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cDNA microarray analysis reveals novel candidate genes expressed in human peripheral blood following exhaustive exercise

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Zieker, Derek, Elvira Fehrenbach, Janko Dietzsch, Judith Fliegner, Marc Waidmann, Kay Nieselt, Peter Gebicke-Haerter, Rainer Spanagel, Perikles Simon, Andreas Michael Niess, and Hinnak Northoff. cDNA microarray analysis reveals novel candidate genes expressed in human peripheral blood following exhaustive exercise. *Physiol Genomics* 23: 287–294, 2005. First published August 23, 2005; doi:10.1152/physiolgenomics.00096.2005.—It is generally accepted that exhausting endurance exercise exhibits strong effects on the immune system. Such effects have been attributed to changes in the cellular composition of peripheral blood as well as to changes in the expression of plausible candidate genes. The list of candidate genes is far from being complete, since this issue has not yet been investigated in a systematic way. In this study, we used a custom-made cDNA microarray focused on inflammation as a screening approach to study gene expression in eight one-half marathon runners before, immediately after, and 24 h after exercise. Significant differential gene expression was verified by quantitative real-time PCR. Linear regression analysis showed that microarray expression analysis of cell type-specific surface molecules reflects the observed individual cellular shifts in peripheral blood cells with high statistical significance. In line with the results of former studies, we observed an upregulation of mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP-K2), L-selectin, and IL-1 receptor antagonist (IL-1ra) after exhaustive exercise. The main results of this study report, for the first time, the downregulation of CD81; the upregulation of thioredoxin, which may play an important part in antioxidative defense; and, surprisingly, the downregulation of the anti-carcinogenic gene glutathione-S-transferase-3 (GSTM3) in peripheral blood. The study shows cDNA microarray expression analysis as a reliable systematic instrument to complete the list of candidate genes that may play a role in exhaustive exercise-induced modulation of the immune response.

thioredoxin; CD81; oxidative; marathon

HIGH TRAINING AND COMPETITION efforts lead to repeated stress responses in the immune system of athletes. This can cause a transient weakening of the immune system, in contrast to moderate endurance training, which is held to have a positive effect on the immune system (40). The reaction of athletes to exercise is a coordinated response of multiple organ systems (4).

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In leukocytes, several different systems are activated or regulated after intensive, exhausting endurance exercise. A variety of genes are known to be involved in this process. These comprise the heat shock proteins as well as the cytokines (10, 15, 55).

After prolonged, exhaustive endurance exercise, an increase in different cytokines, like TNF- α , IL-1, IL-6, IL-8, and IL-1 receptor antagonist (IL-1ra), were observed in plasma or urine of athletes, with levels falling back to normal on the following day (20, 43, 45, 58, 62). Presently, IL-6, which also has major metabolic functions for the working muscle, is considered to be the major indicator for physical strain in sports medicine (45, 52, 57). Considerably elevated levels of cortisol and adrenaline were suggested to be at least partly responsible for these changes (21, 48). On the other hand, after endurance exercise, ex vivo cytokine production by peripheral blood mononuclear cells was shown to be significantly decreased, probably as a result of counterregulation (63).

Exercise also leads to increased generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) (41). Besides their damaging potential, ROS play a physiological role in cellular signaling and in promoting lymphocyte apoptosis (21, 35).

To date, there is no proven knowledge about the molecular mechanisms by which exercise exerts its effects on the immune system. In humans, the most convenient source available to perform measurements of stress parameters on the cellular and molecular level is peripheral blood. cDNA arrays have evolved into widely used tools for comprehensive analysis of gene expression.

Using spotted cDNA chips, we decided to examine blood samples of one-half marathon runners before and after a run to look for changes in gene expression patterns. The aim, using microarrays, was to find hitherto unknown target genes involved in the reaction of the immune system to exercise. It is hoped that this may finally lead to an improved understanding of physiological and metabolic regulatory pathways that are influenced by endurance exercise.

The genes represented on the cDNA microarray were selected on the basis of up-to-date knowledge about their role in inflammation, apoptosis, stress response, and related pathways. It is also known that exhaustive exercise induces major transit changes in subpopulations of the peripheral blood.

