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

Asghar Abbasi[1,4], Elvira Fehrenbach[1], Melanie Hauth[1], Michael Walter[2], Jens Hudemann[3], Veit Wank[4], Andreas M Niess[3], Hinnak Northoff[1]

[1] Division of Molecular Exercise Immunology, Institute of Clinical and Experimental Transfusion Medicine (IKET), University Hospital Tuebingen, Tuebingen, Germany
[2] Department of Medical Genetics, University Hospital Tuebingen, Tübingen, Germany.
[3] Department of Sports Medicine, University Hospital Tuebingen, Tuebingen, Germany
[4] Institute of Sports Science, Eberhard Karls University, Tubingen, Germany.

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

Correspondence to:
Asghar Abbasi, Division of Exercise Immunology & Genetics, Institute of Clinical and Experimental Transfusion Medicine (IKET), University Hospital Tuebingen
Otfried-Mueller -Str.4/1, 72076 Tuebingen, Germany Tel. +4970712981659, Fax. +497071295240, E-mail. Ashgar.Abbasi@med.uni-tuebingen.de

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.

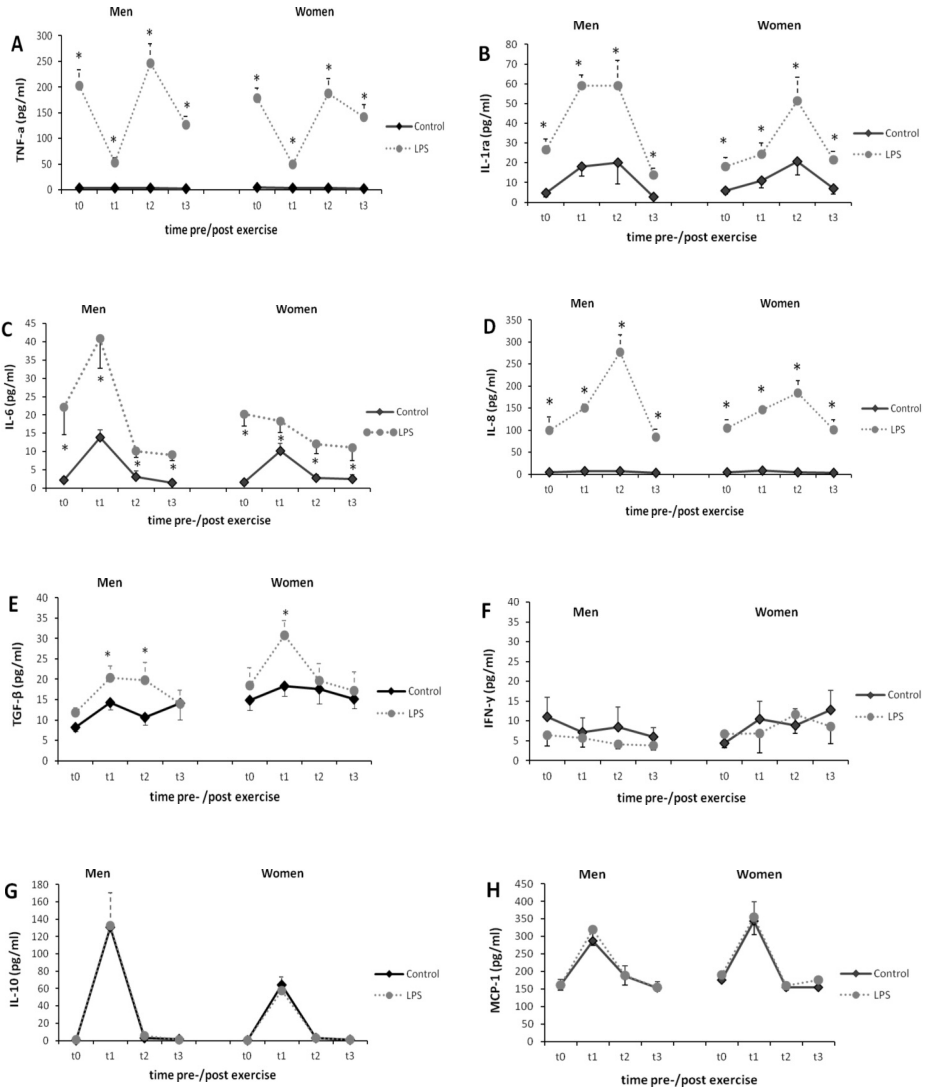
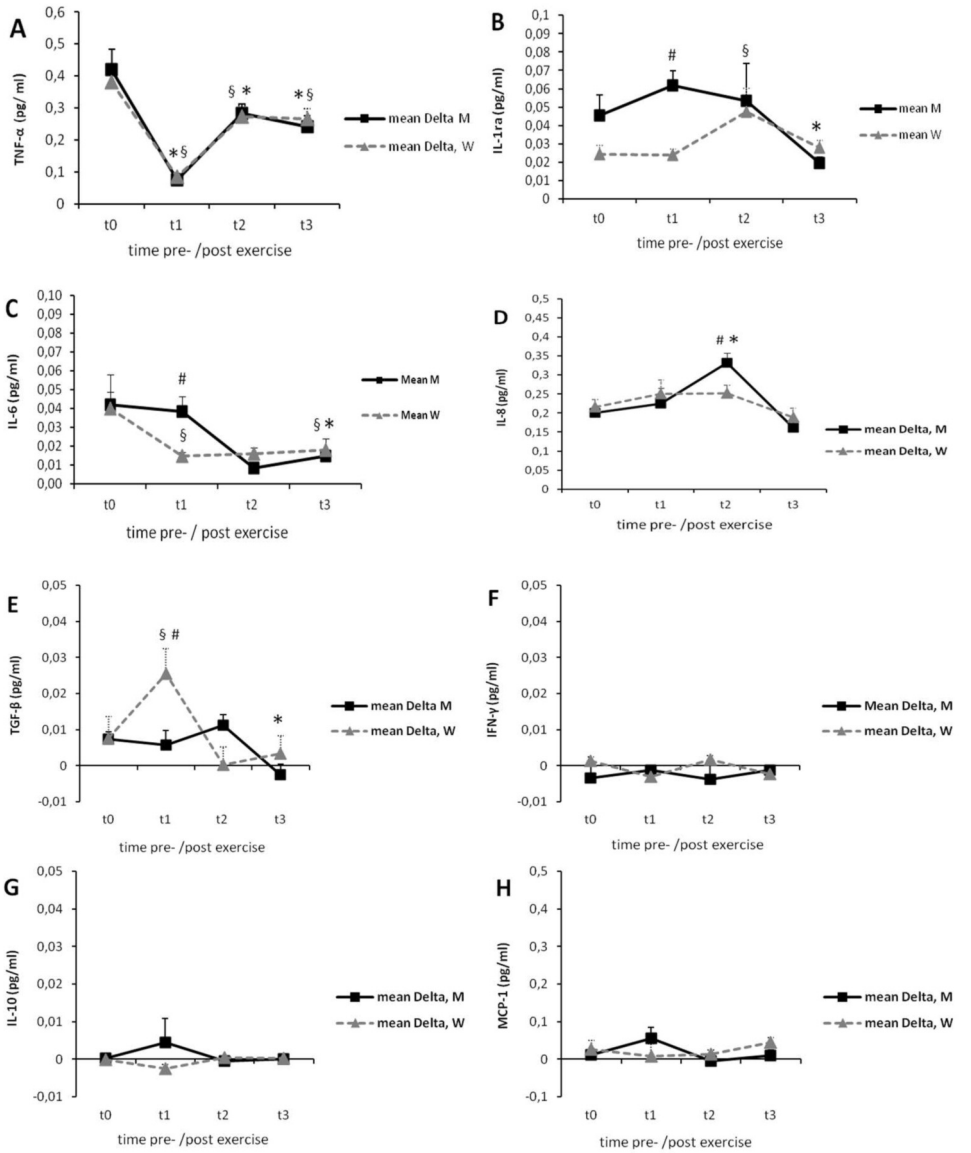


