 min at 5000 *g* to remove cells and insoluble matter, saliva samples were stored frozen at -80ºC prior to analysis. Subsequently, a resting venous blood sample (12 ml) was obtained by venepuncture from an antecubital forearm vein into two vacutainer tubes (Becton Dickinson, Oxford, UK) containing K_3EDTA and lithium heparin. Haematological analysis was immediately carried out on the the EDTA sample (including haemoglobin, haematocrit and total and differential leukocyte counts) using an automated cell-counter (A^c.T™5diff haematology analyser, Beckman Coulter, High Wycombe, UK). Subjects had to have normal haematology to be included in the study. The remaining EDTA blood was centifuged for 10 min at 1500 *g* and 4°C and the plasma stored at -80°C prior to analysis. Heparinised blood was used immediately for the measurement of antigen-stimulated cytokine production.

Study protocol

During the 4-month study period subjects were requested to continue with their

normal training programs. Subjects completed a validated health (URTI symptoms) questionnaire [14] on a daily basis. Subjects were not required to abstain from medication when they were suffering from illness symptoms but they were required, on a weekly basis, to report any unprescribed medications taken, visits to the doctor or any prescribed medications.

The illness symptoms listed on the questionnaire were: sneezing, headache, malaise, nasal discharge, nasal obstruction, sore throat, cough, ear ache, hoarseness, fever, chilliness and joint aches and pains. The non-numerical severity ratings of mild, moderate and severe of severity of symptoms were scored as 1, 2 or 3, respectively to provide a quantitative means of data analysis and the total symptom score for every subject each day was calculated as a sum of multiplied numbers of symptoms experienced by the numerical severity ratings. A URTI was deemed present when (i) total symptom score was ≥15 on any two consecutive days and (ii) when a subject positively indicated suffering a common cold on ≥ 3 days according to Jackson et al. [14]. Subjects were also asked to rate the impact of illness symptoms on their ability to train (above normal, at the same level, below normal or training stopped). The total number of URTI symptom days was also determined as the number of days with a symptom score of ≥5 according to Predy et al. [26].

Subjects were also asked to fill in a standard short form of International Physical Activity Questionnaire (IPAQ; http://www.ipaq.ki.se/downloads.htm) at weekly intervals, thus providing a quantitative information on training loads in metabolic equivalents (MET)-h/week [6]. Subjects attended the laboratory every 4 weeks following an overnight fast. Subjects were required to abstain from any strenuous physical activity for 24 h before coming to the laboratory. During these visits body mass was recorded and an unstimulated saliva sample was collected. Venous blood samples were collected only at the start and end of the study period. A total of 225 subjects completed the study and provided sufficient blood for routine haematology and vitamin D status analysis at the start of the study and sufficient saliva for analysis of AMPs on all 5 occasions. Plasma samples from 181 subjects were also analysed for Vitamin D status at the end of the study. After vitamin D status analysis of baseline samples, we also analyzed the initial plasma cathelicidin concentration from a subset of 80 subjects with high-level (all subjects with initial plasma 25(OH)D >90 nmol/L, n=26), a random selection of mid-level (33-89 nmol/L, n=27) and low-level (all subjects with <33 nmol/L, n=27) 25(OH)D concentration. After vitamin D status analysis of samples collected at the end of the study, we also measured antigen-stimulated cytokine production in a subset of 48 subjects with high-level (initial plasma 25(OH)D >90 nmol/L, n=24) and low-level (<30 nmol/L, n=24) 25(OH)D concentration.

Saliva analysis

The saliva volume collected was estimated by weighing and the saliva flow rate was calculated. Saliva samples were analysed for secretory immunoglobulin A (SIgA) using an ELISA kit (Salimetrics, Philadelphia, USA) and α-amylase activity was measured as previously described [22]. Salivary lactoferrin and lysozyme were analysed using commercially available ELISA kits (Calbiochem, USA and

Biomedical Technologies, USA, respectively). Secretion rates for each of the salivary AMPs were calculated as the multiple of the saliva flow rate and the AMP concentration. Values obtained from the 5 visits at 4-week intervals were averaged for each subject. All saliva assays were carried out in duplicate. Coefficients of variation (CVs) for the assays were <5% for all salivary AMPs.

Plasma analysis

Vitamin D occurs in two forms: cholecalciferol (D3) which is formed by the action of UV light on the skin and ergocalciferol (D2) from plant food sources. The best measure of vitamin D status is considered to be the sum of the 25-hydroxy metabolites of D2 and D3 ($25(OH)D_3$ and $25(OH)D_2$) and the best way of measuring these is considered to be the high pressure liquid chromatography - tandem mass spectrometer method [31]. Our EDTA plasma samples were analysed for $25(OH)D_3$ and $25(OH)D_2$ with a high pressure liquid chromatography tandem mass spectrometer (Waters Acuity, Manchester, UK) after a maximum of 10 months in storage with no previous freeze-thaw cycles as described previously [29]. Briefly, $25(OH)D_2$, $25(OH)D_3$ and deuterated internal standard were extracted from plasma samples, following protein precipitation, using Isolute C18 solid phase extraction cartridges. Potential interfering compounds were removed by initial elution with 50% methanol followed by elution of the vitamins using 10% tetrahydrofuran in acetonitrile. Dried extracts were reconstituted prior to injection into a high performance liquid chromatography tandem mass spectrometer in the multiple reaction mode (MRM). The MRM transitions (*m/z*) used were 413.2 > 395.3, 401.1 > 383.3 and 407.5 > 107.2 for $25(OH)D_2$, $25(OH)D_3$ and hexa-deuterated$(OH)D_3$ (internal standard), respectively. Intra-assay CVs were <10% across a working range of 2.5–624 nmol/L for both $25(OH)D_3$ and $25(OH)D_2$. Measurements were performed in a laboratory meeting the performance target set by the Vitamin D External Quality Assessment Scheme (DEQAS) Advisory Panel for 25(OH)D assays.

EDTA plasma was assayed for cathelicidin concentration using a commercially available ELISA kit (Hycult Biotech, Uden, The Netherlands) according to the manufacturers' instructions. The intra-assay CV was 3%.

Antigen-stimulated cytokine production

Stimulated whole blood culture production of cytokines (interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-4, IL-6 and IL-10) was determined as described previously [11]. The stimulant was a commercially available multi-antigen vaccine (Pediacel Vaccine, Sanofi Pasteur, UK) containing diphtheria, tetanus, acellular pertussis, poliomyelitis and haemophilus influenzae type b antigens. Briefly, 0.25 mL of heparinized whole blood was added to 0.75 mL of RPMI 1640 medium (Sigma Chemicals, Poole, UK) with an added 40 μL of Pediacel vaccine cocktail (Sanofi Pasteur msd Limited, Maidenhead, UK) at a dilution of 1:100, before being incubated at 37°C and 5% CO_2 for 24 h. The stimulant dilution of 1:100 used in this study was based on a separate experiment (unpublished data), which established the dose–response curve for the measured cytokines over the dilution range of 1:100–1:20 000. Samples were then centrifuged at 15000 rpm for 4 min at 4°C, following which the supernatant

fluid was harvested and stored at -80°C prior to analysis of cytokine concentrations using an Evidence Investigator System using the high sensitivity cytokine biochip array EV3513 (Randox, County Antrim, UK). The intra-assay CV for all measured cytokines was less than 5.0%. The measured cytokine concentrations for the monocyte-derived cytokines (TNF-α, IL-1β and IL-6) and lymphocyte-derived cytokines (IL-2, IL-4 and IFN-γ) were divided by the monocyte and lymphocyte counts, respectively to give cytokine production per 10^6 cells.

Statistical analysis

The Shapiro-Wilk test was used to determine if data sets were normally distributed. The Kruskal-Wallis test (nonparametric equivalent of one-way ANOVA) with post-hoc Mann-Whitney test was used to examine differences in the salivary variables, blood leukocyte counts and the total number of URTI episodes and symptom days among groups classified by vitamin D status using the following ranges for plasma total 25(OH)D: 12-30 nmol/L (deficient); 30-50 nmol/L (inadequate), 50-120 nmol/L (adequate) and >120 nmol/L (optimal) [4]. For subjects with URTI symptoms, the symptom-severity score and the duration of URTI episodes among the 4 groups were also assessed using the Kruskal-Wallis test with post-hoc Mann-Whitney test. The plasma total 25(OH)D concentrations were compared between male and female subjects, and indoor and outdoor training locations using the Mann-Whitney test. The total plasma 25(OH)D concentrations at the start and the end of the study were compared by Wilcoxon signed-rank test. The difference in proportion of subjects who presented with symptoms of URTI during the trial between the vitamin D optimal and deficient groups was assessed by the chi-squared test. Correlation between the number of URTI episodes and the plasma 25(OH)D concentration as well as the plasma cathelicidin and 25(OH)D concentration was done using Spearman's rank correlation coefficient. One-way ANOVA with post-hoc Bonferroni test was used to examine differences in the plasma cathelicidin among high, middle and low level vitamin D status groups. Differences in antigen-stimulated cytokine production between high and low vitamin D status groups were compared with the Mann-Whitney test. We also evaluated the impact of URTI episodes on training volume by comparing physical activity levels (MET-h/week) on weeks when an URTI episode was present with the average MET-h/week when the subjects were healthy. Data are presented as mean (±SD) for data sets that were normally distributed; for data sets that were not normally distributed, the median and interquartile range (IQR) are shown. The accepted level of significance was $P<0.05$.

Results

Adherence to the study

Of the 267 subjects, 239 subjects (169 males, 70 females) completed the full 16 weeks of the study. Reasons for dropout included overseas travel, injury or persistent non-respiratory illness (preventing them from performing training) or due to undisclosed reasons. Saliva samples were obtained on all 5 visits from 236 subjects. Plasma samples were analysed for total 25(OH)D from 225 subjects

because there were 11 subjects having insufficient plasma volume for this analysis. At the end of the study blood samples with sufficient volume for analysis of plasma total 25(OH)D were obtained from 181 subjects.

Baseline characteristics and physical activity levels

Baseline characteristics of the 225 subjects who completed the study and for whom vitamin D status was established were (mean ± SD) age: 21 ± 3 years, body mass: 73.4 ± 11.4 kg, height: 176.2 ± 9.1 cm, body mass index: 23.5 ± 2.3 kg/m^2 and self-reported weekly training duration: 9.6 ± 5.2 h/week. Analysis of the IPAQ questionnaires indicated that the training loads were fairly consistent over the 16 weeks of the study. Mean training loads were 67.5 ± 31.2 MET-h/week which is equivalent to about 11 ± 5 hours of moderate-vigorous activity per week.

Vitamin D status

Plasma 25(OH)D_2 was below the detection limit (2.5 nmol/L) in 57% of subjects and the average plasma 25(OH)D_2 concentration was only 4.4 nmol/L (median, 0; IQR, 0-9 nmol/L) . The median (IQR) plasma 25(OH)D_3 concentration at the start of the study was 53 (40-66) nmol/L and the median total 25(OH)D concentration was 57 (44-71) nmol/L. The number (and percentage) of subjects classed as optimal, adequate, inadequate and deficient was 11 (5%), 128 (57%), 68 (30%) and 18 (8%). Plasma 25(OH)D concentration was not significantly different (p=0.478) between males (N=157; median, 56; IQR, 43-69 nmol/l) and females (N=68; median, 58; IQR, 45-72 nmol/L). Plasma 25(OH)D concentration was not significantly different (P = 0.120) between indoor (N=50; median, 64; IQR, 46-73 nmol/L) and outdoor sports (N=175; median, 55; IQR, 43-69 nmol/L). The total plasma 25(OH)D concentration at the end of the study (N=181; median, 47; IQR, 35-68 nmol/L) was significantly lower (P = 0.003) than that at the start of the study. At the start of the 4-month study period 38% of athletes had insufficient or deficient plasma 25(OH)D values (< 50 nmol/L) and by the end of the study 55% of athletes had plasma 25(OH)D values of less than 50 nmol/L.

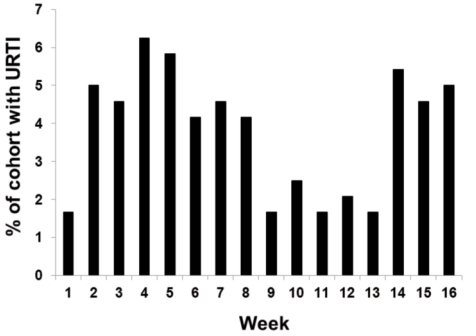


Figure 1. Percentage of the cohort reporting a URTI episode for each week of the study period.

URTI symptom incidence and its impact on training loads

Analysis of the URTI symptom questionnaires indicated that 4.0 ± 1.6% of the cohort experienced a URTI episode each week (Figure 1). One hundred and thirty six subjects did not experience a single URTI episode during the study period whereas 103 subjects experienced at least one URTI episode during the study period.

Table 1. Infection symptom incidence among different vitamin D status groups. Also shown are the severity score and duration of URTI episodes.

INCIDENCE	Optimal N = 11	Adequate N = 128	Inadequate N = 68	Deficient N = 18	P
Number of episodes	0 (0-1) *	0 (0-1)	0 (0-1)	0 (0-1)	0.062
Number of symptom days	1 (0-6)*	4 (0-8)*	4 (1-8)*	9 (3-17)	0.040
WHEN URTI PRESENT	Optimal N = 3	Adequate N = 56	Inadequate N = 27	Deficient N = 12	P
Symptom severity score	43 (38-52)*	47 (40-69)*	62 (46-74)*	102 (67-199)	0.013
Duration (days)	5 (5-7)*	8 (6-9)*	8 (5-14)*	13 (10-17)	0.059

Data are median and interquartile range (IQR). P value from Kruskal-Wallis test is shown in right hand column.

* Significantly different from Deficient (Mann-Whitney U test).

The proportion of subjects whose training was negatively affected when URTI was present was 70%. When mean MET-h/week physical activity during healthy (no URTI symptoms) weeks was compared with mean physical activity during weeks when an URTI episode occurred, there was a significant decrease from 68.3 ± 32.3 to 51.9 ± 40.4 MET-h/week (P = 0.001). Thus, when URTI was present subjects reduced their training load by an average of 24%.

Vitamin D status and URTI incidence, severity and duration

The proportion of subjects in the optimal vitamin D status group who experienced one or more URTI episodes during the trial was significantly lower than for the vitamin D deficient group (optimal 27%, deficient 67%; P = 0.039). There was a significant difference for URTI symptom days among the four vitamin D status groups and the total number of URTI symptom days in the deficient group was significantly higher than the other groups (Table 1). Vitamin D status tended to influence prevalence of URTI episodes but this fell just short of statistical significance (P = 0.061) and there was a tendency for the deficient group to have more episodes (Table 1). For subjects who experienced one or more URTI episodes, there was a significant difference in the median symptom-severity score per URTI episode among the four groups but no significant difference in the median duration of episodes (Table 1) although there was a tendency for episodes to be longer with low vitamin D status. The median symptom-severity score in the deficient group was significantly higher than the other groups.

Plasma cathelicidin concentration

The plasma cathelicidin concentrations were 32.2 ± 11.9, 27.7±10.6 and 24.5±7.5 ng/ml in the high, mid and low level vitamin D status groups, respectively. There was a significant influence of vitamin D status on the plasma cathelicidin concentration (P = 0.027). The plasma cathelicidin concentration in the high-level vitamin D status group was significantly higher than in the low-level group (P = 0.023). In addition, there was a positive correlation between the plasma 25(OH)D and cathelicidin concentrations (r = 0.234, P = 0.036).

Table 2. Salivary concentrations and secretion rates of antimicrobial peptides (AMPs) among different vitamin D status groups.

	Optimal N = 11	Adequate N = 128	Inadequate N = 68	Deficient N = 18	P
Lactoferrin concentration (ng/mL)	2006 (1413-3184)	2187 (1546-3184)	2491 (1606-3633)	2419 (1624-3001)	0.948
Lactoferrin SR (ng/min)	801 (587-1229)	756 (527-1151)	771 (367-1143)	872 (554-1645)	0.568
Lysozyme concentration (μg/mL)	1524 (641-2595)	1524 (926-2808)	1701 (1102-2693)	1361 (883-2437)	0.687
Lysozyme SR (μg/min)	435 (314-906)	517 (315-872)	504 (315-972)	609 (331-837)	0.970
SIgA concentration (μg/mL)	91 (82-130)	66 (49-100)	76 (46-111)	59 (46-78)*	0.103
SIgA SR (μg/min)	38.7 (30.3-48.6)	22.9 (14.2-36.6)*	19.5 (12.7-32.3)*	23.6 (14.8-32.9)*	0.018
Amylase activity (U/L)	131 (60-213)	142 (73-233)	131 (74-202)	121 (64-222)	0.839

Data are median and interquartile range (IQR). P value from Kruskal-Wallis test is shown in right hand column. SR = secretion rate.

* Significantly different from Optimal (Mann-Whitney U test).

Salivary variables

There was a significant difference in SIgA secretion rate among the four vitamin D groups (Figure 2) but no significant difference for the other salivary AMPs (Table 2). The SIgA secretion rate in the optimal vitamin D status group was significantly higher than in the other groups. The SIgA concentration tended to be lowest in the deficient group.

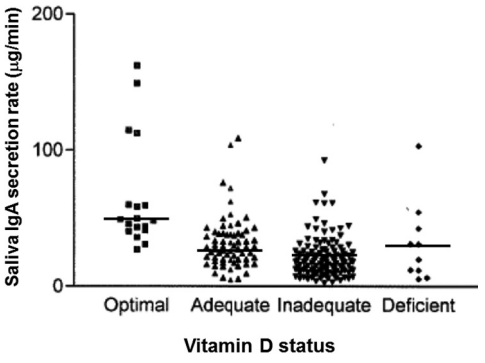


Figure 2. Influence of vitamin D status on the saliva IgA secretion rate. Median values for each group are indicated by the horizontal lines. Kruskal-Wallis test indicated a significant influence of vitamin D status on IgA secretion rate (P = 0.018). Saliva IgA section rate was significantly higher in the Optimal group than in the other groups (P < 0.05).

Blood leukocyte counts

Based on analysis of blood samples collected at the start of the study there was no influence of vitamin D status on circulating numbers of total leukocytes, neutrophils, lymphocytes or monocytes (Table 3).

Antigen-stimulated cytokine production

Both blood monocyte and lymphocyte counts were not significantly different in the subset of subjects for whom antigen-stimulated cytokine production was determined. However, production of all the monocyte-derived cytokines (TNF-α, IL-1β and IL-6) were significantly lower in the vitamin D deficient subjects compared with those with high vitamin D status (all P < 0.005; Figure 3). Production of the lymphocyte-derived pro-inflammatory cytokine IFN-γ was significantly lower in the vitamin D deficient subjects compared with those with high vitamin D status (P < 0.01; Figure 4) and

Table 3. Blood total and differential leukocyte counts among different vitamin D status groups.

	Optimal N = 11	Adequate N = 128	Inadequate N = 68	Deficient N = 18	P
Leukocytes (x10^9 cells/L)	5.9 (5.3-6.6)	5.9 (5.2-6.9)	5.9 (5.2-6.9)	6.5 (5.8-7.8)	0.495
Neutrophils (x10^9 cells/L)	2.8 (2.3-3.7)	2.9 (2.4-3.9)	3.0 (2.3-3.5)	3.4 (2.9-4.2)	0.352
Lymphocytes (x10^9 cells/L)	1.9 (1.7-2.3)	2.1 (1.7-2.3)	2.1 (1.8-2.4)	1.9 (1.8-2.0)	0.555
Monocytes (x10^9 cells/L)	0.5 (0.4-0.6)	0.6 (0.5-0.7)	0.6 (0.5-0.7)	0.6 (0.6-0.8)	0.136

Data are median and interquartile range (IQR). P value from Kruskal-Wallis test is shown in right hand column.

IL-4 also tended to be lower (P = 0.062). Production of IL-2 was not significantly influenced by vitamin D status. The anti-inflammatory cytokine IL-10 is produced by both monocytes and lymphocytes so it is not appropriate to normalise its production by cell counts. The antigen-stimulated production of IL-10 was not significantly different between the high and deficient vitamin D status groups (high: median 4.0; IQR 2.4-8.8 pg/ml *versus* deficient: median 2.8; IQR 2.0-6.0 pg/ml; P = 0.164).

Discussion

This research is of direct relevance to on-going study of the factors that determine illness susceptibility in athletes. The main findings of the present study were as follows: (1) there was a higher proportion of subjects who experienced one or more URTI episodes in the vitamin D deficient status group during the 4-month study period than in the optimal vitamin D group; (2) the total number of URTI symptom days and the median symptom-severity score in the vitamin D deficient group were significantly higher than in the other groups; (3) the plasma cathelicidin concentration was positively associated with vitamin D status; (4) the SIgA secretion rate in the optimal vitamin D status group was significantly higher than in the other groups; (5) pro-inflammatory cytokine production in response to multi- antigen stimulation was substantially lower in vitamin D deficient subjects.

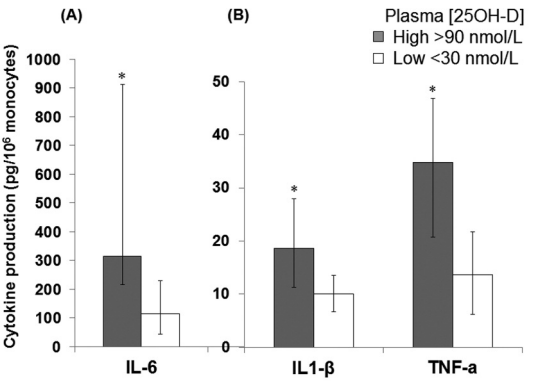
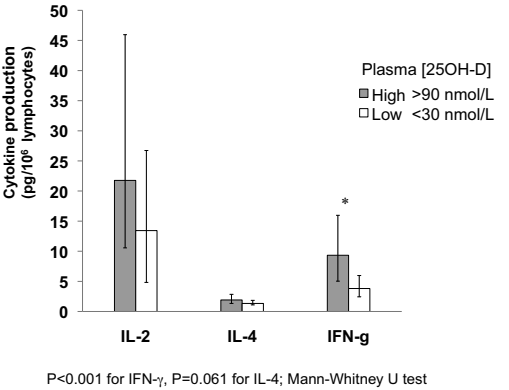


Figure 3. Influence of high or deficient vitamin D status on the antigen-stimulated production of (A) IL-6 and (B) IL-1β and TNF-α by monocytes. Production of all cytokines was significantly higher in the high vitamin D status group compared with the deficient group (all P < 0.005 as indicated by the asterisks).



P<0.001 for IFN-γ, P=0.061 for IL-4; Mann-Whitney U test

Figure 4. Influence of high or deficient vitamin D status on the antigen-stimulated production of cytokines by lymphocytes. * Significant difference between groups (P < 0.05).