METHODS

Eight well-trained male athletes [38.9 ± 11.8 yr, body mass index (BMI) 23.6 ± 1.8 ; see Table 1] performed an official one-half

Table 1. *Anthropometric and training characteristics of the individual subjects*

Athlete	Age, yr	BMI	Running Time, min	Average km/wk	Height, cm	Weight, kg
1	58	26.3	129	25	177	82.5
2	50	25.6	139	35	182	85
3	28	24.1	105	50	169	69
4	25	22.3	82	60	164	60
5	44	23.4	95	50	178	74
6	44	20.8	77	110	170	60
7	30	23	103	45	191	84
8	32	23.1	108	45	192	85

BMI, body mass index.

marathon under competition conditions (21.1 km). None of the athletes suffered from acute or chronic diseases or reported intake of medication, including antioxidants and nicotine abuse. 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 samples (5 × 2.5-ml whole blood) were drawn in PAXgene blood RNA tubes (Qiagen, Hilden, Germany) at rest before (t0), immediately (up to 15 min) after (t1), and 24 h after (t2) exercise in a sitting position. The individuals had been engaged in specific endurance training for at least 2 yr (52.2 ± 25.5 km/wk, running).

These evacuated PAXgene-tubes were prefilled with a “stop”-reagent, which lyses all cells and stabilizes the RNA immediately, avoiding changes of the cellular RNA profile due to storage.

We compared the gene expression pattern of each athlete before and after exhaustive exercise.

Each subject gave written informed consent before participation in the study. The experimental protocols were approved by the Institute’s Human Ethics Committee according to the principles set forth in the Declaration of Helsinki of the World Medical Association.

Preparation of Glass cDNA Microarrays

cDNA microarrays were produced in the Center for Mental Health in Mannheim, Germany. Human clones were purchased from the Ressourcen Zentrum für Genomforschung Library (RZPD) in Berlin, Germany. The final library contained 277 different genes (500–1,500 bp). Insert DNAs of all clones were amplified, each in a 50-µl PCR solution [10× PCR buffer (Qiagen), MgCl₂ (Qiagen), Taq polymerase (Qiagen), RZPD vector primers] in 384-well microtiter plates. The plates were incubated for 3 min at 94°C, before 35 cycles of denaturation at 94°C for 75 s, annealing at 59.5°C for 90 s, and elongation at 72°C for 90 s were performed, followed by a final elongation step at 72°C for 10 min. The quality of the PCR products was routinely checked on a 1.5% agarose gel stained with ethidium bromide.

Each PCR sample was concentrated, and the pellet was taken up in 15 µl of 3× SSC plus 1.5 M betaine (Sigma, Munich, Germany) spotting solution. Stratagene’s SpotReport-10 Array Validation System included human β-actin PCR product (3′-sense and 5′-sense) (Stratagene), poly(dA) oligonucleotide (Stratagene), human COT-1 (Stratagene) and cDNA from Universal RNA (Stratagene), 3× SSC buffer, and 1.5 M betaine spotting solution, spotted as positive and negative controls together with the RZPD gene library, in triplicate, on QMT amino slides (75 × 25 mm) (Quantifoil, Jena, Germany). cDNAs were spotted in triplicate with a MicroGrid I spotter (BioRobotics, Cambridge, UK) supplied with 16 solid microspotting pins (BioRobotics). The slides were UV cross-linked with 250 mJ/cm² and incubated at 80°C for 2 h.

Isolation, Amplification, and Labeling of RNA

Blood was drawn in PAXgene blood RNA tubes (Qiagen). RNA was isolated using the PAXgene blood RNA kit (Qiagen), includ-

ing a DNase I treatment, according to the manufacturer’s recommendations. The quality of the RNA was checked with Agilent’s Lab-on-a-Chip total RNA nanobiosizing assay (Agilent Technologies, Palo Alto, CA). Four micrograms of total RNA from each sample was spiked with Stratagene’s SpotReport-10 Array Validation System RNA spikes (amounts per spike varying from 0.0025 to 2.5 ng) and reverse transcribed. Amplification was performed exactly according to the Eberwine procedure (61) using Ambion’s MessageAmp kit (Ambion, Austin, TX). After in vitro transcription, the indirect labeling method with aminoallyl was used with equal amounts of all samples. The protocol follows exactly the Institute for Genomic Research (TIGR) protocol from Jeremy Haseman called “Aminoallyl labeling of RNA for microarrays” (<http://pga.tigr.org/sop/M004.pdf>), using cyanine-3 (Cy3) and Cy5 ester (Amersham Biosciences, Amersham, UK). For each labeled sample, absorbance was measured at 260 nm, for Cy3 at 550 nm, and for Cy5 at 650 nm, respectively.