Figure 2. LPS-dependent release per 1000 monocyte (LDR) of cytokines (concentration in stimulated cultures minus concentration in un-stimulated cultures). LPS-dependent release of IFN-γ was normalized to 1000 lymphocytes. Data represent the means ± SE of the values. **M** represents men and **W** represents 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

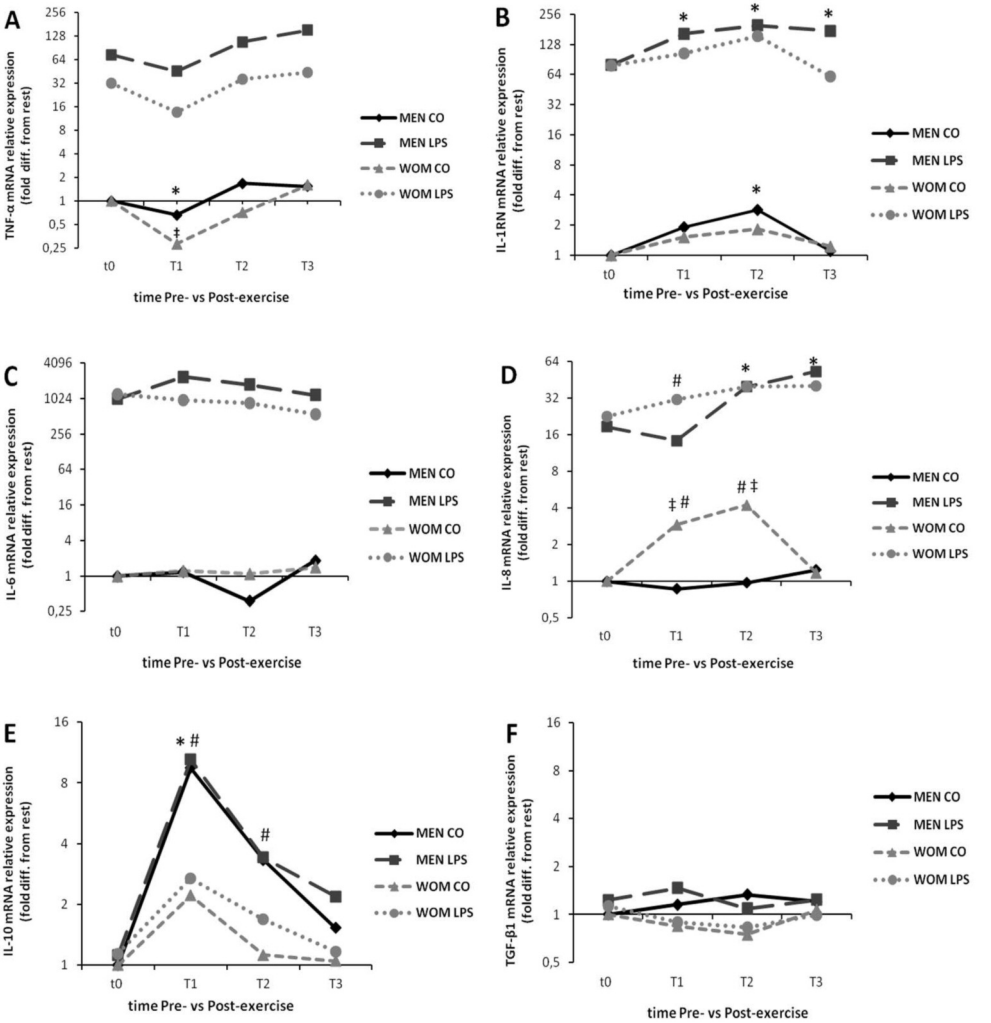
No significant difference was observed between sexes in LDR and unstimulated levels of TNF-α and MCP-1 at either time point (P>0.05) (Figure 2, Table 4). Men showed significantly higher LDR of IL-6 and IL-1ra and also significantly higher unstimulated levels of IL-10 at 30 min post race. As compared 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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