According to the findings from the present study, it seems likely that vitamin D status has an influence on URTI symptom incidence. We found that a higher proportion of subjects (67%) in the vitamin D deficient status group experienced one or more URTI episodes during the 4-month study period than in the optimal vitamin D group (27%). Recent studies have also shown an inverse association between vitamin D status and respiratory infection incidence in young and elderly adults [1,9]. In the Third National Health and Nutrition Examination Survey involving 18,883 participants [9], it was found that individuals with plasma 25(OH)D concentration less than 25 nmol/L had a significantly higher risk of respiratory infections (24%) than those with 25(OH)D levels higher than 75 nmol/L (17%). In addition, a population-based study on 6,789 British adults demonstrated that plasma 25(OH)D concentrations are inversely associated with recent URTI [1]. There was a 7% reduction in the risk of respiratory infections for each 10 nmol/L increase in plasma 25(OH)D.

Furthermore, we also found that the total number of URTI symptom days in the vitamin D deficient group was significantly higher than in the other groups. There was a similar result in a Finnish study in which young male Finnish soldiers with plasma 25(OH)D concentration less than 40 nmol/L had more days of absence from duty due to respiratory infections during the 6-month study period than soldiers with plasma 25(OH)D concentration more than 40 nmol/L [20]. In addition, an observational study has shown that adults with 25(OH)D status less than 95 nmol/L had a longer duration of illness compared with those whose 25(OH)D status was higher than 95 nmol/L [27]. In the present study, it was also shown that there was a tendency for episodes to be longer with low vitamin D status. Moreover, we found that subjects with plasma 25(OH)D higher than 30 nmol/L had less severe symptoms during URTI episodes compared with those having plasma 25(OH)D below 30 nmol/L. Thus, vitamin D might play a role in reducing both the severity and duration of URTI symptoms.

There is a plausible mechanism for the inverse association between plasma 25(OH)D status and risk/severity of URTI episodes: vitamin D is a key link between Toll-like receptor (TLR) activation and antimicrobial responses in innate immunity. Following activation of the TLR signalling cascade in the presence of microbes, vitamin D has a vital role in up-regulating the production of AMPs, such as cathelicidin and β-defensin [23]. These AMPs are produced by both

epithelial cells and macrophages and have a broad range of activities against microorganisms including the direct inactivation of viruses [16]. They are secreted into the biofilm covering the epithelial surface, thereby creating a barrier that is chemically lethal to microbes. One of these AMPs, cathelicidin, enhances the microbicidal capability of monocytes and macrophages by increasing the oxidative burst potential of these phagocytic cells [28] and has a defined vitamin D-dependent mechanism. Pathogenic antigens interact with TLRs on the epithelial cells and macrophages to upregulate the expression of the 1α-hydroxylase enzyme that converts 25(OH)D to the biologically active 1,25-dihydroxyvitamin D. This in turn activates a suite of genes which enhance the production of cathelicidin [19,23]. Our finding that the plasma cathelicidin concentration in the high-level vitamin D status group was significantly higher than in the low-level group and the significant correlation between plasma 25(OH)D and cathelicidin concentrations are in agreement with other studies [2,7,15]. Furthermore, vitamin D has been shown to be essential in activating and controlling the T-cell antigen receptor and thus enhancing the recognition of antigens by T lymphocytes [30].

A novel and potentially important finding of the present study is that vitamin D deficiency was associated with a significant and substantially lower production of pro-inflammatory cytokines by both monocytes and lymphocytes in response to a multi-antigen challenge. The consequence of this could be an impaired immune response to an infectious pathogen, increasing the likelihood of an infection occurring. Our finding is in contrast to other studies that have indicated a reduced pro-inflammatory cytokine response when the biologically active form of vitamin D ($1,25(OH)_2D$) is added in concentrations of 10-100 nmol/L to stimulated cultures of peripheral blood mononuclear cells [17,18]. The reason for this discrepancy is unclear. The stimulant we used was a vaccine containing antigens from a virus and both gram-positive and gram-negative bacteria and we used a whole blood culture. The studies by Khoo and colleagues [17,18] used lipopolysaccharide or *Candida albicans* to stimulate isolated peripheral blood mononuclear cells (PBMC). Inhibition of pro-inflammatory cytokine production in these studies was only observed when PBMC were incubated with $1,25(OH)_2D$ in concentrations that were 50-500-fold above the normal healthy range for plasma $1,25(OH)_2D$ (50-250 pmol/L), so the physiological relevance is questionable. Moreover, the multiple antigen challenge used in our study provides valuable information on cytokine production since not all cytokines respond to the same antigen. The capacity of leukocytes to produce cytokines upon adequate challenge (e.g. with mitogen, antigen, endotoxin or pathogen exposure) has potentially far reaching consequences for the entire functional capacity of the immune system. It is highly likely to reflect the capacity of an individual to defend itself against intruding microorganisms and hence is a suitable measure to examine the impact of nutritional interventions (e.g., vitamin D supplementation) designed to boost immune function. We chose to examine whole blood culture rather than isolated PBMC as the former retains the normal cellular, hormonal and cytokine milieu that the leukocytes are normally exposed to in the circulation. This model probably comes closest to the natural environment avoiding artefacts from cell isolation and preparation and allowing natural interactions between immune components and antigens within the normal hormonal milieu. Essentially it is an *in vitro* method of

simulating responses to an infection. Our findings indicate that low vitamin D status is associated with an impaired ability to mount a pro-inflammatory cytokine response to a multi-antigen challenge, whereas the production of anti-inflammatory cytokines (IL-4 and IL-10) was not significantly influenced by vitamin D status. An impaired pro-inflammatory response to antigen challenge could increase the risk of succumbing to infection and increase severity and/or duration of symptoms of infection.

Another interesting finding in the present study is that the SIgA secretion rate in the optimal vitamin D status group was significantly higher than in the other groups. The mucosal immune system, especially SIgA, functions as the first line of defence against pathogen invasion by preventing antigens and microbes adhering to mucosal surfaces and interrupting replication of intracellular pathogens during transcytosis through epithelial cells [10]. Previous studies have shown an inverse relationship between SIgA values and URTI prevalence. For example, low SIgA values have been reported to be associated with increased incidence of URTI in athletes [8,13,24]. The finding in the present study that a significantly lower proportion of subjects in the vitamin D optimal status group experienced URTI episodes during the 4-month study period could be explained partially by the protective effect of their higher SIgA secretion rate. To our knowledge, this is the first study to report an association between SIgA values and plasma vitamin D status. It would be interesting to know if high dose Vitamin D supplementation could elevate SIgA in people with low SIgA secretion.

The inter-individual variation in vitamin D status within our athlete cohort is most likely due to differences in sunlight exposure rather than diet since the D3 form (derived primarily from synthesis in the skin) made up >90% of total plasma 25(OH)D. None of the subjects were taking vitamin supplements but we did not assess dietary vitamin D intake in this study. Vitamin D insufficiency has been reported to be common in athletes [21] especially if exposure to natural sunlight is limited (e.g. when training in the winter months or when training mostly indoors). On the basis of the present data, we found that 38% of athletes had insufficient plasma 25(OH)D values (< 50 nmol/L) at the start of the 4-month period and 55% of athletes had plasma 25(OH)D values of less than 50 nmol/L at the end of the study. This proportion is slightly lower than previously reported in a study on UK athletes [5] that reported 61% had serum 25(OH)D concentrations of less than 50 nmol/L during the winter months. Moreover, we also found that there was a significant drop in plasma 25(OH)D concentration from the start of the 4-month period to the end of the study. This is most likely due to insufficient UV radiation of appropriate wavelength between November and March in the UK (Loughborough latitude is 53°N) to produce vitamin D in the skin [32]. This probably also explains why there was no difference in plasma 25(OH)D concentration between indoor and outdoor athletes during the winter months in the present study, an observation also reported by other studies [5,13]. Given that the incidence of URTI is generally higher in athletes [10] and that low vitamin D status could be a contributing factor, it seems probable that vitamin D supplementation could be desirable for athletes during the winter months. Our results also provide confirmation that the presence of URTI episodes in athletes results in a significant reduction of their training load.

In conclusion, our study suggests that low vitamin D status could be an important determinant of URTI risk in endurance athletes. Athletes with low vitamin D status may have a higher risk of URTI and suffer more severe symptoms when URTI is present. This may be due to impaired mucosal and systemic immunity as SIgA secretion, cathelicidin levels and antigen-stimulated pro-inflammatory cytokine production appear to be increased by vitamin D-dependent mechanisms. Overall, with regard to URTI and vitamin D status the results indicate that it is not good to be deficient in vitamin D (plasma 25(OH)D < 30 nmol/L) and it is probably best to be optimal (plasma 25(OH)D > 120 nmol/L). Further studies are needed to establish if vitamin D supplementation can improve immunity and reduce URTI risk in athletes.

Acknowledgements

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Butyrylated starch increases colonic butyrate concentration but has limited effects on immunity in healthy physically active individuals.

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Abstract

Background: *Butyrate delivery to the large bowel may positively modulate commensal microbiota and enhance immunity.*
Objective: *To determine the effects of increasing large bowel butyrate concentration through ingestion of butyrylated high amylose maize starch (HAMSB) on faecal biochemistry and microbiota, and markers of immunity in healthy active individuals.*
Design: *Male and female volunteers were assigned randomly to consume either two doses of 20 g HAMSB (n=23; age 37.9 ± 7.8 y; mean ± SD) or a low amylose maize starch (LAMS) (n=18; age 36.9 ± 9.5 y) twice daily for 28 days. Samples were collected on days 0, 10 and 28 for assessment of faecal bacterial groups, faecal biochemistry, serum cytokines and salivary antimicrobial proteins.*
Results: *HAMSB led to relative increases in faecal free (45%; 12-86%; mean; 90% confidence interval; P=0.02), bound (950%; 563-1564%; P<0.01) and total butyrate (260%; 174-373%; P<0.01) and faecal propionate (41%; 12-77%; P=0.02) from day 0 to day 28 compared to LAMS. HAMSB was also associated with a relative 1.6-fold (1.2- to 2.0-fold; P<0.01) and 2.5-fold (1.4- to 4.4-fold; P=0.01) increase in plasma IL-10 and TNF-α but did not alter other indices of immunity. There were relative greater increases in faecal P. distasonis (81-fold (28- to 237-fold; P<0.01) and F. prausnitzii (5.1-fold (2.1- to 12-fold; P<0.01) in the HAMSB group.*

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Conclusions: *HAMSB supplementation in healthy active individuals promotes the growth of bacteria that may improve bowel health and has only limited effects on plasma cytokines.*

Key words: Butyrylated starch, immunity, exercise, short chain fatty acids, faecal microbiota

Introduction

Prolonged physical and psychological stress as experienced by elite athletes (12, 39), nurses (24) and students undertaking examinations (25), can increase susceptibility to illness and disease via perturbations in immune function. Stress can lower the salivary concentrations of immunoglobulin A (IgA), lactoferrin and lysozyme, and other humoral factors of the immune system that provide defence at mucosal surfaces. Lower salivary IgA is correlated with an increased risk of upper respiratory tract illness (URTI) (14) and a recent study also showed that dys-regulated cytokine responses to an exercise challenge test were associated with a higher rate of URTI in active individuals (8). There may be potential to support the immune system through diet and lifestyle changes which would reduce the risk of stress-induced illnesses.

Altering the abundance of beneficial colonic microbiota and increasing the concentration of bacterial-derived metabolites, in particular short-chain fatty acids (SCFAs), has the potential to improve gut health and immune function. A substantial body of research strongly indicates that commensal bacteria play a key role in health and disease through interaction with epithelial and immune cells (26, 29). The colonic microbiota is also responsible for the fermentation of undigested dietary carbohydrates and the production of SCFAs, principally acetate, propionate and butyrate. These SCFAs play a key role in the function of epithelial cells and the mucosal barrier in the colon (5). Butyrate in particular has attracted considerable attention as a major metabolic fuel for colonocytes and has been reported to have a wide range of effects on immune cell functions including chemotaxis, phagocytosis, reactive oxygen species production, cytokine/chemokine release and immune cell migration (2, 36, 37). Butyrate's effects may occur via the activation of G protein coupled receptors (GPR) which are expressed on immune cells, adipocytes and intestinal epithelial cells. GPR41 and 43 have the highest affinity for SCFAs (33) and are both expressed by cells in the colonic epithelium and GPR43 is also highly expressed in circulating immune cells.

Bacterial fermentation of fibre increases the abundance of commensal bacteria and increases the concentration of SCFAs. Resistant starch (RS) is a component of dietary fibre, which, when fermented in the large bowel favors the production of butyrate and increases the abundance of the commensal bacteria *P. distasonis* (7, 9, 28, 35). The ingestion of acylated starches has the capacity to deliver specific SCFAs to the colon (6). Acylated starches in which SCFAs are esterified to a carrier starch to a moderate degree of substitution (DS: the average number of

hydroxyl groups on each D-glucopyranosyl unit derivatized by substituent groups (34) resist small intestinal digestion and pass to the colon where the esterified acids are liberated by bacterial enzymes. The residual starch is fermented with the production of further SCFAs. Butyrylated starch has proven effective at delivering butyrate to the large bowel of healthy humans and increasing colonic SCFA (7). This was, however, in a specific population cohort with low butyrate concentrations and further research in populations with normal faecal butyrate concentrations is necessary.

The aim of this study was to investigate the effects of increasing colonic butyrate through the ingestion of butyrylated starch on gut health and immunity in healthy, active individuals. To achieve this aim a 28 day parallel design, double blind, randomized, controlled trial was undertaken and markers of gut, mucosal and systemic immunity were measured to determine the potential of butyrylated starch to improve the health of physically active individuals.

Methods

Subjects

Following screening 41 healthy active male (n=23; age 37.9 ± 7.8 y, mean ± SD) and female (n=18; age 36.9 ± 9.5 y) cyclists were enrolled to provide 80% statistical power and an alpha of 0.05 on the primary outcome measures based on the literature and the authors' experience. These individuals were recruited from the general cycling community in Canberra Australia by email and personal contact at cycling events. Subjects were required to declare their use of dietary and/or performance enhancing substances that may influence underlying immune function. All subjects consuming immuno-modulatory medications or the regular use of any drug, medication or supplement which could interfere with bowel function were excluded. Inclusion to the study was dependent upon the subjects not taking antibiotics and supplements or foods with probiotics for at least one month prior to and during the study period. The study statistician allocated the participants to treatment groups using minimisation stratified by sex. . Participants and the study team were blinded as to the allocation of treatments. All procedures involving human subjects were approved by the Human Research Ethics Committees of the Australian Institute of Sport (AIS), CSIRO and Griffith University. All participants provided written informed consent. The study was conducted according to the guidelines prescribed in the Declaration of Helsinki and registered with the Australian New Zealand Clinical Trials Registry ACTRN12611000771954.

Study design

The study was a randomized, double blind, parallel controlled trial designed to compare the effects of ingestion of 40g/day of butyrylated high amylose maize starch (HAMSB) with low amylose maize starch (LAMS; control) . The study consisted of a 14-day pre-intervention period where subjects were asked to refrain from eating yoghurt and any supplements that could modulate enteric microbiota. This baseline phase was followed by a 28-day period when subjects consumed the supplements. At the end of the baseline period (day 0) and at day 14 and day 28 of the supplementation period subjects provided samples for the assessment of fae-

cal microbiology and biochemistry, and immune function. Subjects were paired on age and maximal oxygen uptake (VO_2max), and randomly allocated to either experimental (HAMSB) or placebo supplementation by the study statistician who did not have direct contact with the subjects.

During supplementation subjects consumed two specially formulated beverages containing 20g of the test starches daily; one in the morning and one in the evening. All subjects maintained a four-day food diary in the second week of the study that incorporated two weekdays and a weekend to determine total daily energy (kJ) and fibre (g) intakes. Subjects were also required to maintain two two-day food diaries prior to providing the faecal samples on days 0 and 28. These records were used to identify any short term changes in starch consumption which could modify faecal microbiota and SCFA. Dietary analysis was undertaken using FoodWorks professional edition software package (version 3.0, Xyris Software, Brisbane, Australia). At the end of the study subjects completed a gastrointestinal quality of life questionnaire (GIQLI) to examine the effects of supplementation on GI function as described previously (7).

Test Product

The specially formulated beverages comprised 60 g of Protein Plus Protein Powder® (Powerbar Oceania, Rhodes, Australia) with either 20 g HAMSB (Ingredion Incorporated, formerly National Starch and Chemical Company, Bridgewater, NJ) or LAMS (New Zealand Starch, Auckland, New Zealand) in 200 ml of milk or water. HAMSB had a DS of 0.23 as determined by CSIRO Materials Science and Engineering by use of ^{13}C-NMR spectroscopy using a Bruker BioSpin DRX500 NMR spectrometer (Fällanden, Switzerland). Subjects were asked to consume one beverage in the morning and one in the evening. The beverages were consumed with or without food.

Sample collection

Saliva, blood and faecal samples were obtained pre- (day 0), mid- (day 14) and end-of supplementation (day 28) from all subjects. Saliva was collected with an oral eyespear swab (Defries Industries Pty Ltd, Victoria, Australia) for determination of IgA, lactoferrin and lysozyme. The eyespear was placed between the cheek and teeth for 5 min, removed and centrifuged immediately for 5 min at 778 g and then frozen at -80°C until analysis. Albumin concentration was assessed to control for changes in salivary flow rate. All saliva samples were taken at the same time of the day to control for diurnal variation. A blood sample (9 ml) was drawn from the antecubital vein to quantify resting serum cytokine concentrations. Each sample was collected directly into a K_3EDTA tube (Greiner Bio-one; Frickenhausen, Germany) and frozen at -80°C until analysis. Participants provided a faecal sample within 48 h of the blood and saliva sampling. Faeces were collected in a sealable plastic bag and frozen immediately at -20°C in a portable freezer until transfer to laboratory storage at -80°C.

Measures of mucosal immunity

Lactoferrin, lysozyme and SIgA concentrations were measured spectrophotometrically by enzyme linked immunosorbant assay (ELISA) using commercial kits

(lactoferrin – EMD Chemicals, New Jersey, USA; lysozyme - Sapphire Bioscience, Redfern, Australia; SIgA – Salimetrics, IgA –Salimetrics, Philadelphia, USA). Albumin concentration was measured by immunoturbidimetric assay on a Hitachi 911 Chemistry Analyzer (Roche). Osmolality was measured on a Model 3320 Osmometer (Advanced Instruments Inc) as per the manufacturer's instructions. Variability was acceptable at <10% for the low and high positive controls.

Plasma cytokines

Granulocyte macrophage-colony stimulating factor, (GM-CSF), interleukin (IL)-1RA, IL-6, IL-8, IL-10, tumour necrosis factor (TNF)-α and interferon gamma (IFN-γ) cytokines were measured on a Bio-Plex Suspension Array System (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA). The plasma samples were analyzed on custom manufactured Multiplex Cytokine Kits (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA). Plates were read using the Bio-Plex Suspension Array System (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA). A full blood count including white cell count and differential was performed on a haematology analyser (Advia, GMI, Michigan, USA). Results from each assay were accepted if the positive controls were within two standard deviations of their established mean concentration. Each plate included a control.

Illness, training and performance measures

Symptoms of gastrointestinal illness were recorded daily by subjects as described previously (11). Details of exercise or physical training undertaken during the study were also recorded. For each session, training mileage ($km.wk^{-1}$), duration ($h.wk^{-1}$) and intensity (scored on a 1–5 scale: 1, easy; 5, maximal) were recorded. From this data a value for weekly training load can be ascertained by multiplying training duration in hours by intensity. At the start of the study subjects undertook an incremental exercise test to exhaustion to determine peak power output and maximal oxygen uptake (VO_2max). The test was performed on an electromagnetic cycle ergometer (Excalibur Sport, Lode NV Groningen, Netherlands) as described previously (20).

Microbial analysis

DNA extraction

DNA was extracted from faecal samples as previously described (41). Briefly, the protocol combines a mechanical (bead beating) and enzymatic lysis of bacterial cells followed by a cleanup to eliminate contamination from other cell debris.

Phylogenetic profile using a custom microarray

A custom phylogenetic microarray developed and validated for human gut bacteria was used to analyze the microbiota (17). Briefly, extracted DNA from faecal samples from days 0 and 28 were amplified using the prokaryote 16S rRNA gene primer sets 27F (5'– AGAGTTTGATCMTGGCTCAG–3') and T7/1492R (5'–TCTAATACGACTCACTATAGGGGGYTACCTTGTTACGACTT-3') (the underlined region is modified to include a T7 promoter sequence). Detailed methods of cRNA synthesis and labeling, hybridization, image capture and analysis have been previously described (17).

Quantitative real time PCR (Q-PCR)
Q-PCR was performed on samples collected at days 0, 14 and 28 to confirm the findings of the microbial changes identified with the human gut microarray. Total bacteria, *Faecalibacterium prausnitzii* and *Parabacteroides distasonis* were quantified using Q-PCR, which were performed in reaction volumes of 10 or 20 µl (*P. distasonis*) containing 1X SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA) and 0.2 mg/ml BSA. Primers (concentration): Total bacteria (150 nM), 1114f (5`-CGGCAACGAGCGCAACCC-3`) and 1275r (5`-CCATTGTAGCACGTGTGTAGCC-3`) (10); *F. prausnitzii* (500 nM), FPR-1 (5`-AGATGGCCTCGCGTCCGA-3`) and FPR-2 (5`-CCGAAGACCTTCTTCCTCC-3`) (16), and *P. distasonis* (500 nM) BdisF (5`-TGATCCCTTGTGCTGCT-3`) and BdisR (5`-ATCCCCCTCATTCGGA-3`) (21). For quantification a total of 10 ng of template DNA was used and Q-PCR cycling was performed in a Chromo-4 thermocycler (Bio-Rad Laboratories, Hercules, CA). The Q-PCR cycling conditions were as follows: 4 min at 98°C followed by 35 cycles of 98°C for 20 s, 60-62°C for 20 s (total bacteria: 60°C; *F. prausnitzii* and *P. distasonis*: 62°C) and 72°C for 30 s with fluorescent acquisition after each cycle. A final meltcurve analysis was performed after completion of all cycles with fluorescence acquired at 0.5 °C intervals between 55 and 95°C to verify that only the expected fragment was amplified. Q-PCR product was also visualized on a 1.5% agarose gel. Non-template controls were included and assays were performed in triplicate by analyzing the same DNA sample in 3 independent reactions. An 8-series of 10-fold dilutions of a sample derived plasmid construct (Top chemical competent cells, Invitrogen) containing the target amplicon were analyzed in parallel with DNA samples for estimation of absolute abundance and Q-PCR efficiency for all assays. Results were analyzed with the Opticon Monitor 3 software (ver. 3.1) (Bio-Rad Laboratories, Hercules, CA). All Q-PCR data were analyzed as absolute numbers of bacteria in one gram of wet weight faeces.

Short chain fatty acids
Faecal samples were thawed at 4°C and then subsampled for analysis. Weighed portions for the determination of free (unesterified) SCFA were diluted 1:3 w/w with deionized water containing 1.68 mM heptanoic acid as an internal standard (Sigma Chemical Co, St Louis, MO). Unesterified SCFA were analyzed as described previously (4). A three point linear standard curve containing acetic, propionic, isobutyric, butyric, isovaleric, valeric and caproic acids was used for calibration at concentrations spanning the range of those measured in samples from this study. Total SCFA concentrations were determined as described previously (6) with the exception that hydrolysis was undertaken by agitating the samples for 2 h with 0.7 times the sample volume of 6 M NaOH. A subset of seven samples with a wide range of butyrate concentrations were analyzed twice using both methods to ensure consistency and accuracy of the revised method. Total faecal ammonia concentration and pH were determined using previously described methods (3).