Hybridization of Glass cDNA Microarrays

After labeling, the samples were vacuum dried for 3 h. During this time, the cDNA arrays were treated with a prehybridization step according to the Quantifoil prehybridization protocol A (<http://www.quantifoil.com>), followed by a denaturing step, for arrays spotted with PCR probes [1 × 3 min in boiling distilled water (dH₂O; 95–100°C)]. The vacuum-dried Cy3- and Cy5-labeled samples were resuspended together in 10 µl of 10 mM EDTA, incubated at 95°C for 5 min, and followed by incubation at room temperature for another 5 min. Prewarmed Ambion’s Hybridization Buffer No. 1 (35 µl) was added to the samples and was incubated for 10 s at 68°C before given on the microarray. Slides were hybridized in hybridization chambers (TeleChem International, Sunnyvale, CA). Hybridization was done at 55°C for 14–16 h in a water bath. After hybridization, each slide was washed with 2× SSC + 0.2% SDS at 30°C for 10 min, 2× SSC at room temperature for 10 min, and 0.2 × SSC at room temperature for 10 min. Slides were dried immediately with N₂.

To compare the gene expression pattern of each athlete before and after exhausting exercise, we hybridized each slide with samples from t0 vs. t1 and from t0 vs. t2. A dye swap was done with each investigation.

Scanning, Feature Extraction, and Analysis

Slides were scanned in a microarray scanner (Genetix Limited, Hampshire, UK).

Photomultiplier tube voltage was always set to 100% for both red and green channels. The two resulting green and red images were overlaid using ImaGene 5 (BioDiscovery).

Signal and Feature Extraction

As a first step of feature extraction, all spots that were flagged as poor by the ImaGene 5 software (due to signal foreground or background contamination, shape irregularity, or poor spot quality) were excluded.

The modus of the pixel distribution for the signal foreground and background of each spot was selected as a robust estimator of the raw signal values of both channels (see Fig. 1).

Normalization

The normalization was done in three steps (also see Fig. 1). First, a print tipwise Loess correction of the raw values was performed to reduce the spatial dependent intensity bias (6, 9, 64). Then, for each spot the log ratio