Statistical analysis
All data is presented as mean ± standard deviation. Filtered and normalized microarray data were analyzed by the multivariate analysis tool, principal compo-

nent analysis (PCA) using the Genespring program, which determined automatically the number of components in the PCA models. Volcano plot analysis was also performed on the data with more than two-fold differences (> 2 fold) and significant P-value (P=0.05) between different diets (HAMSB and LAMS) and time points (days 0 and 28) in Genespring 7.3 software (Agilent Technologies, Santa

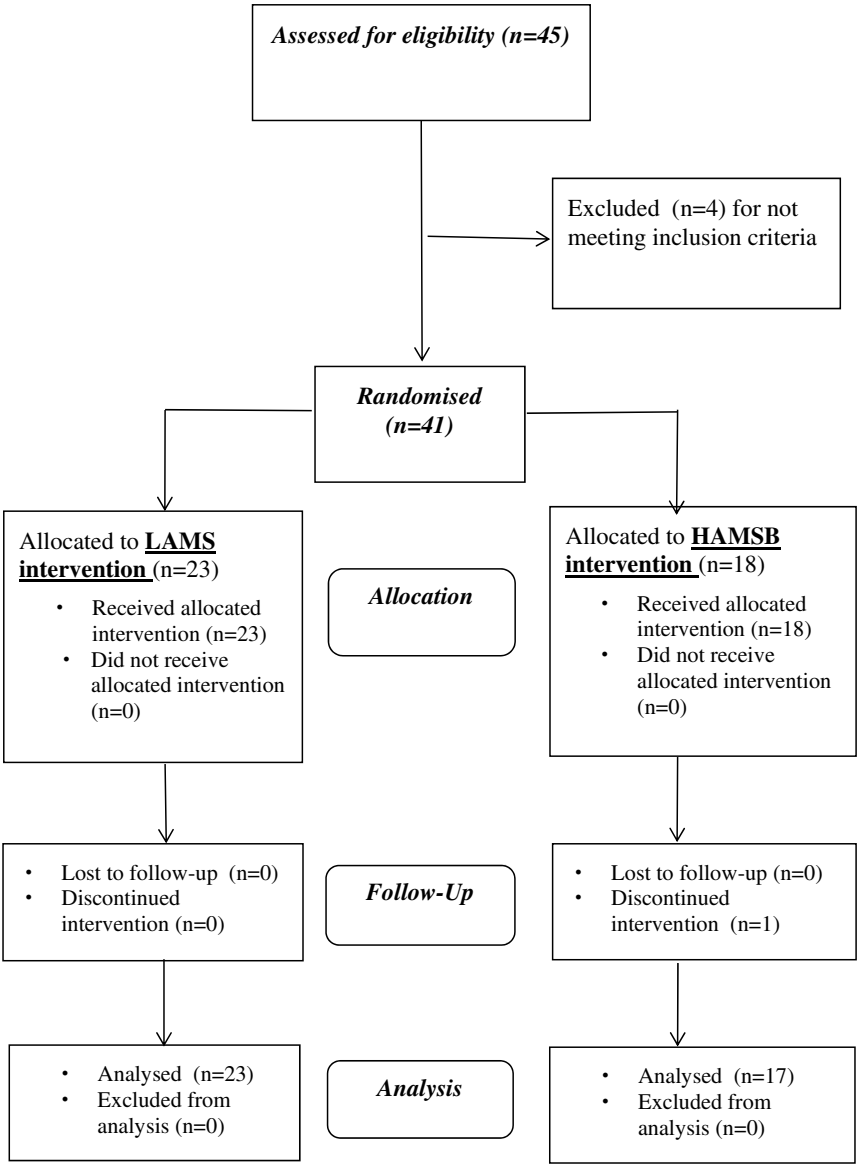

Figure 1. Flow of participants through the study using a Consort Flow Diagram.

Clara, CA, USA). The selected probes filtered by volcano plot analysis were compared by box plot using the same software and differences were confirmed by Q-PCR analysis. Statistical analysis of measures of immunology and enteric microbiota (Q-PCR data) evaluated the magnitude of the difference in the mean change between the treatment groups from day 0 to days 14 (mid-supplementation) and 28 (end of supplementation).

The measures of physical activity, salivary and serum proteins, faecal biochemistry and Q-PCR microbiological data were all log-transformed before analysis to reduce non-uniformity of error and permit the effect of the treatment to be analyzed as a percent. Differences in the change in mean saliva and serum protein concentrations, and Q-PCR faecal data between the groups were analyzed with a Student's t-test for independent samples (unequal variance). Baseline values of the dependent variable were included as a covariate in these analyses to account for regression to the mean. The extent to which changes in bacterial counts accounted for changes in other outcome measures was examined through covariate analysis.

Standardized mean changes were used to characterize differences between groups. A modification of Cohen's effect size (ES) classification system (trivial: 0.0–0.2; small: 0.2–0.6; moderate: 0.6–1.2; large: 1.2–2.0) was used to interpret the magnitude of observed changes (15). The effects of supplementation are shown with 90% confidence limits. Statistical significance was accepted at $P<0.05$.

Results

Subjects

A total of 45 individuals volunteered and were assessed for eligibility. The flow of participants through the study and the consort checklist is shown in Figure 1. Four individuals who did not meet the inclusion criteria were excluded. Of the 41 individuals recruited one male participant in the HAMSB group withdrew from the study within seven days due to the taste of the supplement.

There were no significant differences in baseline characteristics between the groups at allocation (Table 1). Three female and two male volunteers reduced their intake of supplementation from 40 g of supplement to 20 g in the first seven days due to a feeling of fullness. Data were analyzed from all subjects allocated to a treatment group. Dietary analysis exclusive of the supplements found no significant differences between the groups in intakes of total energy (kJ) (HAMSB 8954 ± 2870 versus LAMS 8069 ± 1850) or fibre (g) (HAMSB 19 ± 12 versus LAMS 16 ± 7) during the study. HAMSB supplementation was not associated with a change in symptoms of GI discomfort as measured by the GIQLI (data not shown). Examination of the two 2-day food diaries showed no instances of volunteers consuming foods high in RS immediately before the faecal collection days of the study. There were no significant differences between the groups in the number of exercise training days per week (HAMSB 4.4 ± 2.0; LAMS 4.5 ± 2.1) or training load (HAMSB 4.26 ± 1.22; LAMS 4.23 ± 1.73).

Table 1. Baseline characteristics of study volunteers

	LAMS	HAMSB	P-value
n	23	18	
Age (y)	37.2 ± 8.5	37.6 ± 8.3	0.87
Mass (kg)	69.0 ± 14.2	66.0 ± 11.2	0.45
VO_2max ($ml.min^{-1}.kg^{-1}$)	57.5 ± 5.0	54.9 ± 7.2	0.18

Data are mean ± SD. VO_2max, maximal oxygen uptake.

Faecal measures

Changes and differences in selected faecal measures between HAMSB and LAMS are shown in Table 2. The change in faecal pH from day 0 to day 14 was lower in the HAMSB group 14 (HAMSB -0.11 ± 0.37 versus LAMS 0.22 ± 0.49, mean ± SD, *P*=0.02) but not from day 0 to day 28 (HAMSB 0.03 ± 0.29 versus LAMS 0.21 ± 0.45, *P*=0.13). There was a substantial reduction in the concentration of ammonia in the first 14 days in the HAMSB group (-33%; -12 to -50%, mean; ±90% confidence interval) but not LAMS (17%; 0 - 36%, *P*=0.01). The difference between the groups in the mean change in faecal ammonia concentration from pre- to post-supplementation was not significant.

Changes in the concentration of total SCFA, acetate, propionate, butyrate are also presented in Table 2. There was a relative 38% (10 - 74%; *P*=0.01) difference in total SCFA concentration between the groups from pre- to mid-study, with HAMSB increasing total SCFA concentration by 14% (-4 to 36%) and LAMS reducing total SCFA concentration by 19% (-32 to -54%). No significant effects were evident in total SCFA concentration between the groups from pre- to post supplementation. There was a moderate difference between the groups in the concentration of acetate (28%; 2 - 60%; *P*=0.07) from pre- to mid-supplement (mean change, HAMSB -1.3%; ±18% versus LAMS -21%; ±20%, mean; ±90% confidence limits) but no substantial difference between the groups in acetate concentration after 28 days. The concentration of propionate increased by 25% (7 - 47%) in the HAMSB group with no substantial change in the LAMS group from pre- to post-supplementation. The ratio of acetate to propionate was reduced by 30% (20-38%; *P*<0.01) in the HAMSB group compared to the LAMS group over the course of the study. Supplementation with HAMSB over 28 days yielded singificantly higher increases of ~45% in free butyrate, ~10-fold in bound butyrate, and, ~2.5 fold in total butyrate compared with LAMS (Table 2).

Covariate analysis did not reveal any clear trends between changes in *P. distasonis* and *F. prausnitzii* and changes in SCFA. Furthermore, no clear relationship between changes in SCFA, individually or in total, was evident with changes in faecal pH or ammonia.

Table 2. The effect of supplementation for 14 and 28 days with LAMS and HAMSB on faecal biomarkers.

	LAMS			HAMSB			Difference in Change (Day 14 - 0)		Difference in Change (Day 28 - 0)	
Parameter	**Day 0 Mean ± SD**	**Day 14 Mean ± SD**	**Day 28 Mean ± SD**	**Day 0 Mean ± SD**	**Day 14 Mean ± SD**	**Day 28 Mean ± SD**	**Mean%; ± 99%CL**	**P-value**	**Mean%; ± 99%CL**	**P-value**
Faecal output (g)	155 ± 94	189 ± 98	135 ± 95	136 ± 54	127 ± 61	183 ± 78	-29; ±33	0.05	65; ±42	0.02
Ammonia µmol.g^{-1}	22 ± 13	22 ± 9	18 ± 8	23 ± 13	16 ± 9	17 ± 7	-36; ±37	0.02	-5; ±25	0.55
Total SCFA µmol.g^{-1}	83 ± 21	71 ± 30	78 ± 40	86 ± 33	99 ± 37	84 ± 27	45; ±27	0.01	-14; ±27	0.38
Acetate µmol.g^{-1}	52 ± 15	44 ± 19	50 ± 23	54 ± 20	54 ± 19	47 ± 15	28; ±26	0.08	-0.4; ±27	0.90
Propionate µmol.g^{-1}	15 ± 4	14 ± 6	15 ± 7	16 ± 7	23 ± 9	20 ± 6	67; ±28	<0.01	41; ±26	0.02
Free butyrate µmol.g^{-1}	16 ± 7	13 ± 6	13 ± 7	16 ± 7	23 ± 11	18 ± 6	77; ±35	<0.01	46; ±29	0.02
Bound butyrate µmol.g^{-1}	6 ± 5	5 ± 4	6 ± 7	6 ± 3	58 ± 32	47 ± 29	618; ±102	<0.01	950; ±58	<0.01
Total butyrate µmol.g^{-1}	21 ± 7	18 ± 8	20 ± 11	21 ± 7	81 ± 41	65 ± 31	192; ±50	<0.01	260; ±31	<0.01

CL – confidence limit; g- gram; µmol – micromole; SD – standard deviation

Faecal microbiology

Microarray analysis comparing microbial profiles between day 0 and day 28 showed clear differences between the responses to HAMSB and LAMS treatments. Principal components analysis (PCA) showed differences in the microbial profiles after treatments of LAMS samples compared to HAMSB (Figure 2). Microarray analysis revealed post-HAMSB treatment group had a higher *P. distasonis* signal than other groups. Similarly samples from some individuals

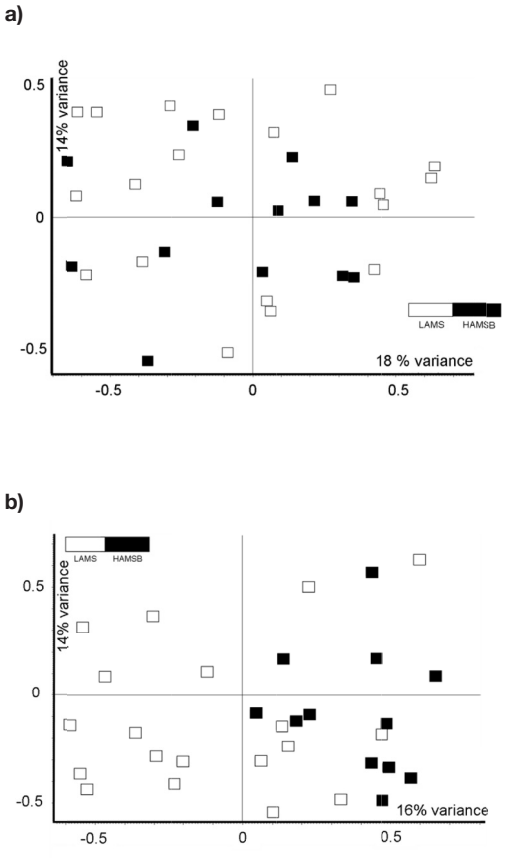

Figure 2. Principal component analysis plots showing the % variation of faecal microbial diversity explained by the x-axis and y-axis. Plot A) shows faecal microbial diversity between HAMSB and LAMS on day 0. Plot B) shows a substantial change in microbial diversity between HAMSB and LAMS on day 28. Individual data points represents a sample with black squares representing HAMSB samples and clear squares represents LAMS samples.

showed a significant increase in signal intensity of the *F. prausnitzii* probes in response to HAMSB but the mean difference was not significant for the whole treatment group (data not shown).

These differences were confirmed by Q-PCR using primers specific for *P. distasonis* and *F. prausnitzii* (Figure 3). HAMSB supplementation elicited an 81-fold increase in *P. distasonis* and a 5.1-fold increase in the abundance of *F. prausnitzii* from day 0 to day 28 compared to LAMS. Differences in total bacteria between the treatment groups were not significant.

Plasma cytokines

The effect of supplementation on resting plasma concentrations of IL-1RA, IL-10 and TNF-α is shown in Figure 4. IL-1RA concentration was 1.9-fold higher in the HAMSB group compared with the LAMS group from day 0 to day 14. However, there was only a trivial difference between the groups from 0 to day 28 of supplementation. The differences between the groups from day 0 to day 14 in IL-1RA were a result of a reduction in IL-1RA concentration in the LAMS group (-43%; -55 to -28; 90% confidence interval). From day 0 to day 28 there was a greater decline in IL-10 in the LAMS group than the HAMSB group (by a factor of 1.6-fold; 1.2- to 2.0-fold; $P<0.01$) while the change in TNF-α concentration was 2.5-fold (1.4- to 4.4-fold; $P=0.01$) higher in the HAMSB group compared with the LAMS group. Starch supplementation did not affect changes in serum IL-6, IL-8 or GM-CSF concentrations. There were no significant effects of supplementation on other plasma cytokines (GM-CSF, IL-6, IL-8, and IFN-γ).

Mucosal immunity

There were no substantial differences between the treatment groups over the course of the study in any of the salivary proteins measured: compared with LAMS the HAMSB group had a 10% (-19% to 50%; *P*=0.61) increase in the concentration of SIgA, a 7% (-43% to 95%; *P*=0.86) increase in salivary lysozyme and a 3% (-35% to 30; *P*=0.80) decrease in the concentration of salivary lactoferrin from day 0 to day 28.

Discussion

This study confirms the findings of previous studies that HAMSB supplementation raises faecal butyrate concentrations (7), which is consistent with the documented ability of acylated starches to deliver SCFAs to the human large bowel (6). The study demonstrated also that supplementation with HAMSB substantially increased the faecal numbers of *P. distasonis* and maintained those of *F. prausnitzii*. Similar increases in the faecal numbers of *P. distasonis* have been reported in humans (7) and animals (1) consuming HAMSB. In contrast, consumption of LAMS substantially lowered the faecal numbers of *P. distasonis* and *F. prausnitzii* and also the concentrations of propionate and butyrate. Collectively these data suggest HAMSB may promote large bowel health in healthy active individuals and that diets containing refined starch (such as LAMS) may have detrimental effects on colonic health. Walker et al (38) reported that substitution of a highly digestible starch for foods containing significant quantities of resistant starch raised

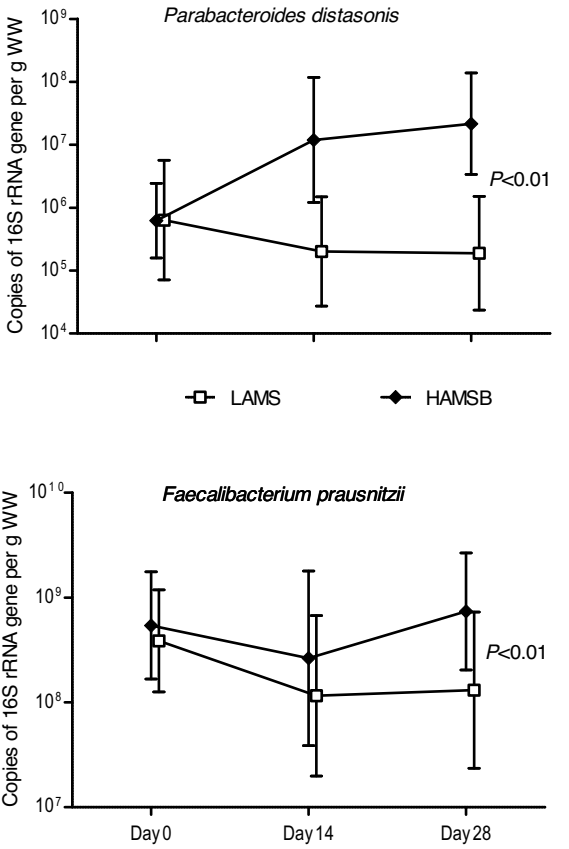

Figure 3. The effect of supplementation with 40 g.day^{-1} HAMSB and LAMS on fecal *P. distasonis* and *F. prausnitzii* (mean ± SD). *significant difference between the treatment groups from day 0 to day 28.

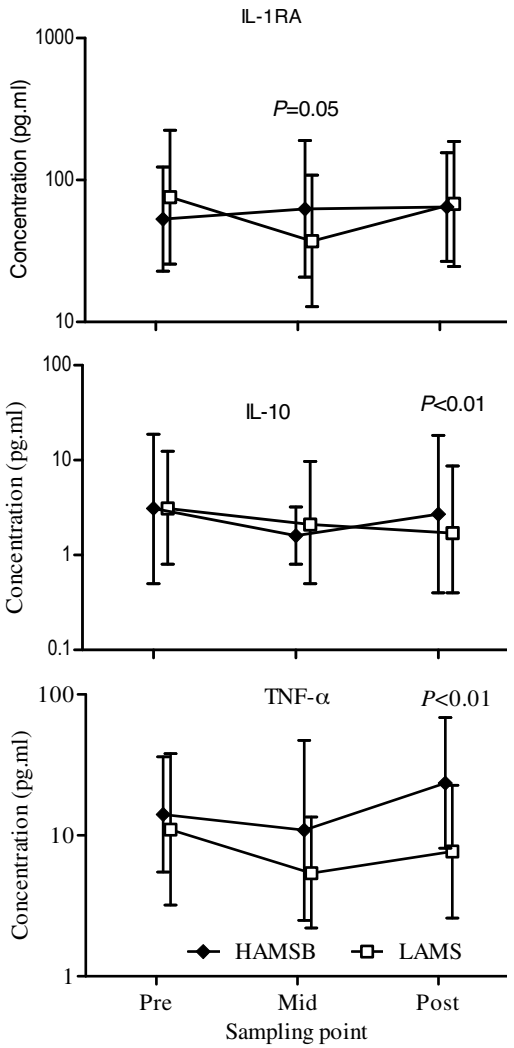

Figure 4. The effect of supplementation on the concentration of IL-1RA, IL-10 and TNF-α from pre- to post-intervention (mean ± SD). *significant difference (P<0.01) between the treatment groups over the course of the study.

faecal pH in African schoolchildren supporting the latter suggestion.

Previous animal experiments have shown that acylated starches raise large bowel SCFA after the release of the esterified SCFA and subsequent fermentation of the residual starch. This combined action explains the higher faecal concentrations of all major SCFAs as well as the reduction in pH values in the HAMSB group in the present study. The latter effect is thought to occur via direct acidification of gut contents by SCFAs and, while animal results are conflicting (1) by lower NH_3 levels as well. In this study covariate analysis found no clear trend between the increasing SCFA and lower NH_3 levels with pH.

The ileal digestibility of LAMS is ~100% and we anticipated its consumption would not alter any of the faecal biomarkers which we measured (6). However, this was not entirely the case as HAMSB and LAMS had divergent effects on faecal propionate, butyrate and pH. As expected, butyrate concentrations (bound, free and total) substantially increased with HAMSB supplementation but increases in free butyrate were only evident to day 14 of the study. Furthermore, there were moderate decreases in the concentration of butyrate in the LAMS group. The explanation for the reduction in butyrate concentration in the HAMSB group from day 14 to day 28 is uncertain given there were no substantial changes in dietary practices and compliance throughout the study, although the increase in feacal bulk in this last 14 days suggests ongoing adaptation in the large bowel. The role of butyrate as a fuel for colonocytes and

in epithelial integrity is well recognized (32) and this study provides support for increased uptake and utilization with prolonged ingestion. The question as to whether the lower butyrate concentration at day 28 reflects increased utilization needs to be the focus of future research. The reason for the difference in total SCFA concentration between the groups to the mid-point of the study relates to both a fall in total SCFA concentration in the LAMS group and a substantial increase in the HAMSB group.

The substantial falls in butyrate and total SCFA concentration and the increases in faecal pH and ammonia in the LAMS group may be related to the replacement of fermentable carbohydrates with LAMS during the study. The continued refinement of starches in modern food manufacturing has resulted in an increasing consumption of simple starches. The findings from this study indicate that these starches may reduce the abundance of beneficial bacteria and concentration of short chain fatty acids.

Commensal microbiota plays an important role in gut and immune development and homeostasis and the prevention of disease (22). Estimates for faecal abundance of *P. distasonis* and *F. prausnitzii* showed no differences between the two groups at entry into the trial. The dose of the HAMSB supplement (40 $g.person^{-1}.day^{-1}$) was sufficient to alter faecal biomarkers, promote an increase in faecal recovery of *P. distasonis*, and maintain the level of faecal *F. prausnitzii*. In contrast, consumption of LAMS lowered substantially the faecal recovery of both bacterial species. *F. prausnitzii* favors butyrate production, which is of considerable importance in light of the putative role of this SCFA in bowel health. Numbers of this bacterium are low in Crohn's disease and irritable bowel syndrome patients compared to healthy controls (17, 31). However a recent report of clinical improvement in Crohn's disease correlated with a substantial decrease in *F. prausnitzii* abundance (16), highlighting the complexity in understanding the role of the microbiota in health and disease. Oral administration of *P. distasonis* reduced the severity of intestinal inflammation in a mouse model of colitis (18). This study provides evidence that HAMSB promotes selective growth of potentially beneficial bacteria in healthy active individuals while consumption of LAMS reduced the number of those organisms.

There were indications of an effect of supplementation on selected circulating immune markers while mucosal (salivary) indices were unchanged. Cytokines have an essential role in regulating and coordinating immune activity. We observed a substantial difference from day 0 to day 28 in the concentration of TNF-α between the groups, due in part to a decrease in TNF-α concentration in the LAMS group from days 0 to 14. Furthermore, the concentration of the anti-inflammatory cytokine IL-10 was maintained in the HAMSB group in comparison to the LAMS group, which had a moderate reduction in IL-10 from days 0 to 28. Other inflammatory markers were unchanged.

An important benefit of exercise is its anti-inflammatory effect in protecting host tissues from micro-trauma and damage to skeletal muscle (27). However, a possible consequence of this down-regulation of inflammation is an increased suscep-

tibility to common infections, particularly upper respiratory tract illness, in people undertaking prolonged intense exercise (13). Evidence indicates that anti-inflammatory cytokines may increase susceptibility to infection (13). In the context of this sample of healthy active adults and the hypothesis that exercise promotes an anti-inflammatory cytokine profile, maintaining serum TNF-α concentration with HAMSB consumption may ameliorate this risk of infection. It should also be noted, however, that the fall in the circulating concentration of the anti-inflammatory cytokine IL-10 in the LAMS group may also moderate the risk of illness associated with an enhanced anti-inflammatory profile from exercise. There is evidence from colitis animal models and *in-vitro* studies of colitis that *P. distasonis* and *F. prausnitzii* modulate cytokine production from resident tissue immune cells, the gut and peripheral blood mononuclear cells (19, 30). *F. prausnitzii* has been demonstrated to induce IL-10 secretion, which may explain the maintenance of this cytokine in the HAMSB group compared to the LAMS group. With the exception of TNF-α, the effect of supplementation with HAMSB on cytokine concentration in this study are consistent with other research which found no substantial effects of 25 g HAMS supplementation per day for four weeks on serum cytokines (40). Serum cytokines are linked with chronic low grade inflammation that underlies the pathogenesis of many chronic developed world diseases (23). In this context determining whether butyrylation of HAMS contributed to the increase in serum TNF-α concentration and also the role of LAMS in reducing anti-inflammatory cytokines is necessary. Given the strengthening link between inflammation, metabolic dys-regulation and chronic disease, understanding the role of HAMSB and LAMS may have important implications for dietary guidelines on starch consumption.

In conclusion, this study confirms the beneficial effects of supplementation with HAMSB on markers of bowel health in healthy physically active adults. Furthermore, supplementation with LAMS had potentially detrimental effects on these parameters and on markers of inflammation. The potential for HAMSB to support the health and performance of healthy active individuals undertaking prolonged, stressful exercise warrants further investigation.

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Effects of Exercise on Immune Function in Patients with Cancer: a Systematic Review

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Abstract

Background *The role of exercise therapy in the rehabilitation of cancer patients and survivors is becoming increasingly important as it is thought to modulate immunity and inflammation. More knowledge about the effects of exercise on immune function in these patients is needed. Our aim is to systematically review changes in immune parameters after acute and chronic exercise in cancer patients.*

Results *Of the 3586 retrieved articles, 21 met the inclusion criteria, and were included in this systematic review. The systematic search yielded 18 articles in adults, and three in children. Six were of low methodological quality, mainly due to lack of blinding of the assessor and high drop-out rates. The effect of chronic exercise on immune function was examined in 18 studies, while two studies evaluated the effects of acute exercise, and one study combined acute and chronic exercise. Following exercise, increases were seen in Natural Killer cytotoxic activity, as well as lymphocyte proliferation and the number of granulocytes. The number of leukocytes, lymphocytes, Natural Killer cells, T lymphocytes, C-reactive protein, and pro- and anti-inflammatory mediators remained stable.*

Limitations *Of the 21 included studies, only three were conducted in the pediatric population, and many studies have included small and heterogeneous samples. Due to the large variety in exercise training protocols and immune parameters, no meta-analysis has been performed.*

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Conclusions *Various immune parameters improved after exercise; however, knowledge of the effects of exercise on immune function in cancer patients is still limited. Additional research is needed to gain insight into the mechanism underlying the effects of exercise on immune function in different populations, and to link these immune parameters to clinical outcomes.*

Key words: Neoplasms, Exercise training, Immune system, Inflammation

Background

In 2008, approximately 3.2 million new cases of cancer have been reported in Europe (18), and currently there are more than 11 million cancer survivors in the US alone (28). Due to improving survival rates, more focus is now placed on post-treatment care as it relates to the adverse short- and long-term effects of cancer and its treatment, including increased fatigue, increased susceptibility to infections due to immunosuppression, reduced physical fitness and lower quality of life (21, 58, 64). Pediatric cancer survivors are also exposed to adverse consequences of treatment on growth and development, including diminished neurological function, altered endocrine function, osteoporosis and obesity (65). Moreover, both pediatric and adult cancer survivors are at an increased risk of disease recurrence or development of a secondary malignancy (17).

In the more recent field of 'exercise – oncology' studies have shown that moderate intensity exercise (e.g. 60% VO_{2peak}) is associated with increased survival rates in breast cancer patients, and/or a reduced risk of postmenopausal breast cancer as a result of favorable changes in body-fat and sex hormone profiles (28, 43, 45). As cancer is a complex disease, multiple mechanisms are operative. The individual characteristics, type of exercise, as well as the cancer diagnosis and stage determine which mechanisms may play a role. Possible beneficial effects of exercise on cancer outcomes are the reduced obesity and adipokine concentrations, reduced levels of insulin, glucose, and sex hormones, increased intestinal motility, decreased inflammation (22), and immunostimulation (5, 22, 41, 42, 47, 58). However, the clinically relevant cut-off points of the exercise-induced immuno-inflammatory response are still largely unknown within cancer. Various factors mediate the relationship between exercise, inflammation and immune function, such as catecholamines and cortisol (22, 45, 51, 52, 60, 63). Both the incidence of cancer and the impairment of the immune system also show negative age-related changes that can partially be counteracted with exercise (6, 31). Furthermore, weight loss by dietary and exercise interventions can partially reverse obesity-related metabolic, endocrinal and inflammatory alterations (14, 45, 47, 51).

These effects, however, will often depend on the type of exercise and its duration. Several authors have proposed an 'Inverted J Hypothesis', where an enhanced immune function and low susceptibility to cancer occur with regular moderate exercise, whereas sedentariness and exhaustive exercise suppress immune func-

tion, and elevate susceptibility to infections (7, 17, 40-42, 52, 60). This is supported by results of animal studies, where exercise-trained animals exhibited a lower inflammatory state compared with the sedentary animals (11, 54). On the other end of the spectrum, it has been reported that, compared with low intensity exercise, high intensity exercise can induce a pro-inflammatory state and higher oxidative DNA damage, both of which are hypothesised to be markers of infection and cancer recurrence (22, 56, 58, 63).

It is indicated that chronic exercise training reduces inflammation in patients with chronic inflammatory diseases, whereas single bouts of exercise elicit a worsened inflammatory response, especially in the more severely affected patients (50). Although exercise-induced alterations in immune function are generally short-lived, changes may accumulate over time (60), making it crucial to identify the optimal 'exercise dose' since the main objective in treating patients with chronic inflammatory disease or cancer is either to suppress inflammation when it is elevated (50) or to boost the immune surveillance when it is compromised (17). Exercise seems to have the most beneficial effects on host defence and disease susceptibility or severity if the individual has a compromised immune function (63).

Potentially relevant immunological biomarkers are the number of neutrophils, Natural Killer (NK) cells, T lymphocytes, and/or their regulating cytokines. Also NK cytotoxic activity (NKCA) and the function of neutrophils, lymphocytes and monocytes represent innate and acquired immune components that play an important role in the defense against tumor cells. The aims of this review are to 1) evaluate the changes in immune parameters after acute and chronic exercise in cancer patients, 2) provide a systematic and comprehensive review of the existing literature examining exercise training and immune function in cancer survivors, and 3) offer a critical analysis of this literature and outline directions for future research.

Methods

Literature search

A systematic literature search was performed in Pubmed (Medline), Embase, Cochrane Library and CINAHL (until April 2011). Titles and abstracts were retrieved and screened by two independent reviewers (MKJ, DR). Additionally, the reference lists of all identified reviews were scanned manually. The search strategy used consisted of a combination of database-specific MeSH terms, free text, 'wild cards' (words truncated by using ''*'') and Boolean operators (''AND'', ''OR'', ''NOT''). The detailed search strategy was performed with the following words: Exercise, Motor activity, Sports, Immunity, Leukocytes, Natural killer cells, C-Reactive protein, Interleukins, Neutrophils, Lymphocytes, Monocytes, Eosinophils, Basophils, Macrophages, Neoplasms, Immunoproliferative disorders, Tumours, Malignancies.

Inclusion criteria of studies

- Study design: randomized and non-randomized controlled and uncontrolled interventions.

- Participants: cancer patients without any age restriction.
- Interventions: acute or chronic aerobic and/or resistance exercise.
- Outcome measures: all parameters of immune function (e.g. cytokines, mediating proteins, cell counts and functions).

Articles were excluded when multiple interventions were described, if they were case reports, or if they were written in a language other than English.

Definitions

In this review a distinction was made between acute and chronic exercise interventions. Acute exercise was defined as a single bout of exercise followed by assessment of immunological parameters. Chronic exercise was repeatedly performed in the form of an exercise training programme. Aerobic training exercise was defined as exercise that requires the heart and lungs to work harder to meet the increased demand of the body's oxygen needs. Examples include running, cycling and swimming. Other studies described resistance training that works to increase muscle strength and endurance by doing repetitive isometric, isotonic, or isokinetic exercises to strengthen or develop the muscles.

Studies were defined as being either randomized controlled trials (RCT), nonrandomized controlled trials (NCT), and other designs (OD), i.e. pre-post tests without controls.

Quality assessment

Two independent reviewers (MKJ, DR) assessed the methodological quality of the articles using a modified PEDro scale based on the scale utilized by Ploeger et al. (8, 50). Nine criteria were evaluated and scored as either yes, no, not applicable or unclear. Among these were: 1) Blinding of the assessor(s); 2) less than 15% drop-out during the study; 3) between group comparison; 4) proper description of the exercise protocol, defined as reporting of the frequency, intensity and duration of the exercise; 5) sufficient exercise training intensity, defined as a training program in which exercise was performed at ≥40% of the patient's VO_{2peak}, for at least two sessions per week, and at least 30 minutes per session, or at least 50% of their maximum voluntary contraction for resistance training exercise; 6) sufficient acute exercise intensity, defined as exercise at ≥40% of their VO_{2peak} for at least 10 minutes, or at least 50% of their maximum voluntary contraction; 7) reliable measurement methods, defined by proper description of methodology, and no microbead immunoassay to detect cytokines; 8) blood sampling was considered sufficient when samples were taken at least before and after exercise; 9) result reporting was sufficient when means and standard deviations were reported in the studies. Studies were considered to be of high quality if at least 75% of the criteria were scored positively.

Data analysis

Study and patient characteristics, and baseline, post-test, and follow-up data of outcome measures were extracted from the studies. Data analysis was performed separately for studies performed in the pediatric and adult populations, as well as for acute and chronic exercise interventions. A large degree of heterogeneity was expected with regard to patient population (e.g. disease characteristics, age cate-

gories, treatment severity), interventions (e.g. timing, duration, intensity, frequency), and outcome assessments (e.g. methods, timing, presentation of results), making it impossible to perform quantitative meta-analyses. As such, we have formulated a best-evidence synthesis – as proposed by Van Tulder et al. (62) – by assigning different levels of evidence to the effects of exercise on immune parameters, while taking into account the methodological quality and the statistical significance of the findings. Evidence was classified as being:

- Strong; defined as consistent, statistically significant findings in outcome measures in at least two high quality RCTs.
- Moderate; defined as statistically significant findings in outcome measures in at least one high quality RCT, and at least one low quality RCT, or high quality NCT.
- Limited; defined as statistically significant findings in outcome measures in at least one high quality RCT, or at least two high quality NCTs.
- Indicative; defined as statistically significant findings in at least one high quality NCT or low quality RCT, or at least two high quality ODs.
- No evidence; defined as study results that do not meet the criteria for one of the above-mentioned levels of evidence.

Outcome measures

Immune parameters were divided into four categories: leukocyte types, lymphocyte subsets, immune cells functions and the soluble inflammatory mediators (Table 1).

Table 1: Overview of immune parameters divided into four categories

1. Leukocyte types	
Leukocytes, lymphocytes, monocytes, granulocytes, neutrophils, basophils, eosinophils, dendritic cells	
2. Lymphocyte subsets	
CD 3^+ T lymphocytes, $CD4^+$ or $CD8^+$ T lymphocytes, $CD4^+/CD8^+$ ratio, $CD19^+$ or $CD20^+$ B lymphocytes, $CD25^+$ or $CD122^+$ T lymphocytes, $CD56^+$ NK, NK T lymphocytes	
3. Immune cell functions	
NK cytotoxic activity, neutrophil oxidative burst, lymphocyte proliferation or cytolytic activity, lymphocyte activation ($CD69^+$)	
4. Soluble inflammatory mediators	
Pro-inflammatory cytokines	Intercellular Adhesion Molecule 1, IL-1α, IL-1β, IL-6, sIL-6R, IL-8, Interferon-γ, Monocyte chemotactic protein-1, Macrophage Inflammatory Protein-1α, TNF-α, soluble glycoprotein 130 (IL-6 receptor)
Anti-inflammatory cytokines	IL-1ra, IL-1ra/IL-1β ratio, IL-1ra/IL-6 ratio, IL-1ra/TNF-α ratio, IL-4, IL-10, sTNF I/II, Transforming growth factor-β1
C-reactive protein	

CD = Cluster of differentiation; IL = Interleukin; NK = Natural Killer cells; R = Receptor; RA = Receptor antagonist; sIL = soluble interleukin; TNF = Tumor necrosis factor

Results

Retrieved articles and screening

In total, 3586 articles were identified during the systematic search. After screening of titles, abstracts and full texts, 21 relevant articles were included in this review (Figure1).

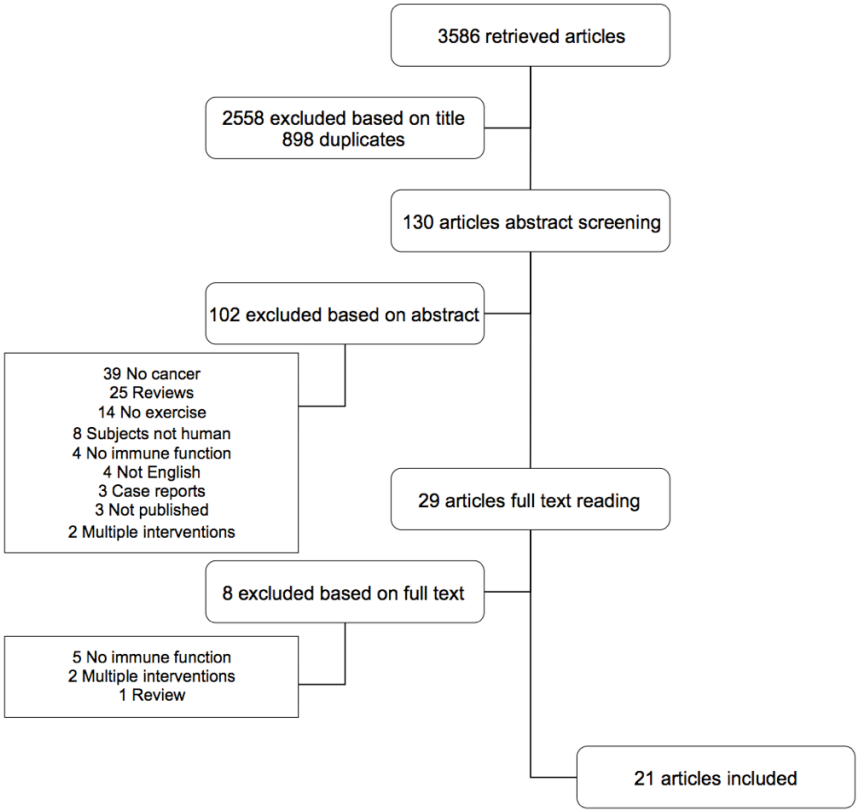

Figure 1: This flowchart shows the in- and exclusion of studies in this systematic review.

Characteristics of the included studies

A total of 10 RCTs, six NCTs and five ODs were identified. Three of the 21 included studies were focused on the pediatric population (9, 37, 57), and 18 on adult cancer patients (1, 4, 13, 15, 16, 19, 20, 25, 26, 30, 32, 35, 36, 44, 46, 48, 49, 59). Only three studies measured the effects of an acute bout of exercise (19, 32, 37), whereas the majority of studies reported the effects of chronic exercise. Only one study has applied resistance training (19), whereas aerobic training has been applied by 11 studies (1, 13, 15, 16, 30, 32, 35-37, 44, 48, 49), and a combination of resistance and aerobic training by eight studies (4, 9, 20, 25, 26, 46, 57, 59).

Table 2: Patient and disease characteristics of the included studies

Primary author	N	Sex	Cancer diagnoses	Disease stage	Age (Mean±SD, or Median;range)	Current treatment protocol	Timing of intervention
Acute exercise in children							
Ladha et al. 2006	E=4 C=6	M	E=Pre-B ALL C=Healthy	ns	E=11.3±5.3y C=10.8±4.6y	Antimetabolites, MTX, 6-MP	During maintenance treatment ALL
Acute exercise in adults							
Galvão et al. 2008[b]	E=10	M	E=PC	ns	?	ADT: LHRHa or CA treatment	More than 2 months after starting with ADT
Jönsson	E=6 C=5	M+F	E=CML C=Healthy	CCgR	E=59; 42-17y C=29; 22-44y	Imatinib	During Imatinib treatment
Chronic exercise in children							
Chamorro-Viña et al. 2010	E=7 C=13	M+F	E=RMS, B-ALL, T-ALL, AML, NB C=Historical	High Risk	E=8±4y C=7±3y	GVHD Prophylaxis: ACV, CsA, MTX, Conditioning therapy: T, F, DXM, BU alHSCT	During hospitalization for alHSCT, from conditioning phase until end of neutropenic phase (± 15 days post-HSCT)
Shore et al. 1999	E=6 C=11	M+F	E=ALL, other C=Historical healthy	ns	E completed (3)=14±0.6y E not completed (3)=13±3.1y C=?	ns	Max 4 weeks after completion of induction phase
Chronic exercise in adults							
Allgayer et al. 2004	E_1=13 E_2=10	M+F	RC, CC	UICC stage II or III	E_1=49, 36-60y E_2=60, 59-67y	No current treatment	At least 4 weeks after completion of primary therapy (e.g. surgery, CT, and/or RT)
Battaglini et al. 2009	E=10	M+F	AML	ns	E=35.7±8.9y	ns	Newly diagnosed or relapsed, receiving (re)induction therapy
Dimeo et al. 1997	E=33 C=37	M+F	E= AC, BC, GCC, MBC, NB, , NSCLC Sarc, SCLC C=Usual care	ECOG score 0-2	E=39±10y C=40±11y	1-4 CT-cycles: ETO, IFF, CIS, (EPI), G-CSF auPBST	Week after first high-dose CT and after auPBST, during hospitalization
Fairey et al. 2005/2005[a]	E=25 C=28	F	E=BC C=BC	I - IIIB	E=59±6y C=58±6y	46% TMX or ANA, 54% no current treatment	After surgery, radiotherapy and/or CT
Galvão et al. 2008[b]	E=10	M	E=PC	ns	?	ADT: LHRHa or CA treatment	More than 2 months after starting with ADT
Galvão et al. 2010	E=29 C=28	M	E=PC C=PC	ns	E=69.5±7.3y C=70.1±7.3y	ADT: LHRHa or CA treatment, 25% RT	More than 2 months after starting with ADT
Hayes et al. 2003	E=6 C_1=6 C_2=?	M+F	E= ALL, BC, NHL, RMS C_1=Matched AML, BC, MM, NHL C_2=Normative healthy	2 high risk stage II +1 stage IV BC	E=39.5y C_1=54.5y C_2=32±8y	CT-cycles, auPBST	During high-dose CT, after auPBST
Hutnick et al. 2005	E=28 C=21	F	E=BC C=BC	I–III (5 not staged)	E=48.5±10.6y C=52.3±9.2y	No current treatment	At least 2 weeks after

							completion of treatment (CT or RT)
Jones et al. 2009	E=12	M+F	E= NSCLC, SCLC, other	stage I-IIIA	E=67±8y	ns	Before primary surgery, until surgical resection
Kim et al. 2005/2006[a]	E=18 C=17	M+F	E=ALL, AML, SAA C=ALL, AML, SAA	ns	E=32.9±7.0y C=34.3±7.8y	alHSCT	During hospitalization with alHSCT
Na et al. 2000	E=17 C=18	M+F	E=SC C=Usual care	ns	E=57.8±12.1y C=52.2±10.3y	ns	From post-operative day 2, after curative surgery
Nieman et al. 1995	E=6 C=6	F	E=BC C=Usual care	ns	E=60.8±4.0y C=51.2±4.7y	No current treatment	After surgery, CT and/or RT (within 4 previous years)
Peters et al. 1994/1995[a]	E=24	F	E=BC	I-II	E=49.3±6.4y	No current treatment	More than 6 months after surgery
Sprod et al. 2010	E=19 C=19	M+F	E=BC, PC C=BC, PC	Mean Karnofsky status: 95.0 ± 8.6	E=56.6±13.7y C=63.3±9.4y	RT, 8% hormone therapy	After primary diagnosis, starting RT of at least 6 weeks

[a] = Patients described in two articles; [b] = Author has investigated both acute and chronic exercise effects E= Exercise group; E_1, E_2= Different exercise protocols; C= Control group; M= Males; F= Females; ns= Not specified

Diseases: AC= Adenocarcinoma; ALL= Acute lymphoblastic leukemia; AML= Acute myelogenous leukemia; B-ALL= B-cell ALL; BC= Breast cancer; CC= Colon carcinoma; CT= Colorectal tumor; ET= Esophagus tumor; GCC= Germ cell cancer; LC= Lung cancer; MBC= Metastatic breast cancer; NB= Neuroblastoma; NHL= Non-Hodgkin's lymphoma; NSCLC= Non-small cell lung carcinoma; PC= Prostate cancer; Pre-B ALL= Precursor B-cell ALL; PT= Pancreas tumor; RC= Rectal carcinoma; RMS= Rhabdomyosarcoma; SAA= Severe aplastic anaemia; Sarc= Sarcoma; SC= Stomach cancer; SCLC= Small cell lung carcinoma; T-ALL= T-cell ALL; VT= Ventriculum tumor