$$M = \log_2 \left(\frac{Ch^{\text{red}}}{Ch^{\text{green}}} \right)$$

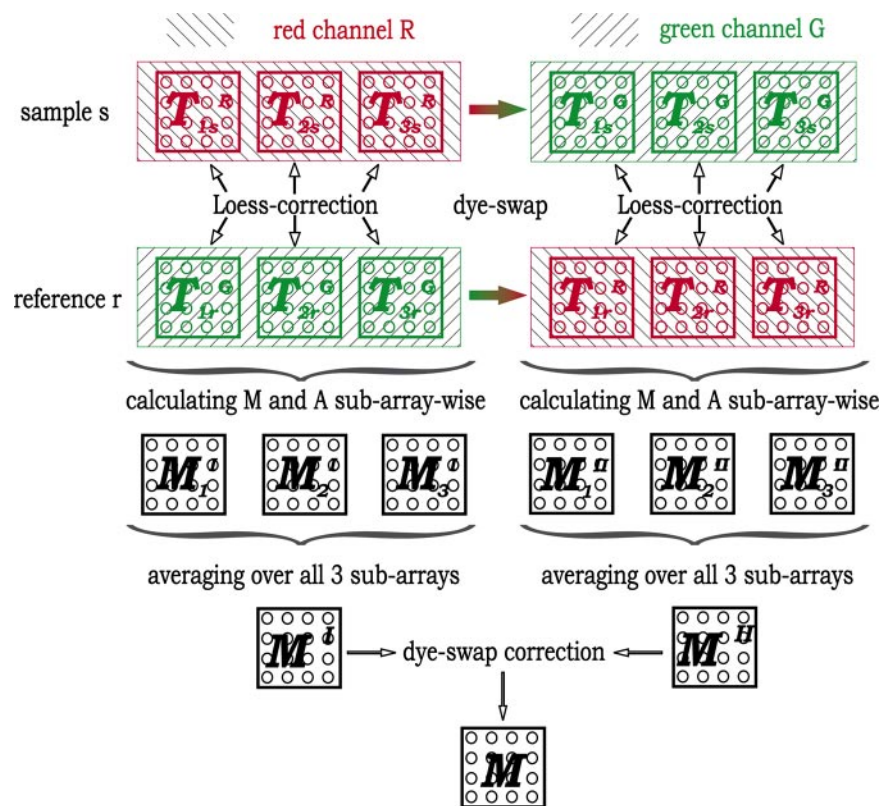


Fig. 1. Flow chart of the normalization procedure (see METHODS, *Normalization*). The dye-swapped arrays are shown at top. At left, the sample is in the red and the reference in the green channel; at right, the channels are flipped. After subarray-wise Loess correction, the log ratios M'_x, M''_x of the sample and reference intensities per spot were calculated. M', M'' and A', A'' were gained by averaging over all 3 identically spotted subarrays. Both estimators M' and M'' were used to calculate the finally used estimator for M via a dye-swap correction.

and the logarithmic average

$$A = \log_2 \sqrt{Ch^{\text{red}} \cdot Ch^{\text{green}}}$$

of the intensities of the red (Ch^{red}) and the green (Ch^{green}) channels, respectively (6, 13), were calculated as the basis for the next normalization steps. Due to the dye-swap design, one array carries the sample s in the red channel and the reference r in the green channel, and the other array has the sample s in the green channel and the reference r in the red channel. This leads to two M values for each spot

$$M' = \log_2 \left(\frac{s^{\text{red}}}{r^{\text{green}}} \right), M'' = \log_2 \left(\frac{r^{\text{red}}}{s^{\text{green}}} \right).$$

The use of the log (base 2) transformation has several advantages, like symmetrizing the spot intensity data, stabilizing the variances, and reducing the skewness of skewed distributions (14, 56), and it allows a natural interpretation of the resulting values as the logarithm of the fold change (FC), $M = \log_2(\text{FC})$. All three replicates (subarrays) of one sample and dye configuration were averaged by taking the arithmetical mean of the three estimators for M', M'' and A', A'' per transcript (see Fig. 1). Finally, the two arrays per sample with flipped dyes were used to execute a dye-swap correction of the log ratios M to remove remaining dye effects as follows (53, 60, 64)

$$M = \log_2 \left(\frac{s}{r} \right) \cong \frac{1}{2} \cdot (M' - M'') = \frac{1}{2} \cdot \left[\log_2 \left(\frac{s^{\text{red}}}{r^{\text{green}}} \right) - \log_2 \left(\frac{r^{\text{red}}}{s^{\text{green}}} \right) \right] \\ = \frac{1}{2} \cdot \left[\log_2 \left(\frac{s^{\text{red}}}{r^{\text{red}}} \right) + \log_2 \left(\frac{s^{\text{green}}}{r^{\text{green}}} \right) \right]$$

Differentially Expressed Genes

Due to the direct design of the experiment, only log ratios M that lie statistically significantly above or below 0 mark correspond to differentially expressed genes. To investigate this, a one-sample t -test procedure against the mean 0 was used (13). The problem of multiple

testing was addressed by controlling the family-wise error rate (FWER) and the false discovery rate (FDR) (13, 17). A gene was called differentially expressed if its Bonferroni-corrected (7) P value was <0.05 (5%). In addition, other correction methods like Benjamini and Hochberg (2), Benjamini and Yekutieli (3), Holm (22), and Sidak (54) were also applied and the results compared. The data discussed in this publication have been deposited in the National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE2532. A subset of interesting genes was selected for a further evaluation by real-time PCR.

Software

All parts of the feature extraction and analysis procedures were computationally done inside R 1.8.1 (50) with a mixture of Bioconductor structures (18) and self-written functions.

Quantitative real-time PCR. Selected cDNAs were quantified by real-time PCR on a LightCycler instrument (Roche, Mannheim, Germany). For PCR-assisted amplification, the LightCycler Fast Start DNA Master SYBR Green I-kit (Roche) was used according to the manufacturer's instructions. Final concentrations of primers and magnesium were optimized for each set of primers (for primer sequences, see Table 2).

Specificity of the PCR conditions was assured by determining the melting temperature of the respective amplicon previously shown to correspond to the expected product size by agarose gel analysis.