Treatment-related: ACV= Acyclovir; ADT= Androgen deprivation therapy; alHSCT= Allogeneic hematopoietic stem cell transplantation; ANA= Anastrozole; auPBST= Autologous peripheral blood stem cell transplantation; BU= Busulphan; CA= Cyproterone acetate; CCgR= Complete cytogenetic response; CIS= Cisplatinum; CsA= Cyclosporine A; CT= Chemotherapy; DXM= Dexamethasone; ECOG= Eastern Cooperative Oncology Group; EPI= Epirubicine; ETO= Etoposide; F= Fludarabine; GVHD= Graft-versus-Host Disease; IFF= Ifosfamide; LHRHa= Lutenizing hormone-releasing hormone agonist; 6-MP= 6-Mercaptopurine; MTX= Methotrexate; RT= Radiation therapy; T= Thiotepa; TMX= Tamoxifen; UICC= Union for International Cancer Control

Table 3: Description of exercise interventions in the included studies

Primary author	Design	Study duration	Frequency	Aerobic/Resistance exercise	Duration, sets, reps	Intensity
Acute exercise in children with cancer						
Ladha et al. 2006	NCT: Pre-ex Post-ex 1hr post-ex 2hr post-ex	-	-	Aer: intermittent run-walk on treadmill with warming-up and cooling-down	30 min: Aer: 5 min warm 5 min run, 10 min walk, 10 min run, 5 min cool	Run: 85% HR_{peak} Walk: 70% HR_{peak}
Acute exercise in adults with cancer						
Galvão et al. 2008[b]	Pretest-posttest Pre-ex Post-ex	-	-	Res1: hydraulic ex bout Res2: Isotonic ex bout	Res: 4 sets, 8 reps	Res: 6 RM
Jönsson et al. 2011	NCT 3mth pre-ex Pre-ex Post-ex 15min post-ex 45min post-ex 75min post-ex 120min post-ex 180min post-ex 240min post-ex 3mth post-ex	-	-	Aer: Maximal ex test on cycle ergometer (±60 RPM)	-	Aer: Until maximal exhaustion
Chronic exercise in children with cancer						
Chamorro-Viña et al. 2009	NCT Pre-tr 15d post-tr 30d post-tr and Post-ex	ns	Aer: 3x/wk Aer+Res: 2x/wk	In-hospital ex training program: Aer: Cycle ergometer Res: Each muscle group Stretch: Start and end	50 min: Aer: 25-30 min Res: 12-15 reps per ex	Aer: 50-70% HR_{peak}
Shore et al. 1999	NCT Pre-ex Post-ex 7d post-ex	12 wk	Aer: 3x/wk	Aer: Cycling, soccer, skating, skiing, swimming or combination Stretch: Before ex	Aer: 30 min Stretch: 2-3 min	Aer: 70-85% HR_{peak}
Chronic exercise in adults with cancer						
Allgayer et al. 2004	RCT Pre-ex Post-ex	2 wk	Aer: Daily	Maximal Aer ex test on cycle ergometer Mod.Aer group or Low Aer group	Aer: 40 min	Mod.Aer: 55-65% IAP Low Aer: 30-40% IAP
Battaglini et al. 2009	Pretest-posttest Pre-ex 3-4wk ex Post-ex	Hospitalization + 2 wk recovery	Aer+Res: 3-4x/wk 2x/d Recovery: 3x/wk	Baseline and post-ex: Cycle ergometer and squat and biceps curl ex In-hospital ex training: Stretch Aer: Cycle or walk Res: upper and lower body Recovery: Walk	30 min: Stretch: 3-5 min Aer: 5-10 min Res: 5-15 min + 5-10 min core ex Recovery: 10-30 min	Aer: 40-50% HRR Res: RPE 5
Dimeo et al. 1997	RCT Pre-ex Post-ex	Hospitalization	Aer: daily	Baseline and post-ex: treadmill stress-test Aer: Bed cycle ergometer	Aer: 30 min: 15 reps, 1 min, 1 min pause	Aer: 50% CR at 32±5W
Fairey et al. 2005/2005[a]	RCT Pre-ex Post-ex	15 wk	Aer: 3x/wk	Aer: Cycle ergometer with warming-up and cooling-down	Aer: 5 min warm, 15 min (+5 min each 3 wk → 35 min at end), 5 min cool	Aer: 70-75% VO_{2peak} Start + end: 50% VO_{2peak}
Galvão 2008[b]	Pretest-posttest Pre-ex Post-ex	Res1: 10 wk Res2: 10 wk	Res1/2: 2x/wk	Res1: hydraulic ex training Res2: isotonic ex training	Wk 1-2: 2 reps Wk 3-4: 3 reps Wk 5-7: 3 reps Wk 8-10: 4 reps	Wk 1-2: 12RM Wk 3-4: 10RM Wk 5-7: 8RM Wk 8-10: 6RM
Galvão et al. 2010	RCT Pre-ex Post-ex	12 wk	Aer+Res: 2x/wk	Aer: Progressive cycling and walking Res: Progressive upper and lower body ex	Aer: 15-20 min Res: 8 ex, 2-4 sets	Aer: 65-80% HR_{peak} and 11-13 RPE Res: 12-6RM
Hayes et al. 2003	NCT PI=pre-tr PII=post-tr, and pre-ex I1=1mth ex I2=2mth ex PIII=3mth post-tr and post-ex	12 wk	Aer: 3x/wk Res: 2x/wk Con: 3x/wk	Maximal graded ex treadmill test (PII) Aer: treadmill walk and cycle ergometer Res: machine and free weight Con: stretch major muscle groups	Aer: 20-40 min Res: 3-6 ex,15-20 reps (start) and 8-12 reps (end) until failure Con: 20-30 reps, 15-30 sec per stretch	Aer: 70-90% HR_{peak}
Hutnick et al. 2005	NCT T1=pre-tr T2=post-tr and pre-ex	24 wk	Aer+Res: 3x/wk	Aer+Res: First 12 wk: one-on-one sessions (N=28); Second 12 wk: with trainer (N=10); at home (N=6); quit training	40-90 min: Aer: 5 min warm + 10-20 min Res: 4 ex, 1-4 sets (start-end), 8-12	Aer: 60-75% FC

	T3=12wk ex T4=post-ex			(N=12) Aer: Warming-up + treadmill for outdoor running and walking Res: flexbands	reps	
Jones et al. 2009	Pretest-posttest Pre-ex Post-ex	4-6 wk	Aer: 5x/wk	Aer: cycle ergometry with warming-up and cooling-down Wk 1-3: Progressive cycling Wk 4-6: Progressive cycling and interval training	Aer: 20-30 min: Wk 1: 20-30 min Wk 2-3: 4 sessions 25-30 min and 5th session 20-25 min Wk 4-6: 3 sessions 30 min, 4th session 20-30 min, and 5th session 10-15 reps of 30 sec + 60 sec recovery 5 min warm and cool	Aer: Wk 1: 5x 60-65% VO_{2peak} Wk 2-3: 4x 60-65% VO_{2peak} + 1x VT Wk 4-6: 3x 60-65% VO_{2peak} + 1x VT + 1x VO_{2peak}
Kim et al. 2005/2006[a]	RCT Pre-ex Post-ex	6 wk	Aer: Daily	Relaxation breathing bed ex: preliminary ex, relaxation breathing, finish ex	30 min: preliminary ex 10 min, relaxation breathing 10 min, finish ex 10 min	-
Na et al. 2000	RCT 1d post-tr 7d post-tr, ex 14d post-tr and post-ex	2 wk	Bed: 3x/d Aer: 5x/wk 2x/d	Bed: 'Active range of motion' ex Aer: When ambulatory: arm and cycle ergometers	Bed: 30 min Aer: 30 min	Bed: Moderate Aer: 60% HR_{peak}
Nieman et al. 1995	RCT Pre-ex Post-ex	8 wk	Aer+Res: 3x/wk	Maximal ex test on treadmill, 6-min walking test, leg extension strength test Aer: Walking Res: Weights	Aer: 30 min Res: 7 ex, 2 sets, 12 reps	Aer: 75% HR_{peak} Res: Weight increasing
Peters et al. 1994/1995[a]	Pretest-posttest Pre-ex Post-ex 6mth post-ex	5 wk	Aer: 5x/wk Self-report: 2-3x/wk	Aer: Cycle ergometer during hospitalization Self-reported cycling after hospitalization	Aer: 30-40 min	Aer: 60% HR_{peak} Self-report: Moderate
Sprod et al. 2010	RCT Pre-ex Post-ex 3mth post-ex	4 wk	Aer+Res: daily	Aer: Walking Res: Upper body ex	Aer: daily increasing with 5-20% of steps Res: 11 ex, increasing towards 4 sets 15 reps	Aer: 3-5 RPE Res: Low-moderate

[a] = Patients described in two articles; [b] = Author has investigated both acute and chronic exercise effects; Aer= Aerobic exercise; Bed= Bed exercise; Con= Controls; CR= Cardiac reserve; D= Days; Ex= Exercise; FC= Functional capacity; HRR= Heart rate reserve; HR_{peak}= Peak Heart Rate; IAP= Individual aerobic power; Mth= Month; NCT= Nonrandomized Controlled Trial; RCT= Randomized Controlled Trial; Reps= Repetitions; Res= Resistance exercise; RM= Repetition maximum; RPE= Rating of perceived exertion; Tr= Treatment; VO_2= Oxygen uptake; VT= Ventilatory threshold; Wk= Week(s)

Table 2 depicts the patient and disease characteristics of the included studies, while Table 3 describes the exercise interventions of the studies.

Methodological quality

Full consensus was reached when assessing the methodological quality of the included studies (Table 4). In total, 15 studies were identified as having high methodological quality, and six studies were deemed to be of low methodological quality. Strengths of the studies were the adequate description of exercise protocols (100% scored positively), sufficient cell measurement techniques (100%), blood sampling time points (100%) and statistical result reporting (95%). Weaknesses of these studies were the lack of a blinded assessor (24%), and the high drop-out rates (43%). Fairey et al. (15, 16), Kim et al. (35, 36) and Peters et al. (48, 49) have published more than one article about their patient population. For these studies, the methodological quality and the populations were only described once.

Exercise in children

Three high methodological quality NCTs (9, 37, 57) were conducted in children. Each study included four to seven patients, and six to 13 historical (9, 57) or healthy controls (37). Tables 5 and 6 summarize the effects of acute and chronic exercise interventions, respectively.

Table 4: Quality assessment of the studies with a modified PEDro scale[a]

Included studies		Design[b]	Blinding of assessors	Drop-outs <15%	Between group comparison	Exercise protocol described	Exercise intensity sufficient	Measuring methods sufficient	Blood sampling sufficient	Result reporting sufficient	Methodological quality[c]
Acute exercise performed in children											
1	Ladha et al. (2006)	NCT	?	N	Y	Y	Y	Y	Y	Y	High (75%)
Chronic exercise performed in children											
2	Chamorro-Viña et al. (2010)(10)	NCT	Y	Y	Y	Y	Y	Y	Y	Y	High (100%)
3	Shore & Shephard (1999)	NCT	?	N	Y	Y	Y	Y	Y	Y	High (75%)
Acute exercise performed in adults											
4[d]	Galvão et al. (2008)	OD	?	?	N	Y	Y	Y	Y	Y	Low (67%)
5	Jönsson et al. (2011)	NCT	?	Y	Y	Y	Y	Y	Y	Y	High (88%)
Chronic exercise performed in adults											
6	Allgayer et al. (2004)	RCT	N	?	Y	Y	Y	Y	Y	N	Low (63%)
7	Battaglini et al. (2009)	OD	?	N	N	Y	Y	Y	Y	Y	Low (63%)
8	Dimeo et al. (1997)	RCT	Y	Y	Y	Y	Y	Y	Y	Y	High (100%)
9/10	Fairey et al. (2005/2005)*	2 RCT	Y	Y	Y	Y	N	Y	Y	Y	2 High (88%)
4[d]	Galvão et al. (2008)	OD	?	?	N	Y	Y	Y	Y	Y	Low (67%)
11	Galvão et al. (2010)	RCT	Y	Y	Y	Y	Y	Y	Y	Y	High (100%)
12	Hayes et al. (2003)	NCT	?	?	Y	Y	Y	Y	Y	Y	High (75%)
13	Hutnick et al. (2005)	NCT	?	N	Y	Y	Y	Y	Y	Y	High (75%)
14	Jones et al. (2009)	OD	?	N	N	Y	Y	Y	Y	Y	Low (63%)
15/16	Kim et al. (2005/2006)*	2 RCT	?	N	Y	Y	N	Y	Y	Y	2 Low (63%)
17	Na et al. (2000)	RCT	?	?	Y	Y	Y	Y	Y	Y	High (75%)
18	Nieman et al. (1995)	RCT	?	N	Y	Y	Y	Y	Y	Y	High (75%)
19/20	Peters et al. (1994/1995)*	2 OD	?	Y	N	Y	Y	Y	Y	Y	2 High (75%)
21	Sprod et al. (2010)	RCT	?	Y	Y	Y	N	Y	Y	Y	High (75%)

[a] Y = Yes; N = No; ? = Unclear; n/a = not applicable
[b] RCT = Randomized controlled trial; NCT = Nonrandomized controlled trial; OD = Other design
[c] Methodological quality high when ≥ 75% answered Yes
[d] = Galvão et al. (2008) have performed both acute and chronic exercise interventions
[e] = Same patient population

Leukocytes
All three studies in the pediatric population measured the effects of exercise on leukocytes. There is limited evidence that leukocyte numbers increase after acute (37) and chronic exercise (9), although one study reported decreases after chronic exercise (57), and there is limited evidence that the number of lymphocytes and monocytes remain constant (9, 37, 57). We found indicative evidence of an increase in the number of neutrophils (37) after an acute exercise bout, whereas the number of DC (9) and eosinophils (37) decreased after chronic and acute exercise, respectively. The number of basophils (37) and granulocytes (57) remained constant after acute and chronic exercise, respectively.

Lymphocyte subsets
Two studies (9, 57) focused on the lymphocyte subsets in children after chronic exercise. Based on these results, there is limited evidence that $CD8^+$ T lymphocyte and NK numbers remain stable after exercise (9, 57), whereas the number of $CD3^+$ T lymphocytes was reported to either increase (57) or decrease (9) with exercise. One study found that $CD4^+$ and NK T lymphocyte numbers decreased after hematopoietic stem cell transplantation and normalized after 30 days of chronic exercise (9), whereas another study found that $CD4^+$ lymphocyte numbers decreased after chronic exercise (57). Shore et al. reported that chronic exercise did not affect the $CD4^+/CD8^+$ ratio, $CD19^+$ B and $CD122^+$ T lymphocyte numbers, whereas $CD25^+$ T lymphocyte numbers decreased (57).

Cell functions
Two studies have measured immune cell function (37, 57). Acute exercise did not change the oxidative capacity of neutrophils (37), and the proliferative and cytolytic activity of lymphocytes (57) were found to be unaffected by chronic exercise.

Inflammatory mediators
No study has investigated changes in soluble inflammatory mediators in children with cancer.

Exercise in adults
In adults, 12 high quality studies (13, 15, 16, 20, 25, 26, 32, 44, 46, 48, 49, 59) and six low quality studies (1, 4, 19, 30, 35, 36) have been performed. These studies included six to 33 patients and five to 37 controls. Three were identified as being NCTs (25, 26, 32) and 10 were RCTs (1, 13, 15, 16, 20, 35, 36, 44, 46, 59) with either usual care controls (13, 15, 16, 20, 26, 35, 36, 44, 46, 59), healthy matched controls (25, 32), or a low intensity exercise control group (1). The remaining five studies were ODs without controls (4, 19, 30, 48, 49). Tables 4 and 5 summarize the effects of acute and chronic exercise interventions, respectively.

Leukocytes
Six high quality studies (25, 32, 46, 48, 49) and three low quality studies (19, 35, 36) have investigated leukocyte responses in adults. Based on these trials, there is moderate evidence that the number of granulocytes increases after acute (32) and

Table 5: Effects of acute exercise interventions on immune parameters in children and adults with cancer

Primary author	Immune parameters + units	Patients				Controls			
		Pre-bout	Post-bout	Follow-up1	Follow-up2	Pre-ex bout	Post-bout	Follow-up1	Follow-up2
Exercise interventions in children with cancer									
Ladha et al. 2006		**Pre**	**Post**	**1-hr post**	**2-hrs post**	**Pre**	**Post**	**1-hr post**	**2-hrs post**
$\times 10^9$/L	Basophils	0.05 (0.03)	0.08 (0.08)	0.04 (0.02)	0.05 (0.04)	0.07 (0.03)	0.13 (0.07)	0.09 (0.04)	0.11 (0.07)
$\times 10^9$/L	Eosinophils	0.23 (0.08)[d]	0.24 (0.09)	0.13 (0.04)[e]	0.15 (0.09)	0.37 (0.08)[d]	0.40 (0.10)[e]	0.33 (0.08)	0.32 (0.07)
$\times 10^9$/L	Leukocytes	4.70 (2.00)	6.10 (2.30)[e]	4.50 (1.40)[e]	5.00 (1.60)[e]	5.00 (1.50)	6.80 (1.70)[e]	5.70 (1.40)[e]	6.20 (1.80)[e]
$\times 10^9$/L	Lymphocytes	0.82 (0.15)[d]	1.20 (0.37)[e]	0.78 (0.18)[e]	0.89 (0.20)	2.09 (0.48)[d]	2.74 (0.42)[e]	2.09 (0.31)[e]	2.28 (0.14)
$\times 10^9$/L	Monocytes	0.30 (0.06)	0.46 (0.17)	0.32 (0.12)	0.39 (0.12)	0.37 (0.08)	0.54 (0.12)	0.45 (0.10)	0.46 (0.10)
$\times 10^9$/L	Neutrophils[b]	3.3	4.2[e]	3.1[e]	3.4[e]	2.1	3[e]	2.7[e]	2.9[e]
Ratio	Active neutrophils 5 min	33.8 (25.7)[d]	27.3 (18.8)[e]	24.4 (13.1)[e]	18.9 (20.1)[e]	6.0 (5.9)[d]	4.4 (3.9)[e]	7.4 (4.1)[e]	7.2 (4.6)[e]
Ratio	Active neutrophils 10 min	94.4 (81.1)	55.3 (38.9)	49.8 (33.9)	35.2 (30.7)	10.7 (11.0)	4.8 (3.4)	13.1 (8.1)	13.0 (11.6)
Ratio	Active neutrophils 15 min	82.6 (59.1)[d]	63.2 (47.6)[e]	54.8 (36.9)[e]	44.2 (29.4)[e f]	8.5 (9.1)[d]	4.7 (2.6)[e]	13.8 (6.2)[e]	14.6 (10.5)[e f]
RF	Neutrophil function[b]	14[d]	18[e]	20[e]	20[e]	45[d]	52[e]	39[e]	47[e]
Exercise interventions in adults with cancer									
Galvâo et al. 2008[a]		**Pre-bout1**	**Post-bout1**			**Pre-bout2**	**Post-bout2**		
$\times 10^9$/L	Leukocytes	6.7 (0.7)[d]	7.7 (0.9)[e]			6.0 (0.5)[d]	7.3 (0.7)[e]		
$\times 10^9$/L	Lymphocytes	2.0 (0.3)[d]	2.2 (0.3)			1.7 (0.2)	2.2 (0.3)[e]		
$\times 10^9$/L	Monocytes	0.65 (0.09)	0.71 (0.10)			0.56 (0.06)	0.72 (0.07)[e]		
$\times 10^9$/L	Neutrophils	4.0 (0.5)	4.5 (0.6)[e]			3.5 (0.4)	4.2 (0.5)[e]		
Pg/mL	Serum CRP	1.0 (0.55)	0.71 (0.25)			0.63 (0.25)	0.64 (0.24)		
Pg/mL	Serum IL-1ra	301.8 (41.8)	344.8 (47.8)			286.2 (41.6)	307.1 (39.1)		
Pg/mL	Serum IL-6	1.5 (0.2)	1.8 (0.2)			1.6 (0.5)	2.6 (0.5)[e]		
Pg/mL	Serum IL-8	7.6 (1.1)[d]	10.2 (1.9)			10.6 (1.7)[d]	11.4 (1.8)		
Pg/mL	Serum TNF-α	1.7 (0.2)	1.9 (0.4)			1.6 (0.2)	1.8 (0.2)[e]		
Jönsson et al. 2011		**Pre-bout**	**15 min**	**45 min**	**75 min**	**120 min**	**180 min**	**240 min**	
$\times 10^9$/L	Granulocytes Patients[b]	2.5	3.2[e]	2.5	3	3.4[e]	3.6	3.2	
$\times 10^9$/L	Controls[b]	2.5	3.5[e]	2.8	3.5	5.2[e]	5.2	5	
$\times 10^9$/L	Lymphocytes Patients[b]	1.2	2.6[e]	1.2	1	1	1.2	1.4	
$\times 10^9$/L	Controls[b]	2	4[e]	1.6	1.4	1.5	1.6	1.9	

[a] = Author has investigated both acute and chronic exercise effects; [b] = Estimated from graphs and figures; [d] Significant difference between patients and controls (at baseline); [e] Significant difference over time; [f] Significant difference between patients and controls;
Bout1= Hydraulic resistance exercise bout; Bout2= Isotonic resistance exercise bout; CRP = C-reactive Protein; Ex = Exercise; Hrs= Hours; IL = Interleukin; Ra = Receptor antagonist; RF = Relative fluorescence; TNF = Tumor Necrosis Factor

chronic exercise (49), although Fairey et al. did not report any changes after chronic exercise (15). There is also moderate evidence that the number of leukocytes remained stable (25, 46, 49), although some studies reported increases in leukocyte numbers after acute (19) and chronic exercise (19, 36). Similarly, there is moderate evidence that the number of lymphocytes remained stable after acute (19) and chronic exercise (35, 46); however, select studies reported increased numbers after acute (32) and chronic exercise (19, 25), while one study showed a decreased lymphocyte count after chronic exercise (49). There is limited evidence that the number of monocytes remained stable after acute (19) and chronic exercise (15, 19), with only one study reporting a decrease in monocytes (49). Similarly, there is limited evidence that the number of neutrophils remained stable after chronic exercise (19, 46), with one study reporting an increase in neutrophil counts after acute exercise (19), and the duration of neutropenia is reported to be shorter after chronic exercise (13).

Table 6: Effects of chronic exercise interventions on immune parameters in children and adults with cancer

Primary author	Immune parameters + units	Patients			Controls		
		Pre-train	Post-train	Follow-up	Pre-train	Post-train	Follow-up
Exercise interventions in children with cancer							
Chamorro-Viña et al. 2010		**Pre-HSCT**	**15d Post-HSCT**	**30d Post-HSCT**	**Pre-HSCT**	**15d Post-HSCT**	**30d Post-HSCT**
Cells/μL	Dendritic cells	5.4 (2.4)	2.0 (2.2)	2.6 (1.6)	10.6 (8.7)	1.4 (2.3)	1.7 (4.1)
$\times10^9$/L	Leukocytes	2.39 (2.52)	1.27 (1.85)	5.16 (4.08)	2.31 (2.40)	0.86 (1.16)	2.15 (2.85)
$\times10^9$/L	Lymphocytes	1.91 (2.48)	0.23 (0.31)	0.57 (0.28)	1.19 (0.82)	0.16 (0.22)	0.78 (8.60)
$\times10^9$/L	Monocytes	0.63 (0.19)	0.62 (0.31)	0.86 (0.60)	0.42 (0.31)	0.52 (0.51)	0.73 (1.10)
$\times10^9$/L	NK	0.20 (0.18)	0.22 (0.17)	0.37 (0.23)	0.20 (0.15)	0.10 (0.13)	0.33 (0.36)
Cells/μL	NK T lymphocytes	12.7 (17.3)	2.4 (3.2)	4.6 (6.6)	9.3 (11.0)	1.9 (2.7)	7.7 (16.1)
$\times10^9$/L	T lymphocytes	1.27 (1.59)	0.09 (0.12)	0.31 (0.21)	0.93 (0.70)	0.08 (0.10)	0.40 (0.33)
$\times10^9$/L	$CD4^+$	0.64 (0.89)	0.04 (0.05)	0.08 (0.03)	0.40 (0.37)	0.05 (0.07)	0.18 (0.17)
$\times10^9$/L	$CD8^+$	0.53 (0.65)	0.05 (0.07)	0.15 (0.16)	0.53 (0.55)	0.03 (0.04)	0.20 (0.18)
Shore et al. 2010		**Pre-train**	**Post-train**		**Pre-train**	**Post-train**	
$\times10^9$/L	Granulocytes	2.0	1.17 (0.09)		3.0 (0.2)	2.5 (0.2)	
$\times10^9$/L	Leukocytes	2.7 (0.6)[d]	2.4 (0.7)[f]		5.9 (0.5)[d]	5.0 (0.4)[f]	
$\times10^9$/L	Lymphocytes	1.06 (0.43)[d]	0.6 (0.13)		2.6 (0.2)[d]	2.1 (0.2)	
$\times10^9$/L	Monocytes	0.21	0.14 (0.05)		0.33 (0.06)	0.42 (0.06)	
$\times10^9$/L	$CD3^+$	0.51 (0.17)[d]	0.28 (0.07)[f]		1.8 (0.2)[d]	1.4 (0.2)[f]	
$\times10^9$/L	$CD4^+$	0.31 (0.12)[d]	0.13 (0.03)		0.90 (0.01)[d]	0.95 (0.30)	
$\times10^9$/L	$CD8^+$	0.27 (0.07)[d]	0.20 (0.19)		0.85 (0.10)[d]	0.73 (0.10)	
Ratio	$CD4^+/CD8^+$	1.1 (0.2)	0.9 (0.3)		1.3 (0.1)	1.4 (0.1)	
$\times10^9$/L	$CD19^+$	0.01 (0.0)	0.07 (0.06)		0.23 (0.03)	0.25 (0.05)	
$\times10^9$/L	$CD56^+$	0.13 (0.08)	0.10 (0.06)		0.29 (0.03)	0.27 (0.03)	
$\times10^9$/L	$CD25^+$	0.08 (0.03)[d]	0.02 (0.00)[f]		0.15 (0.02)[d]	0.09 (0.02)[f]	
$\times10^9$/L	$CD122^+$	0.08 (0.06)	0.10 (0.08)		0.22 (0.04)	0.17 (0.04)	
$\times10^9$/min	LP (PHA-induced)	12.2 (5.1)[d]	6.2 (2.2)		32.0 (4.7)[d]	35.6 (5.1)	
$\times10^9$/min	LP (PWM-induced)	6.8 (2.4)	3.8 (2.5)		10.9 (1.8)	11.1 (2.6)	
Units/10^6	Cytolytic activity (spontaneous)	2.2 (1.5)	5.1 (3.7)		8.1 (3.4)	4.6 (1.6)	
Units/10^6	Cytolytic activity (IL-2 induced)	3.9 (3.2)	5.0 (2.4)		10.6 (3.7)	6.9 (1.7)	
Exercise interventions in adults with cancer							
Allgayer et al. 2004		**Pre-ME D**	**1 wk**	**2 wk**	**Pre-LE D**	**1 wk**	**2 wk**
Ng/mL	WB IL-1β (LPS)	6.94 (0.82–18.52)	NS/UC	NS/UC	2.23 (1.32–9.81)	NS/UC	NS/UC
Ng/mL	WB IL-1ra (LPS)	28.60 (1.60–30.03)	18.03 (5.04-52.57)[e]	22.89 (6.38-34.73)[e]	27.22 (20.88–29.07)	NS/UC	24[c]
Ratio	WB IL-1ra/IL-1β (LPS)	4.1 (0.09-37.0)	NS/UC	3.7 (0.84-10.3)[e]	9.3 (3.5-21.0)	NS/UC	NS/UC
Ratio	WB IL-1ra/IL-6 (LPS)	2.51 (0.59-6.50)	NS/UC	1.41 (0.29-2.60)[e]	3.3 (3.2-4.3)	NS/UC	NS/UC
Ratio	WB IL-1ra/TNF-α (LPS)	NS/UC	NS/UC	NS/UC	NS/UC	NS/UC	NS/UC
Ng/mL	WB IL-6 (LPS)	12.64 (5.80–30.79)[d]	NS/UC	NS/UC	8.34 (4.83–8.66)[d]	NS/UC	NS/UC
Ng/mL	WB sTNFRI (LPS)	1.30 (0.82–2.51)	NS/UC	NS/UC	1.35 (1.24–1.43)	NS/UC	NS/UC
Ng/mL	WB sTNFRII (LPS)	3.69 (2.42–7.17)	NS/UC	NS/UC	3.28 (3.27–4.17)	NS/UC	NS/UC
Ng/mL	WB TNF-α (LPS)	4.55 (1.94–32.79)	NS/UC	NS/UC	3.64 (2.62–8.06)	NS/UC	NS/UC
Battaglini et al. 2009		**Pre-ex**	**Mid-Ex**	**Post-Ex**			
Pg/mL	WB IFN-γ[c]	33	35	37			
Pg/mL	WB IL-6[c]	58	46	18			
Pg/mL	WB IL-10[c]	28	66	91			
Dimeo et al. 1997							
Days	Duration neutropenia	6.6 (1.5)[f]			7.6 (1.6)[f]		
Fairey et al. 2005[a]		**Pre-train**	**Post-train**		**Pre-train**	**Post-train**	
Mg/L	Serum CRP	5.19 (3.56)	3.79 (2.30)		4.28 (3.05)	4.39 (3.87)	
Fairey et al. 2005[a]		**Pre-train**	**Post-train**		**Pre-train**	**Post-train**	
	Granulocytes	NS/ND	NS/ND		NS/ND	NS/ND	
	$CD3^+$	NS/ND	NS/ND		NS/ND	NS/ND	
	$CD4^+$	NS/ND	NS/ND		NS/ND	NS/ND	
	$CD8^+$	NS/ND	NS/ND		NS/ND	NS/ND	

	$CD14^+$	NS/ND	NS/ND		NS/ND	NS/ND	
	$CD20^+$	NS/ND	NS/ND		NS/ND	NS/ND	
	$CD25^+$	NS/ND	NS/ND		NS/ND	NS/ND	
	$CD56^+$	NS/ND	NS/ND		NS/ND	NS/ND	
RF	Neutrophil function	NS/ND	NS/ND		NS/ND	NS/ND	
%	NKCA (3.125:1 E/T)	7.2 (5.1)	12.4 (6.6)[f]		5.8 (4.5)	5.7 (4.2)[f]	
$/10^3$ cells	Total LU	11.98 (6.76)	8.60 (3.40)[f]		12.72 (8.19)	11.68 (6.00)[f]	
$\times10^9$/min	Spontaneous LP	863 (425)	1042 (290)[f]		776 (417)	811 (247)[f]	
$\times10^9$/min	PHA-induced LP	90098 (49890)	79500 (32218)		91279 (54302)	69487 (31540)	
	PBMC-produced IL-1α	NS/ND	NS/ND		NS/ND	NS/ND	
	PBMC-produced IL-4	NS/ND	NS/ND		NS/ND	NS/ND	
	PBMC-produced IL-6	NS/ND	NS/ND		NS/ND	NS/ND	
	PBMC-produced IL-10	NS/ND	NS/ND		NS/ND	NS/ND	
	PBMC-produced TGF-β1	NS/ND	NS/ND		NS/ND	NS/ND	
	PBMC-produced TNF-α	NS/ND	NS/ND		NS/ND	NS/ND	
Galvão et al. 2008[b]		**Pre-ex**	**10 wk**	**20 wk (post-ex)**			
$\times10^9$/L	Leukocytes	6.4 (0.7)	6.7 (0.7)[e]	6.0 (0.5)[e]			
$\times10^9$/L	Lymphocytes	1.7 (0.2)	2.0 (0.3)[e]	1.7 (0.2)			
$\times10^9$/L	Monocytes	0.56 (0.73)	0.65 (0.09)	0.56 (0.06)			
$\times10^9$/L	Neutrophils	3.9 (0.5)	4.0 (0.5)	3.5 (0.4)			
Pg/mL	Serum CRP	0.91 (0.31)	1.0 (0.55)	0.63 (0.25)			
Pg/mL	Serum IL-1ra	286.5 (39.2)	301.8 (41.8)	286.2 (41.6)			
Pg/mL	Serum IL-6	1.8 (0.3)	1.5 (0.2)	1.6 (0.5)			
Pg/mL	Serum IL-8	8.2 (0.8)	7.6 (1.1)[e]	10.6 (1.7)[e]			
Pg/mL	Serum TNF-α	1.8 (0.2)	1.7 (0.2)	1.6 (0.2)			
Galvão et al. 2010		**Pre-train**	**Post-train**		**Pre-train**	**Post-train**	
Mg/L	Serum CRP	2.7 (3.2)	1.8 (1.1)[f]		2.3 (2.6)	4.5 (6.9)[f]	
Hayes et al. 2003		**PI (pre-Tr)**	**PII (post-Tr)**	**I1 (1mth ex)**	**I2 (2mth ex)**	**PIII (3 mth ex)**	**Norm values**
$\times10^9$/L	Leukocytes	5.46 (0.90)	3.47 (0.78)	5.99 (0.57)	4.70 (0.68)	5.43 (0.47)	5.91 (0.03)
$\times10^9$/L	Lymphocytes	1.14 (0.29)	0.38 (0.14)	1.72 (0.43)[e]	1.49 (0.39)[e]	1.17 (0.17)[e]	2.13 (0.09)
$\times10^9$/L	$CD3^+$	0.65 (0.16)	0.32 (0.15)	1.31 (0.39)[e]	1.10 (0.36)[e]	0.68 (0.16)	1.43 (0.11)
$\times10^9$/L	$CD4^+$	0.37 (0.10)	0.16 (0.08)	0.22 (0.07)[e]	0.24 (0.08)	0.16 (0.03)[e]	0.80 (0.03)
$\times10^9$/L	$CD8^+$	0.28 (0.09)	0.15 (0.07)	1.08 (0.32)[e]	0.87 (0.28)[e]	0.52 (0.15)	0.71 (0.03)
Ratio	$CD4^+/CD8^+$	1.88 (0.54)	1.13 (0.32)	0.29 (0.06)[e]	0.32 (0.03)[e]	0.47 (0.09)[e]	1.21 (0.03)
Ratio	Prolif. Index[c]	1.25	1.0	1.2	1.25	1.1	2.0
Ratio/%	Prolif. Index/$CD3^+$ [c]	2.25	1.35	2.0	2.0	2.8[e]	3.25
Hutnick et al. 2005		**Post-Tr (T2)**	**T3 (3mth)**	**T4 (6mth)**	**Post-Tr (T2)**	**T3 (3mth)**	**T4 (6mth)**
	B lymphocytes	NS/ND	NS/ND	NS/ND	NS/ND	NS/ND	NS/ND
	NK	NS/ND	NS/ND	NS/ND	NS/ND	NS/ND	NS/ND
	$CD3^+$	NS/ND	NS/ND	NS/ND	NS/ND	NS/ND	NS/ND
	$CD4^+$	NS/ND	NS/ND	NS/ND	NS/ND	NS/ND	NS/ND
%	$CD4^+CD69^+$ activation	1.40 (4.00)	0.37 (0.29)	0.51 (0.46)[f]	0.63 (0.82)	0.41 (0.61)[e]	0.34 (0.61)[e f]
	$CD8^+$	NS/ND	NS/ND	NS/ND	NS/ND	NS/ND	NS/ND
CPM	LP 25mg/mL Con-A	14128 (10437)	16352 (16079)	17445 (9587)[f]	10289 (8038)	12771 (14661)	9669 (6274)[f]
CPM	LP 50mg/mL PHA	34600 (27277)	39285 (30853)	39321 (21207)[f]	23694 (17630)	28770 (23537)	26444 (18296)[f]
CPM	LP 5mg/mL PWM	4345 (3077)	5213 (3872)	6754 (5426)[f]	5501 (10184)	4581 (4097)	4192 (2741)[f]
Ng/mL	Lymphocyte-produced IFN-γ (PHA)	24.5 (48.2)	6.6 (11.0)	6.0 (11.0)	15.4 (18.6)	19.3 (44.3)	13.4 (16.6)
Ratio	Lymphocyte-produced IFN-γ/ IL-6 (PHA)	0.2 (0.6)	7.8 (35.5)	0.2 (0.7)	0.3 (0.4)	1.2 (3.6)	0.5 (0.5)
Pg/mL	Lymphocyte-produced IL-6 (PHA)	339.8 (1060.9)	72.5 (114.4)	49.8 (71.3)	72.8 (179.7)	53.8 (87.3)	886.2 (2918.4)
Ng/mL	Plasma IFN-γ	215.4 (490.5)	202.7 (481.6)	279.2 (546.2)	133.2 (236.1)	331.1 (561.6)	239.6 (596.8)
Pg/mL	Plasma IL-6	384.4 (1103.8)	495.2 (1948.8)	690.9 (2333.5)	204.4 (321.6)	176.1 (312.9)	227.9 (388.5)
Ng/mL	Plasma sIL6-R	22.2 (10.7)	26.8 (15.5)	23.9 (20.5)	23.5 (8.0)	21.6 (6.2)	18.3 (12.4)
Ng/mL	Plasma sgp-130	234.5 (118.9)	280.4 (91.5)	309.8 (93.5)	276.0 (74.7)	264.9 (89.3)	282.8 (93.8)
Pg/mL	Plasma BAIL-6	14.7 (34.6)	87.4 (361.4)	133.4 (461.0)	15.3 (28.2)	9.5 (14.6)	18.4 (29.3)
Ratio	Plasma IFN-γ/IL-6	1.31 (0.85)[f]	1.19 (1.09)	1.59 (1.69)	0.70 (0.45)[f]	1.29 (0.90)	3.74 (9.69)

Jones et al. 2009		**Pre-train**	**Post-train**				
Mg/L	Plasma CRP	8.2 (9.3)	6.9 (9.0)				
Ng/mL	Plasma ICAM-1	132.2 (34.9)	120.6 (30.7)[e]				
Pg/mL	Plasma IL-6	6.5 (5.7)	6.3 (4.2)				
Pg/mL	Plasma IL-8	22.9 (22.3)	16.5 (12.7)				
Pg/mL	Plasma MCP-1	214.5 (52.2)	205.2 (36.5)				
Pg/mL	Plasma MIP-1α	35.6 (3.9)	34.4 (3.9)				
Pg/mL	Plasma TNF-α	3.7 (4.8)	4.3 (6.0)				
Kim et al. 2005[a]		**Pre-train**	**Post-train**		**Pre-train**	**Post-train**	
$\times 10^9$/L	Leukocytes	3.92 (1.37)	7.16 (4.18)[e]		4.35 (2.00)	4.64 (2.34)	
Kim et al. 2006[a]		**Pre-train**	**Post-train**		**Pre-train**	**Post-train**	
$\times 10^9$/L	Lymphocytes	1.05 (0.45)	1.09 (0.64)		1.42 (0.62)	0.78 (0.61)[e]	
%	$CD3^+$	50.6 (18.0)	45.0 (29.6)		49.6 (21.3)	47.2 (28.1)	
%	$CD4^+$	20.9 (13.3)	8.9 (8.9)[e]		20.5 (12.9)	8.0 (5.6)[e]	
Ratio	$CD4^+/CD8^+$	0.9 (0.6)	0.6 (1.2)		0.8 (0.4)	0.3 (0.2)	
%	$CD8^+$	24.7 (9.4)	33.0 (25.1)[e]		29.3 (13.2)	40.4 (22.6)[e]	
Na et al. 2000		**Post-Tr d1**	**Post-Tr d7**	**Post-Tr d14**	**Post-Tr d1**	**Post-Tr d7**	**Post-Tr d14**
%	NKCA 50:1 E/T	16.2 (11.4)	14.6	27.9[f]	19.7 (19.6)	17.9	13.3[f]
Nieman et al. 1995		**Pre-train**	**Post-train**		**Pre-train**	**Post-train**	
$\times 10^9$/L	Leukocytes	5.7 (0.3)	4.9 (0.4)		5.9 (0.9)	6.1 (0.9)	
$\times 10^9$/L	Lymphocytes	1.4 (0.2)	1.1 (0.2)		1.4 (0.2)	1.6 (0.3)	
$\times 10^9$/L	Neutrophils	3.7 (0.3)	3.0 (0.4)		3.8 (0.7)	3.9 (0.8)	
$\times 10^9$/L	NK cells	0.3 (0.1)	0.3 (0.1)		0.2 (0.1)	0.2 (0.1)	
$\times 10^9$/L	T lymphocytes	0.9 (0.1)	0.9 (0.1)		1.0 (0.2)	1.2 (0.2)	
%	NKCA 40:1 E/T	39.7 (6.3)	44.3 (4.6)		24.6 (2.6)	38.0 (3.3)	
%	NKCA 20:1 E/T	28.9 (5.8)	41.3 (4.8)		16.0 (2.0)	30.7 (3.4)	
Peters et al. 1994[a]		**Start**	**5 wk**	**7mth**			
$\times 10^9$/L	NK	0.14 (0.12)	0.16 (0.10)	0.16 (0.07)			
%	NK	10.4 (8.7)	12.6 (11.3)	12.9 (5.2)			
%	NKCA 25:1 E/T	18.5 (15.1)	22.4 (18.2)	28.3 (16.0)[e]			
Peters et al. 1995[a]		**Start**	**5 wk**	**7mth**			
$\times 10^9$/L	Granulocytes	3.24 (1.56)	3.21 (1.33)	3.44 (1.60)			
%	Granulocytes	61.3 (7.9)	60.0 (9.7)[e]	65.4 (6.8)[e]			
$\times 10^9$/L	Leukocytes	5.25 (2.08)	5.28 (1.78)	5.19 (2.09)			
$\times 10^9$/L	Lymphocytes	1.49 (0.69)	1.60 (0.73)[e]	1.33 (0.53)[e]			
%	Lymphocytes	28.8 (8.2)	30.6 (9.9)[e]	26.7 (6.6)[e]			
$\times 10^9$/L	Monocytes	0.37 (0.16)	0.35 (0.12)	0.31 (0.17)			
%	Monocytes	7.2 (2.2)	6.8 (1.6)[e]	6.1 (2.2)[e]			
%	PC sheep erythro	44.9 (18.4)	58.3 (18.6)[e]	67.0 (17.5)[e]			
Ratio	PI sheep erythro	1.71 (0.3)	1.91 (0.4)[e]	2.19 (0.4)[e]			
%	PC human erythro	76.6 (8.8)	76.6 (10.3)	78.8 (9.2)			
Ratio	PI human erythro	1.98 (0.3)	1.92 (0.3)	2.02 (0.3)			
Sprod et al. 2010		**Pre-train**	**Post-train**		**Pre-train**	**Post-train**	
Pg/mL	Plasma IL-6	5.74 (0.83–48.10)	6.33 (0.61–24.05)		6.28 (0.08–12.47)	9.26 (1.79–16.74)	
Pg/mL	Serum IL-6	1.08 (0.06–2.97)	1.38 (0.29–6.41)		3.60 (0.00–8.81)	3.75 (0.00–7.76)	
Pg/mL	Serum sTNF-R	760.62 (448.64–1476.21)	680.52 (361.68–1319.53)		766.30 (598.72–933.87)	783.98 (600.99–966.97)	
Pg/mL	Serum TNF-α	0.57 (0.00–4.18)	2.82 (0.00–35.99)		9.43 (0.00–28.84)	9.58 (0.00–29.18)	

[a] = Patients described in two articles; [b] = Author has investigated both acute and chronic exercise effects; [c] = Estimated from graphs and figures; [d] Significant difference between patients and controls (at baseline); [e] Significant difference over time; [f] Significant difference between patients and controls;

CD = Cluster of differentiation; Con-A= Concanavalin A; CPM= Counts per minute; CRP= C-reactive Protein; D= Day(s); E/T= Effector/Target cell ratio; HSCT= Hematopoietic stem cell transplantation; ICAM= Intercellular Adhesion Molecule; IFN= Interferon; IL= Interleukin; LED= Light-intensity exercise; LP= Lymphocyte proliferation; LPS= Lipopolysaccharide stimulation; LU= Lytic Units represent the number of effector cells required to cause 30% lysis of target cells; MCP= Monocyte chemotactic protein; MED= Moderate-intensity exercise; MIP= Macrophage Inflammatory Protein; Mth= Month(s); ND= No difference between groups; NK(CA)= Natural Killer cell cytotoxic activity; NS= Not shown; PBMC= Peripheral Blood Mononuclear Cells; PC= Phagocytosis; PHA= Phytohemagglutin; PI= Phagocytosis Index; Prolif. Index= Proliferation Index; PWM= Pokeweed mitogen; R= Receptor; RF= Relative fluorescence; sgp= soluble glycoprotein; (s)TNF= (soluble) Tumor Necrosis Factor; TGF= Transforming growth factor; Tr= Treatment; UC= Unchanged over time; WB= Whole blood; Wk= Week(s)

Lymphocyte subsets

Five high quality (15, 25, 26, 46, 48) and one low quality study (35) have examined lymphocyte subsets. Strong evidence was found that chronic exercise did not alter $CD3^+$ T lymphocyte (15, 25, 26, 35, 46) and $CD56^+$ NK cell numbers (15, 26, 46, 48). Furthermore, there is moderate evidence that $CD4^+$ T (15, 25, 26),

$CD8^+$ T (15, 25, 26), and $CD20^+$ B lymphocyte numbers (15, 26) are also not altered by chronic exercise, although one study reported that the number of $CD4^+$ T lymphocytes decreased and the number of $CD8^+$ T lymphocytes increased (35). The $CD4^+/CD8^+$ ratio was reported to be constant in one study (35), and decreased in another study (25). We also found that the number of $CD25^+$ T lymphocytes was stable after chronic exercise, but the level of evidence was limited (15).

Cell functions
Seven high quality studies (15, 25, 26, 44, 46, 48, 49) have investigated the function of immune cells. There is strong evidence that NKCA increases as a result of chronic exercise (15, 44, 46, 48), and the total number of lytic units required to cause 30% lysis of target cells decreased (15). There is also moderate evidence that the proliferation of lymphocytes increased due to exercise (15, 26), although one study did not see any changes after chronic exercise (25). Furthermore, there is limited evidence that exercise did not alter neutrophil oxidative burst (15). One high quality NCT analysed the activation marker CD69 on T lymphocytes, and found that the number of $CD4^+$ $CD69^+$ cells did not change after exercise, although the percentage of $CD4^+$ cells expressing CD69 was higher in the exercising group, combined with a significant decrease in the controls (26). Lastly, one study found increased phagocytic activity in monocytes after chronic exercise (49).

Inflammatory mediators
Six high (15, 16, 19, 20, 26, 59) and three low quality studies (1, 4, 30) have examined soluble inflammatory mediators in the adult population. Strong evidence is available that both pro-inflammatory (1, 4, 15, 19, 26, 30, 59) and anti-inflammatory cytokines (1, 4, 15, 19, 30) are unaltered. There is also strong evidence that CRP remains unaltered after acute (19) and chronic exercise (16, 19, 30).

Discussion

The primary aim of this review was to summarize the changes in specific immune parameters after acute and chronic exercise in pediatric and adult cancer patients and survivors. Exercise showed either an increase or no change in the majority of examined immune parameters. For example, the number of leukocytes, lymphocytes and neutrophils increased after acute bouts of exercise in both children and adults. Cytokines, however, did not show a shift towards either a pro- or anti-inflammatory profile in adults. The amount of $CD4^+$ seemingly decreases with exercise, while $CD8^+$ T lymphocytes increase, ultimately resulting in no visible shift in the $CD4^+/CD8^+$ ratio.

We found strong evidence to support an increase in the cytotoxicity of NK cells, and that the number of NK cells and T lymphocytes remained stable, along with the cytokine profile, after exercise. These findings are in line with results from studies in well-trained subjects (34), and asthma patients (40). However, a study in healthy individuals showed an increase after acute bouts of exercise due to higher NK counts (60). These discrepancies across studies may be related to the

different types of exercise performed by participants. More specifically, the increased number of NK cells and NK cytotoxicity seen in healthy participants were reported after acute exercise (53, 55, 61), while the studies in cancer patients have only examined these cells after chronic exercise interventions. Unfortunately, some studies have not reported the exact timing of blood sampling after the exercise session, making it difficult to separate immediate versus longer-term effects of exercise on immune function.

The exact mechanism underlying the effect of exercise on the immune function in cancer patients requires further study. It has been suggested that exercise has anti-inflammatory effects, and therefore, in the long term, regular physical activity can protect against the development of chronic inflammation-associated diseases (22, 33). Exercise may exert its anti-inflammatory effects through systemic mechanisms (such as reduced body fat and a reduced inflammatory status (14, 47) or site-specific mechanisms (release of anti-inflammatory cytokines from contracting muscles, catecholamines, and the inhibition of pro-inflammatory cytokine production). Hereby, a clear distinction has to be made between the role of chronic low-grade inflammation (2, 23, 27), and low numbers of immune components in the increased cancer risk (29). A shift towards an anti-inflammatory state may be favorable for cancer prevention, but may also result in a hampered response of immune cells in the tumor microenvironment. Moreover, the boundaries are still unknown between the beneficial anti-inflammatory effects and the beneficial immune boosting effects of exercise. Immune cells in the tumor microenvironment respond to different signals, such as cytokines, causing them to either display tumor-promoting or tumor-suppressing phenotypes. These cytokines are responsive to acute or chronic bouts of exercise, and may represent an "immune" signature for exercise-induced immunomodulation in the cancer microenvironment. In other words, the balance of these cytokines may indirectly reflect changes in the immune cell phenotype in the tumor microenvironment (24).

Previous studies have also reported that the effects of exercise might be dependent on the intensity, duration and type of exercise (22). We could not confirm this due to the large variety of intervention programmes. We have identified studies that have applied multiple types of exercise interventions, ranging from two to 24 weeks of duration, consisting of different intensities of exercise, and resistance, aerobic as well as combined interventions. Although it is easier to explain the beneficial effects of longer exercise duration, no clear conclusions could be drawn from our findings regarding resistance or aerobic exercise. Previous studies have reported that aerobic exercise had a stronger effect on energy balance than resistance training (3), although the latter might have a stronger effect on bone mineral density (12), and the combination of the two positively influences cardiovascular fitness, body composition, and body fat (38, 39). Future studies could apply a cross-over design in which patients receive both aerobic and resistance training separately in order to compare the effects of these training types on biomarkers and clinical outcomes.

This review highlights the current knowledge of exercise immunology in cancer patients, although a few limitations need to be considered. It is important to

acknowledge that first, only three studies were performed in children, and 18 studies in adult patients. Second, the studies examined consisted primarily of small and heterogeneous samples. Third, there was a large variability in exercise training protocols between studies, making comparison and pooling of data very difficult. Furthermore, it remains unclear whether circulating immune cells and inflammatory mediators reflect the levels at the local microenvironment. Finally, six out of 21 studies were classified as low quality studies due to high drop-out rates, and the lack of assessor blinding. Despite these limitations, this review is strengthened by the systematic and thorough search in various large databases, as well as the independent quality assessment by two reviewers. The study of immunological effects of exercise in humans is a growing area of research (22). We believe that an overview of the studies in exercise immunology in cancer patients is helpful for clinicians and researchers, especially since the last review in this area was carried out a decade ago (17), and 16 additional studies have been published since the time of this original review.

Recommendations for future studies

Based on the currently available literature, future studies should involve more homogeneous populations, preferably including a control group. The interpretation of exercise effects can be improved by detailed reporting of baseline physical fitness characteristics, as well as reports of adherence to the intervention, subject behaviour before blood collection (i.e. medications, dietary intake, and smoking status), the exact timing of blood collection, and the naturally occurring fluctuations in immune parameters. It is also important to monitor and report physical fitness after chronic exercise training.

To gain more insight into exercise-induced alterations in immune function, mediating mechanisms should be examined, outcomes should be measured at more time points during and following exercise, and they should be related to cancer outcomes (i.e. toxicity of treatments, risk of recurrence, and onset of late effects). Ultimately, the aim of research in this area should be to establish the mode, intensity and duration of exercise required to optimize the anti-inflammatory effects in cancer patients (22). Moving into an epoch focused on the practice of personalized cancer care, the study of exercise immunology might help with the development of a personalized training protocol for each cancer patient in order to diminish the side effects of the cancer treatment and to reduce the added risk of cancer recurrence. Our systematic review has focussed on the effects of a wide variety of exercise interventions on inflammation and immune function, but future studies could also look at the (long-term) clinical outcomes in these patients.

Conclusion

Many of the health benefits of regular exercise are thought to be related to its short-term boost of the immune system and long-term anti-inflammatory effects. In this systematic review, we found that Natural Killer cytotoxic activity increased after exercise in cancer patients, along with lymphocyte proliferation and granulocyte cell counts. The number of leukocytes, lymphocytes, Natural Killer cells, T lymphocytes, C-reactive protein, and pro- and anti-inflammatory mediators remained stable in response to exercise. Additional research is needed to gain insight into the mechanism linking exercise and immune function in different populations, as well as to better understand the association between these immune parameters and clinical outcomes.

Acknowledgments

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Spinal cord injury: Known and possible influences on the immune response to exercise

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ABSTRACT

A spinal cord injury (SCI) can increase the risk of infection by impacting on many aspects of immune function; one particularly well-documented observation is a reduction in lymphocyte numbers. The vast majority of lymphoid cells express adrenergic receptors. Therefore, autonomic function loss and concomitant alterations in resting and post-exercise catecholamine concentrations, particularly so in individuals with a tetraplegia, may impact directly on immune cells and depress immunity. Other factors are further likely to contribute, examples including altered muscular, endocrine and cardiovascular function following SCI. However, some alterations, such as increases in natural killer cell cytotoxicity following exercise in those with a tetraplegia, are unrelated to the catecholamine response. Likewise, mucosal immunity in individuals with a tetraplegia appears to be similarly influenced by exercise as in the able-bodied population. Indeed, rehabilitation therapy and exercise can increase some measures of immunity and autonomic function in those with an SCI. It is therefore possible that compensatory mechanisms offset disability-related detriments. This may be by way of sympathetic reflex activity, receptor hypersensitivity, or parasympathetic and neuroendocrine adjustments. Future work needs to explore these mechanisms further to clarify the implications of an SCI on the immune response to exercise and susceptibility to infection.

In this article, we review the impacts of an SCI on immune, and specifically, exercise immune function. The relevant anatomical and physiological foundations of the immune system are first briefly laid out in order to understand the potential impacts of neural and neuroendocrine dysfunction on the immune system. With the limited number of human studies available, we have then aimed specifically to gather all relevant existing literature on exercise immunology in individuals with an SCI in patient, recreationally active and athlete populations. We believe that an understanding of the impacts of exercise can provide a tool to help maintain or improve health in individuals with an SCI.

A comprehensive literature search was conducted using the search engines PubMed, SPORTDiscus, Web of Science and Zetoc, search period June 2012 – February 2013. Key words employed included spinal cord injury, immunology, exercise, paraplegic, tetraplegic, upper body exercise, interleukin, immunoglobulin, sympathetic, and parasympathetic. All articles and articles derived from their reference lists were checked for their suitability.

Key words: Catecholamines, cytokines, natural killer cells, autonomic nervous system, mucosal immunity

1 INTRODUCTION

An SCI increases the risk of infection, and complications from infection are among the leading causes of re-hospitalization and death in the post-acute phase

following SCI (12, 76). Specifically, pneumonia, influenza, or other respiratory complications accounted for the majority of deaths in a large scale study conducted on 886 individuals with SCI between 1943 and 1990 (22). Heightened infection and illness susceptibility are acknowledged sequelae of acute, subacute and chronic SCI that challenge the activity, satisfaction, productivity, and health of its survivors (59). Apart from the deleterious consequences for the suffering individual, there are economic consequences and strain on health care providers.

Therapeutic exercise for individuals with SCI is actively encouraged and interest in wheelchair sports is increasing, particularly with the legacy of the London 2012 Paralympic Games. The advancements in wheelchair design (1), combined with greater funding opportunities and sports professionalism have resulted in a greater number of wheelchair athletes performing on recreational (36) and professional levels (31); likewise, the quality of the sports performance has improved. This is supported by analysis of objective markers of physical performance by both sports scientists and coaching support staff, which, when investigating peak oxygen uptake as an example, has increased around two-fold within 30 years (32, 38).

The able-bodied literature reports a higher prevalence of symptoms of respiratory illness in athletic than non-athletic populations, with a marked number of these infectious in nature (16, 77), which underlines the practical importance to analyse immune function in athletes with an SCI. Understanding the influence of exercise on immune functions is potentially critical for the management of infections in individuals with SCI, given the substantial impacts of exercise on immune functions (87). Furthermore, from a mechanistic point of view, SCI provides exercise immunologists with an ideal *in vivo* model with which to investigate the influences of the autonomic nervous system on the immune response to exercise.

2 PHYSIOLOGICAL BACKGROUND: COMMUNICATION BETWEEN THE IMMUNE SYSTEM AND THE BRAIN

2.1 Role of the autonomic nervous system

The central nervous system receives messages from the immune system and, vice versa, messages from the brain modulate immune functions (21). Some of these unconscious actions are modulated by the autonomic nervous system. Moynihan *et al.* (57) and Elenkov *et al.* (21) summarize three lines of evidence supporting sympathetic nervous system (SNS) involvement in immune regulation:

1. Lymphoid organs are innervated by sympathetic noradrenergic nerve fibres.
2. The vast majority of lymphoid cells express adrenergic receptors.
3. Noradrenaline, an important neurotransmitter of sympathetic nerves, is released in lymphoid organs following immunization.

The rapid, "real-time" brain control of innate immune mechanisms underlying inflammation is thought to be based on autonomic neuronal projections to sites of inflammation (65). Evidence accumulated in the last decades indicates that,

peripherally, both noradrenaline released from the non-synaptic sympathetic nerve terminals and adrenaline (and to a lesser extent, noradrenaline) released from the adrenal medulla are involved in immunomodulation (21), resulting in both activation and suppression of immune parameters (Table 1).

Table 1: Evidence of catecholamine action on immune cells.

References	**Findings**
Kappel *et al.*, 1991; Tonnesen *et al.*, 1987	Natural killer cell activity is increased following both infusion of adrenaline and exercise, which naturally increases catecholamine concentration.
Keller *et al.*, 1983	Stress-induced lymphopenia is adrenal-dependent: adrenaline correlates inversely with lymphocyte number, as shown in animal stress experiments (stressor: electrical shocks).
Landmann *et al.*, 1984	Even moderate physical stress (15 min, increasing up to 75% of maximum power output) results in an increase of adrenaline, which correlates negatively with the $T_{helper}/T_{suppressor}$ cell ratio. These authors suggest that adrenaline plays a role in the mobilisation of immunocompetent cells and may lead to a distribution pattern favouring immunosuppression during stress.
Nash, 1994	Adrenaline administration causes a transitory leuko- and moncytosis. Sympathectomy can lead to decreases in natural killer cell cytotoxicity, and reduce the $T_{cytolytic}/T_{suppressor}$ cell ratio and B cell numbers.
McHale & Thornbury, 1990	Sympathetic nerve stimulation increases lymphocyte output in anaesthetised sheep.

In addition to the sympathetic strand of the autonomic nervous system, efferent fibres of the vagus nerves are involved in immune responses. For example, they inhibit the release of pro-inflammatory cytokines and regulate inflammation, coining the term cholinergic anti-inflammatory pathway (65). Experimentally, peripheral vagus nerve stimulation in vagotomized rats prevents the development of acute inflammation (6). The importance of intact innervation on immune control is also evident in humans, as denervated skin has a greatly reduced leukocyte infiltration following local damage, which is associated with a ~70% reduction in the rate of wound healing compared with normal skin (20). In chronic SCI, it has further been suggested that those with low fibronectin levels may present impaired wound healing, whilst lower zinc levels have been found in those presenting pressure ulcers (17).

Salivary glands are involved in the defence of the mucosa, and salivary secretory immunoglobulin A (SIgA) is the predominant immunoglobulin in saliva. It plays an important role in mucosal immunity and has therefore been described as “first line of defence” against pathogens and antigens presented at the mucosa, such as cold-causing viruses (4, 87). In analogy to immune cells as described above, salivary glands are innervated by autonomic nerve fibres. These originate from the upper thoracic segments, although it remains unclear precisely where in this region (69). In rats, sympathectomy results in a decreased SIgA secretion (70). Conversely, both parasympathetic and sympathetic stimulation of rat salivary glands can increase salivary gland blood flow, saliva flow rate, and SIgA secretion (13, 14, 69). Similarly, infusion of sympathetic and parasympathetic agonists can increase SIgA secretion rate (71).

The autonomic nervous system is not only involved in the execution of immune responses, but has also a sensory component. Afferent vagus nerve fibres rapidly signal the brain to trigger immunomodulatory responses in the early phases of inflammation (65). Peripheral sensory nerves are further part of reflex pathways to contribute to proinflammatory function, which includes vasodilation and mast cell activation (20). These reflex pathways consist of sensory receptors, afferent pathways, integration centers in the central nervous system, efferent pathways, and effector organs (29). Reflex pathways may also be activated by causes other than injury or inflammation; for example, they are involved during static muscle contraction, which increases adrenal sympathetic nerve activity in rats (86).

2.2 Role of humoral factors

In addition to the neural pathways, humoral factors are involved in the communication between the brain and the immune system. One pathway that has been explored extensively is the hypothalamus-pituitary-adrenal (HPA) axis, where adrenocorticotropic hormone (ACTH) released from the pituitary stimulates glucocorticoid secretion in the adrenal gland (30, 65). One of the most prominent glucocorticoids is cortisol, with generally immunosuppressive and anti-inflammatory effects (21). Importantly, humoral feedback mechanisms can inform the brain on immunologic actions in the body, and hence, modify its behaviour. For example, cytokines and other soluble factors secreted in response to infection or inflammation (such as the tumour necrosis factor, TNF-α), produced by immune cells, do not only attract and modulate other immune cells locally, but can act on sensory neurons or the brain directly. In the context of this review, it is important to note that not only immune cells can trigger a cascade of immune actions, also cytokines released by working muscle (myokines) in response to exercise are capable of triggering an immunologic response (67).

3 SPINAL CORD INJURY: IMPACTS ON AUTONOMIC FUNCTION

As outlined above, an important route of communication between the brain and the immune system is via autonomic pathways descending the spinal cord. Given that sympathetic neurons exit the spinal cord at the thoracic (T) and high lumbar (L) level (T1-L2) (29, 49), a complete SCI at the T level (resulting in paraplegia) partly interrupts sympathetic pathways, while a complete cervical SCI (resulting in tetraplegia) completely abolishes sympathetic communication between brain and effector cells/organs (Fig. 1). Autopsy findings in patients with cervical SCI show a marked loss of axons in the dorsal aspects of the lateral funiculus, which is thought to be the location of the descending vasomotor pathways (29). Only injuries below L1 have minimal effects of SNS dysregulation (29). Hence, sympathetically governed function is impaired in individuals with a tetraplegia (TETRA), and one obvious observation in a sporting context includes the reduced maximum heart rate, which is in the range of around 130 beats per minute in these individuals (3). Spinal segments T2 to T4 supply sweat glands of the head and neck, T2 to T8 of the upper limbs, T6 to T10 of the trunk, and T11 to L2 of the lower extremities (29), explaining the reduced ability to sweat in TETRA. Fur-

ther, basal systolic and diastolic blood pressure in TETRA is about 15 mm Hg lower than that in able-bodied subjects (29). This accompanies the loss of motor and sensory control below the level of lesion. These adaptations lead to reductions in peak oxygen uptake in TETRA, even though the fittest individuals with a complete tetraplegia still reach scores of over 30 $mL \cdot kg^{-1} \cdot min^{-1}$ (51).

There is also evidence for an altered HPA axis function in SCI. Animal experiments show that an SCI acutely activates the HPA axis in mice, resulting in elevated circulating cortisol levels, even though cortisol returns to baseline values by 3 days post injury (54). It has been suggested that the acute trauma-induced activation of the HPA axis and the SNS (resulting in increased noradrenaline) axis helps prevent pathological autoimmune reactions, or prevents hyperactivation of

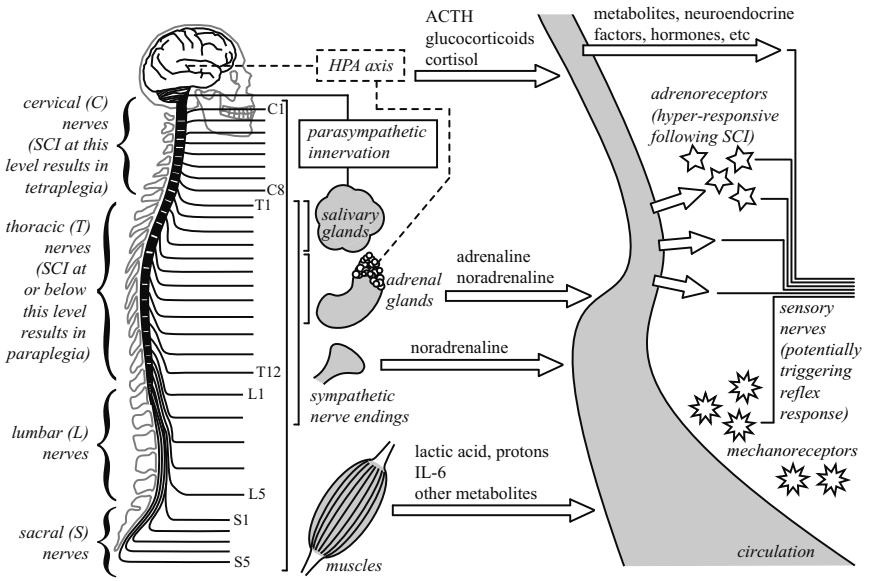

Figure 1: Anatomical and physiological basis for immunomodulatory processes potentially influenced by a spinal cord injury and/or exercise. For glands, sympathetic nerve endings and muscles, the origin of their nerves is shown. ACTH, adrenocorticotropic hormone; HPA, hypothalamus pituary adrenal; IL-6, immunoglobulin-6; SCI, spinal cord injury.

the immune system (55). In chronic SCI, a number of studies in humans document an altered pattern of markers of the HPA axis (8, 37, 48, 89), discussed in detail in section 4.1.

4 SPINAL CORD INJURY: IMMUNE FUNCTION AND THE IMPACTS OF EXERCISE

The section above shows that individuals with an SCI present altered autonomic control due to a dysfunctional SNS and, potentially, an altered HPA axis, two sys-

tems which are known to regulate immune responses. It is therefore legitimate to assume that this is one of the reasons for the increased infection risk in SCI and has led researchers to measure markers of immune function, the autonomic nervous system and/or the HPA axis in a range of contexts. Immune responses have initially been investigated in resting conditions in individuals with an SCI. However, the amount of research on exercise immune function has grown steadily in recent years, not least as the association between resting immune dysfunction and low levels of fitness in persons without SCI (60) implies a negative influence of a sedentary lifestyle. Consequently, exercise has been suggested already two decades ago to be one intervention to reverse the negative immune alterations in TETRA (11).

4.1 Cortisol

4.1.1 Resting responses

Even though adaptations after an SCI include an increase in cortisol immediately after injury, urinary free cortisol concentration falls in normal range after 6 months post injury (17, 46). Kliesch et al. (46) further found a positive association between ACTH and cortisol in TETRA and in individuals with a paraplegia (PARA), indicating the normal function of the HPA axis despite an SCI. This work confirms the endocrine nature of ACTH action on cortisol secretion without the need of "hard-wired" neuronal mechanisms. Other researchers found a normal circadian rhythm of plasma cortisol concentration in TETRA, which further supports the concept of normal HPA axis function in this population (90).

However, there is evidence of a disturbed HPA axis following SCI, as indicated by elevated resting plasma cortisol levels in TETRA and PARA when compared with able-bodied controls, despite no differences in plasma ACTH (8). An impaired cortisol response to a corticotropin-releasing hormone (CRH) bolus in TETRA and PARA has further been observed (37). It must be noted, though, that this external CRH administration may cause a different response when compared with physiological changes of influencing metabolites, as, for example, found following exercise. The researchers of this project indeed point out that non-CRH or non-ACTH-dependent pathways may exist and compensate for their suggested HPA axis dysfunction in patients with an SCI (37).

4.1.2 Exercise responses

The plasma cortisol concentration in athletic TETRA is increased following strenuous exercise, such as simulated race conditions (88). Some studies suggest a minimal exercise duration required for cortisol concentration to change. A full marathon (26) increases plasma cortisol concentration in PARA, whereas a half-marathon does not alter plasma cortisol levels in either TETRA and PARA (2, 27). However, this contrasts other reports where increases in cortisol have been observed after exercise durations as little as 20 minutes (15, 48). The similar rise in plasma cortisol following exercise between TETRA and able-bodied controls (15, 48) further support the concept that cortisol is mainly governed by the HPA axis, with no or only little contribution of the SNS. It has also been suggested that the myokine interleukin-6 (IL-6) may give rise to increased cortisol production

(30), as documented by plasma cortisol rises following IL-6 infusion (62, 79). It has been proposed that this may be by way of secretion modulation via a neuroendocrine-immune loop involving the HPA axis (68). However, IL-6 does not seem to be the main modulator of cortisol: Brief acute exercise increases plasma cortisol in TETRA, PARA and non-spinal cord injured individuals, yet IL-6 levels only increase in PARA and non-spinal cord injured individuals, but not in TETRA (64).

Recently, it has been suggested that one of the benefits of exercise is the creation of an anti-inflammatory environment (30). As exercise gives rise to a range of anti-inflammatory cytokines, it can reduce the "reactivity" of immune cells, for example by downregulating TOLL-like receptor expression on immune cells. Exercise can also reduce fat mass, itself a producer of inflammatory agents (30). Cortisol, with its potent anti-inflammatory effects, has been shown to remain elevated for longer following exercise in TETRA when compared with able-bodied controls (48, 89) thus creating an anti-inflammatory environment for a longer duration. Again, this may be because of secretion modulation via a neuroendocrine-immune loop involving the HPA axis (68), which may be altered in TETRA.

4.2 Catecholamines

Sympathetic neurons exit the spinal cord between T1 and L2 (29, 49), and the majority of sympathetic neurons innervating the adrenal medulla originate from T5-T9 (29). Unsurprisingly, due to the abolition of neural pathways to the adrenal gland and dysfunction of sympathetic pathways, catecholamine release is therefore affected in individuals with a high-level SCI.

Resting plasma catecholamine concentrations are lower in TETRA when compared with able-bodied individuals and controls with a low paraplegia, and the exercise-induced increase is smaller in TETRA (47, 73, 74) or not present at all (48, 89). Plasma adrenaline and noradrenaline remain unchanged in TETRA following simulated racing conditions (88) and graded exercise tests to exhaustion (23, 64, 88), and plasma adrenaline following a half marathon does not increase in TETRA, whereas it does increase in individuals with T4-L1 lesions (2). Individuals with a lesion in the T1-T6 area present a reduced catecholamine response to a graded exercise test to exhaustion when compared to those with a T7-T12 lesion (81). Serum noradrenaline is significantly elevated after exercise in both these groups, whereas adrenaline is only elevated in the T7-T12 group (81), underpinning the physiological relevance of intact adrenal gland innervation.

It is worth noting that in contrast to volitional exercise, exercise-induced increases in adrenaline and noradrenaline have been observed in both TETRA and PARA when electrically stimulating paralysed muscles, even though the relative increase to resting levels was lower than in able-bodied individuals (5). Spinal reflexes have been thought earlier to be the potential candidate for these catecholamine responses (5, 74). It therefore appears that despite the central abolition of neural pathways, ways remain to exert responses normally centrally governed by the SNS. Exploring methods to exert catecholamine responses in those with an impaired SNS could hence be a promising way to modulate and improve immune

function in this population. Catecholamines are known to exhibit both immunodepressive and -stimulating characteristics (Table 1). However, Nash (60) suggests that periods of autonomic dysreflexia in SCI with the concomitant catecholamine boost and spikes in circulating glucocorticoids may be primarily immunodepressive, creating a "window of opportunity" in which opportunistic infections ensue. It has been suggested that suppressed immune function following autonomic dysreflexia (due to lymphocyte apoptosis) may explain why individuals with high level SCI are at high risk for recurrent infections throughout their lifetime (55). Indeed, a number of articles compiled by Nash (60) show the relationship between repeated catecholamine overstimulation and depression of immune function. Nash (60) further points out that adrenergic stress in experimental animals suppresses lymphocyte mitogen proliferation and natural killer cell (NK) and phagocytic activity and diminishes interferon producing capacity, a pattern of immune irregularities nearly matching that of humans with SCI.

Table 2: Adaptations of cellular immunity following an SCI and effect of level of injury

References	Findings
Campagnolo *et al.*, 2000; Cruse *et al.*, 2000; Kliesch *et al.*, 1996	PARA below the T10 level exhibit normal NK cell cytotoxicity. It has hence been suggested that the effects of adrenal hormones and neurotransmitters on NK cell function represent critical targets for future investigation (17). Likewise, neutrophil phagocytic function is decreased in TETRA but not in PARA below the T10 level (9).
Campagnolo *et al.*, 1994; Campagnolo *et al.*, 2000	Despite depressed NK cell levels in SCI, no difference in leucocyte counts, but increases in T and T_{helper} cells in SCI.
Campagnolo *et al.*, 1994	Depressed lymphocyte proliferative response in TETRA when compared to AB controls.
Held *et al.*, 2010	Increased susceptibility to a virus load and reduced macrophage activation and virus-specific T cells that control virus replication in animal SCI models.
Ibarra *et al.*, 2007	The T cell response to mitogen and antibody titre to antigen is reduced in animals with a chronic SCI (both at the T1 and T12 level). The immunosuppressive effect on both T cell and antibody reactions is stronger and lasts longer in the case of high (T1 level) and severe contusions, which gives rise to a higher risk to develop infectious diseases.
Iversen et al., 2000	Reduced immunoglobulin G levels in TETRA and PARA when compared to AB controls.

AB, able-bodied; NK, natural killer cell; PARA, individuals with a paraplegia; SCI, spinal cord injury; T, thoracic; TETRA, individuals with a tetraplegia.

4.3 Leukocytes

4.3.1 Resting responses

Animal experiments show that an SCI at the T3 level acutely reduces the antigen-specific immunoglobulin following vaccination, and reduces spleen weight, dendritic, B, and T cell numbers (54). In humans, a reduced NK count has been

reported in both TETRA (89) and PARA (60, 84). It has been proposed that the NK number depression in TETRA is due to a production problem in normally sympathetically innervated bone marrow (10). NK cytotoxicity can also show reductions in SCI, especially in high level SCI (above T6, affecting autonomic innervation), with NK cytotoxicity in TETRA being about 40-60% of normal (9-11, 40, 46, 61). Further dysfunction in SCI has been reported for a variety of other resting immune measures (Table 2).

4.3.2 Exercise responses

It seems that exercise does not destroy NKs; rather, they are temporarily relocated to reservoir sites such as the walls of peripheral veins in response to the exercise-induced secretion of catecholamines (87) - the concomitant downmodulation of adhesion molecules releases them into the circulation (58). Since individuals with a high level SCI have an impaired sympatheticoadrenal activity, with lower catecholamine concentrations measured at rest and following exercise, these studies support the concept that catecholamines are responsible for recruitment of leukocytes to the circulation at rest and during exercise. Depressed leukocyte number elevation has also been demonstrated in persons without disability who exercise under beta-adrenergic blockade, underpinning this suggestion (78).

NK number and cytotoxicity and other aspects of immunity are influenced by acute exercise of various intensities and durations, and numerous studies have shown a depressed response in TETRA (Table 3). However, it has been shown early that rehabilitation therapy, including strength, endurance and mobility training, improves NK cytotoxicity in patient TETRA and PARA, whereas NK cytotoxicity stays at a low level in those not receiving therapy (46). Interestingly, increases in NK cytotoxicity in TETRA are unrelated to changes in NK number (2, 47, 89). Mechanisms other than catecholamine activation must be considered as responsible, as for example, NK cytotoxicity in both PARA and TETRA is elevated after a half-marathon despite no increases in adrenaline (2). It is hence possible that the altered, elevated cortisol response in TETRA (48, 89) modulates leukocyte function following exercise.

4.4 Cytokines

Appreciable numbers of individuals with SCI have abnormally high levels of pro-inflammatory cytokines whether or not they are symptomatic for infection. It is not surprising that these pro-inflammatory markers are further elevated in those with medical complications, such as urinary tract infection or pressure ulcers (18). Specifically, elevated plasma IL-6 concentrations and elevated soluble IL-2 receptor concentrations have been reported (60). Importantly, plasma IL-6 concentrations are related to reduced pulmonary function in SCI (28) and therefore may have some predictive power of health measures.

However, the manner of the acute cytokine response to strenuous exercise appears again to be lesion level dependent. Plasma IL-6 concentrations have been shown to increase in response to 20 minutes of upper body exercise (48) or a test to exhaustion (64) in PARA and AB, whereas it remains unaffected in TETRA (48, 64). It must be noted that IL-6 production is dependent on the duration of exer-

Table 3: Acute exercise effects on leukocyte number and activity in individuals with a spinal cord injury. Reported changes compare pre exercise with immediately after exercise values.

References	N	Impairment	Activity level	Exercise intervention	Immune measure	Change in immune measure
Banno *et al.*, 2012	6	TETRA (C6-C8)	recreational athletes	half marathon	NK cell number and cytotoxicity	number: no change (TETRA), 1.7-fold increase (PARA); cytotoxicity: 1.3-fold increase in both groups
	7	PARA (T5-L4)	recreational athletes		lymphocyte number	no change
					leukocyte number	1.8-fold increase in both groups
Furusawa *et al.*, 2003	7	PARA (T7-L1)	recreational athletes	half marathon	NK cell number and cytotoxicity	number: no change, cytotoxicity: 1.2-fold increase
					lymphocyte number	no change
					leukocyte number	2-fold increase
Furusawa *et al.*, 1998	9	PARA (T5-T12)	elite athletes	marathon	NK cell number and cytotoxicity	number: 2.4-fold decrease, cytotoxicity: 1.1-fold decrease
					lymphocyte number	no change
					leukocyte number	2.5-fold increase
Kawashima *et al.*, 2004	10	PARA (T5-T12)	patients	20 min orthotic gait exercise	NK cell number and cytotoxicity	number: no change, cytotoxicity: 1.4-fold increase
Klokker *et al.*, 1998	6	TETRA (C5-C7)	participating in on-going training programme	30 min functional electrical stimulation (maximal tolerable load for this duration)	NK cell number and cytotoxicity	number: no change (TETRA), 2-fold increase (PARA); cytotoxicity: increase in both groups (extent not stated)
	5	PARA (T4-T7)			lymphocyte number	increase in both groups (extent not stated)
					leukocyte number	increase in both groups (extent not stated)
Kouda *et al.*, 2011	8	TETRA (C6-C7)	regularly active	20 min arm cranking at 60% $\dot{V}O_{2max}$	lymphocyte number	no change (TETRA), 1.3-fold increase (AB)
					leukocyte number	no change (TETRA), 1.5-fold increase (AB)
	8	AB	not stated		prostaglandin E_2	1.5-fold increase (TETRA), no change (AB)

Nash, 1994	8	TETRA (no details given)	not stated	functional electrical stimulation elevating $\dot{V}O_2$ five-fold (duration not stated)	NK cell number and cytotoxicity lymphocyte and leukocyte numbers	number: 1.8-fold increase; cytotoxicity: 2.5-fold increase no change
Ueta *et al.*, 2008	7	PARA (T11-L4)	regularly active	120 min arm cranking at 60% $\dot{V}O_{2max}$	NK cell number and cytotoxicity	number: no change in both groups; cytotoxicity: 1.1-fold decrease (PARA), 1.2-fold increase (AB)
	6	AB	not stated		lymphocyte number leukocyte number prostaglandin E_2	1.9-fold increase (PARA), no change (AB) 1.6-fold increase in both groups 6-fold change (PARA), no change (AB)
Yamanaka *et al.*, 2010	8	TETRA (C6-C7)	regularly active	20 min arm cranking at 60% $\dot{V}O_{2max}$	NK cell number and cytotoxicity	number: no change (TETRA), 1.8-fold increase (AB); cytotoxicity: no change (TETRA), 1.4-fold increase (AB)
	6	AB	not stated		lymphocyte number leukocyte number	no change (TETRA), 1.4-fold increase (AB) no change (TETRA), 1.3-fold increase (AB)

AB, able-bodied; C, cervical; L, lumbar; NK, natural killer cell; PARA, individuals with a paraplegia; T, thoracic; TETRA, individuals with a tetraplegia.

cise, with longer lasting activities resulting in more pronounced IL-6 level increases (30). Further, the IL-6 response is sensitive to the exercise intensity, directly representing the muscle mass involved, which decreases the higher the SCI lesion level. Upper body exercise per se (and even more pronounced when performed by persons with high level SCI), may therefore be insufficient to increase IL-6 above resting levels (68). Additionally, the before mentioned dysfunction of autonomic innervation could impact on IL-6 appearance in the blood, as catecholamines have been shown to influence cytokine production (65). Indeed, infusion of catecholamines can partly explain some of the rise in plasma concentrations of IL-6 found following exercise (24, 80), and administration of adrenergic antagonists can block adrenaline-stimulated IL-6 expression (24). This supports our finding that the plasma IL-6 response to strenuous exercise is associated with that of plasma adrenaline; responses of both are impaired in TETRA, but not in PARA or AB (64). This is an important issue as several immune responses are mediated by cytokines. For example, IL-6 infusion (amounts corresponding to levels of strenuous exercise) results in acute increases in neutrophils, cortisol, IL-10 and IL-1 receptor antagonist in healthy able-bodied individuals (79). Taken together, the combination of autonomic dysfunction impacting on cytokine production and less active, cytokine producing, muscle mass are the most likely reasons for the depressed production of IL-6 in TETRA. Given the messenger actions of IL-6, depressed levels may then impact on the production of other

cytokines/messenger molecules or alter immune cell number and function directly. Furthermore, an impaired IL-6 response to exercise may have downstream influences on metabolic responses, given the known glucoregulatory functions of IL-6 (68).

4.5 Salivary markers and upper respiratory symptoms

Mucosal immune function and its modulation by exercise have only recently been investigated in the SCI population. SIgA has been analysed in a number of studies to document the impact of chronic (53) and acute (51, 52) exercise in athletes with SCI. It was found that whilst slight differences in the SIgA response to exercise between TETRA and the control groups (PARA and able-bodied) exist (52), the overall pattern of the SIgA response is comparable between these groups. It was therefore suggested that the impact of sympathetic dysfunction on SIgA secretion in TETRA may be compensated by mechanisms such as reflex activity, by the parasympathetic nervous system, or by hypersensitivity of receptors (51).

A reduced SIgA secretion rate during periods of heavy training (53) is consistent with the positive relationship between post-race self-reported upper respiratory tract infections and training volume in athletes with an SCI (25). Epidemiological data from a large scale (N=18,693) study reveal that despite upper respiratory tract infections being the most prevalent acute respiratory condition in SCI, the annual outpatient visit rate is only 68/1000 – surprisingly, a lower rate when compared with the general population (155/1000) (75). Another epidemiological study investigating both TETRA and PARA reports a higher importance of pulmonary function, history of illness and smoking for chest illness than level or completeness of injury (82). In the case of pulmonary function, this may suggest a parameter that is trainable and that training may therefore decrease the risk of illness – indeed, very low incidences of upper respiratory symptoms were reported in wheelchair rugby athletes with tetraplegia during an observational study, with only 3 out of 14 athletes presenting light symptoms over 5 months (53).

5 IMPACTS OF MUSCULAR, VASCULAR, AND PSYCHOLOGICAL CHANGES AFTER SPINAL CORD INJURY

Apart from SNS and HPA dysfunction, it should be acknowledged that other disability-related factors may impact on immune function and further contribute to heightened illness susceptibility. For example, the colony-forming potential of progenitor cells from the bone marrow is reduced in SCI, which may be explained by inactivity characterising these individuals, possibly impairing blood flow through decentralized bone marrow (40). However, as long term adaptations to SCI include reductions in muscle mass, vessel diameter and blood flow below the level of lesion (63), this suggestion may not be limited to inactive individuals with an SCI but concern the SCI community as a whole. Furthermore, lesion level dependent paralysis of respiratory muscles can reduce respiratory function and the ability to cough and clear secretions that arise from respiratory infections (7). Respiratory training can improve respiratory parameters in SCI, such as maximum inspiratory and/or expiratory strength, vital capacity and maximum volun-

tary ventilation (19, 33, 85). Due to an improved ability to clear secretions, this may potentially reduce the risk of secondary complications, especially in individuals with high lesion levels. Finally, psychological stress and depression have known depressive effects on immune function, most likely via autonomic nervous and neuroendocrine system modulation (11). Given the psychological effects of SCI, this is likely to contribute to immune alterations in this population.

6 POTENTIAL COMPENSATORY MECHANISMS AFTER SPINAL CORD INJURY

Compensation of function loss is an intriguing field in exercise immunology in SCI populations. For example, receptors can adapt to an SCI and become hyperresponsive, as confirmed by adrenaline and noradrenaline injection in sympathectomized rats (66). It has also been observed that following SCI, spinal circuits are capable of generating some sympathetic activity, and a peripheral α-adrenoreceptor hyper-responsiveness may help to maintain normal function despite depressed circulating levels of catecholamines (29). Further studies during anaesthesia document increases in catecholamines, ACTH, and cortisol following functional electrical stimulation of anaesthetized limbs (45), suggesting that spinal reflexes and humoral feedback can potently regulate immune responses during exercise. It has also been suggested that the blockade of IL-6 signalling after SCI in mice inhibits classic pathways and promotes an alternative pathway of macrophage activation (34). Likewise, the reported higher levels of dehydroepiandrosterone sulphate (which enhances IL-2 and has anti-glucocorticoid effects) in TETRA may be a compensatory response by the neurohormonal-immune axis to augment an injury-related impairment in the SNS or the immune response itself (8, 9) On a cellular level, increased fractions of T and T_{helper} cells in TETRA and PARA may be a compensatory change related to reduced numbers of NKs (10). Other mechanisms that may compensate for loss of sympathetic function include serotonin (synthesis known to increase during exercise and influence T cells, macrophages and NKs), vasoactive intestinal peptide, substance P or neuropeptide Y, all known to have immunomodulatory capacity (41). Yet for many of these substances, the link between exercise and altered immune function in humans or indeed individuals with an SCI has not been studied in detail (41).

7 CONCLUSION

An SCI, particularly above the T6 level, impacts on immune function, partly caused by the dysfunction of sympathetic pathways and possibly an altered HPA axis. Reduced resting levels and depressed exercise responses of the end products of the SNS and a number of immune measures have consistently been found in TETRA. However, even though a number of studies report depressed immune measures or depressed levels of immunomodulatory substances in response to exercise in high level SCI, there is very little empirical (rather than anecdotal) evidence reporting illness rates in TETRA athletes. The impact of high level SCI on immune responses to exercise is likely to be influenced by the redundancy of the

bidirectional talk between central nervous and the immune system, which seems to be able to compensate some of the lost function. Compensatory mechanisms are likely to include reflex activity, receptor hypersensitivity, or parasympathetic and neuroendocrine adjustments. Future work should address the influence of time since injury on the impact of exercise on immune measures (and infection frequency) as such compensatory mechanisms will likely develop over a period of months, or even years. Further, as participation in wheelchair exercise involves all levels of activity, from the recently injured to the Paralympian, the effect of activity on immune functions and the immune response to exercise should be considered when investigating optimal exercise intensities for both health and performance in SCI. Future work may further investigate the possibility of lesion-level/impairment-dependent individual exercise prescription to help counteract depressed immune function following SCI. Finally, individuals with SCI may serve as experimental models to better understand the impact of various levels of lesion and therefore help elucidate the mechanisms underlying the immune response to exercise.

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Open letter to all authorities and institutions involved in managing curricula of physical education in Brazil

Ladies and gentlemen,
Dear editors and readers of EIR

Brazil is just about to experience a unique moment in its history, as greatest sports events worldwide (i.e., the World Cup Football-2014 and the Olympic Games-2016) will be carried out here. These events could be a sort of free advertisement making possible a significant dissemination of physical exercise among the population, which may improve the Brazilians' health. The Brazilian government, by launching the program "Academia da Saúde" (Health Fitness Center) in 2011, pointed out that regular physical exercise is going to be promoted as a tool to fight many diseases, among them type 2 Diabetes Mellitus (DM-2), cancer, obesity and high blood pressure. To complete this whole positive scenario, the first Brazilian Symposium of Sport Immunology has happened in the beginning of 2013. In this perspective, Physical Education curriculums may be outdated concerning exercise immunology.

In history, the Brazilian curriculum of Physical Education always has been influenced by socio-political-economical aspects. While Exercise Immunology, since its emergence as new discipline more than 20 years ago has been drawing ever growing attention and recognition worldwide, it has failed to make its way into the teaching programs of our physical education students. In a recent survey we found less than 0.6% (4/695) of all relevant curricula to include Exercise Immunology. Given the enormous role Exercise Immunology has in exploring the beneficial effects of exercise in prevention and therapy of epidemical diseases like DM-2 or others, we think that this situation needs to change fundamentally and that the moment to propagate entering Exercise Immunology into the curricula of the relevant institutions in Brazil is just now.

Let’s not forget that lack of exercise in patients having one or more of the above mentioned exercise responsive diseases is associated with enormous direct and indirect costs for patients, insurances and government and with drastically reduced quality of life.

Epidemiological studies carried out in projects involving humans, as well as animal models, point out relevant findings, as the production of different interleukins during physical exercise (aerobic and or anaerobic) by the skeletal muscle. Also, there is an increase in the efficiency of antigen presentation by dendritic cells and macrophages, increasing the production of IL-12 (2) and expression of MHC-II (3). At the same time, lymphocytes and macrophages from aerobic exercised mice improve their efficiency by producing more pro-inflammatory cytokines (e.g.: IFN-γ, IL-12, TNF-α) when stimulated by LPS and/or Leishmania major (2). Concerning cancer studies related to physical exercise, it was verified that, after performing two weeks training of moderate aerobic physical exer-

cise and inoculation with Ehrlich tumor cells, mice presented lower weight and volume of the tumor and less macrophage infiltration and neutrophil accumulation than animals that did not perform any kind of physical exercise and/or performed high intensity physical exercise (1).

These immunological features acquired in consequence of physical exercise give the organism considerable power to improve physiological mechanisms against cancer cell and pathogen susceptibility.

Thus, in Brazil we see the necessity to increase research and detailed teaching of the benefits of regular exercise in promoting health and protective immunological responses. This should enable and encourage physical education students to drive this knowledge to their practices. In this context, the inclusion of Exercise Immunology as subject into the curriculum of Physical Education students seems mandatory, especially for those enrolled in bachelor programs.

Sincerely,

Miguel J.S. Bortolini
Ismair T. Reis
José R. Mineo

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EIR usually solicits papers from authors with acknowledged expertise in the field to be covered. Unsolicited papers will be considered and can also be accepted. All papers are subject to a peer review process.

Usually the manuscripts will fit into one of two major categories: i. a review which thoroughly covers the area indicated in the heading and includes structuring and critical discussion of existing knowledge and, if possible, the ideas of the authors about potential practical consequences and future developments. Mere mentioning and listing of existing literature is not considered to be a good review. The review can be long, if necessary, or short, if the field covered by the heading is relatively new or very focussed. ii. a paper showing original data accompanied by an extended, review-type discussion.

The general format of the review is somewhat flexible. A review must however have an abstract, an introduction and a conclusion around the main sections. Reviews with three or more sections should list the headings of the sections in form of a bullet point table at the end of the introduction. Longer sections should also give a short interim summary at their end.
If substantial amounts of the authors‘ own new data are to be shown, a section on methods and on results must be included. Data will only be accepted, if methods are stated clearly and appropriate statistical evaluation of results is given.

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