Relative quantification of gene expression levels was done by running an external standard curve together with the samples of interest. Standards were prepared by serially diluting a post-PCR amplicon of the respective templates. The linear range was determined for each PCR run by blotting the crossing point (CP) value calculated by the LightCycler software against the dilution factor of these standards, thus allowing the quantitative comparison of the samples. For samples within the linear range of the standard curve, the

Table 2. Primer Sequences

Target	Primer Sequences	Amplicon Size, bp
cd1c	Ffwd 5'-TGAAGCGCTCACAGAAACAG	199
	Bkwd 5'-CCAAACAGGCTTTGGGTAGA	
cd244	Ffwd 5'-GGTACAGAGGGAGCAAGCTG	253
	Bkwd 5'-CTCCTCCACACACAGAAGCA	
cd81	Ffwd 5'-TCATCGCTGTTGCCTGTGAG	270
	Bkwd 5'-CCTCCTTGAAGAGGTTGCTG	
gstm3	Ffwd 5'-CCTGGATGGGAAGAACAAGA	142
	Bkwd 5'-TTGTGTGCGGAAATCCATTA	
icam2	Ffwd 5'-CAAAGATCGCCATGACAG	163
	Bkwd 5'-CTGCACTCAATGGTGAAGGA	
il-1-ra	Ffwd 5'-GGAATCCATGGAGGAAGAT	245
	Bkwd 5'-CCTTCGTACAGGCATATTGGT	
itgax	Ffwd 5'-ATGCAATGGGGTTGGATTA	282
	Bkwd 5'-CTCCAGACCAGGTGAAGCTC	
sell	Ffwd 5'-AAACCCATGAAGTGGCAAAG	250
	Bkwd 5'-CGCAGTCCCTGTTCTTCTTC	
mapkapK2	Ffwd 5'-TCATGAAGAGCATCGGTGAG	165
	Bkwd 5'-TCAAAGAGTTGTGGCTGGTG	
trx	Ffwd 5'-CTGCTTTTCAGGAAGCCTTG	203
	Bkwd 5'-TGTTGGCATGCATTTGACT	

Ffwd, forward; Bkwd, backward (reverse).

respective value was calculated using regression analysis. The ratio of specific sample to β -actin values allowed the relative quantification of gene expression.

RESULTS

The average running time of the one-half marathon was 104.75 ± 21.24 min (see Table 1). Cell counts were significantly changed directly after the run and had returned to preexercise levels 24 h later (see Table 3).

We found a number of genes that were regulated up or down immediately after the run. Comparison of preexercise cDNA microarray expression levels to 24 h postexercise levels revealed no significance for all transcripts that had been significantly altered immediately after exercise. All genes that were regulated with ($P < 1$) are listed in Table 4. The one-sample *t*-test in conjunction with the adjusted *P* values resulted in a set of six genes, which we refer to as differentially expressed on a stringent statistical level (adjusted $P < 0.05$). These genes were L-selectin, which was upregulated, and CD81, CD244, glutathione-S-transferase-3 (GSTM3), ICAM2, and integrin- α x, which were downregulated. Stringent statistics was used to minimize false-positive findings. We are aware of the fact that, by doing so, we increase the rate of false negatives.

The six highly significant genes ($P < 0.05$) and four additional genes were selected for an evaluation using real-time PCR. The results are shown in Table 5 and Fig. 2. All of these genes achieved significance in real-time PCR except ICAM2 and CD244.

Table 3. Changes of cell counts before and immediately and 24 h after one-half marathon

Time Point	Average Leukocytes Count/1 μ l of Blood	Average Granulocytes Count/1 μ l of Blood	Average Lymphocytes Count/1 μ l of Blood	Average Monocytes Count/1 μ l of Blood
t0	6,237 \pm 1,520	3,498 \pm 939	2,050 \pm 565	445 \pm 84
t1	16,825 \pm 3,930	14,846 \pm 3,780	1,140 \pm 412	741 \pm 335
t2	6,270 \pm 1,205	3,682 \pm 799	1,865 \pm 493	517 \pm 134

t0, before; t1, immediately after; and t2, 24 h after one-half marathon.

Table 4. Fold changes and *P* values of differentially expressed genes detected by analysis of arrays

Gene Name	Log ₂ (FC)	FC	<i>P</i> Value (Bonferroni Adj.)
CD 81 (cd81)	-0.9	0.5	0.00
CD 244 (cd244)	-0.6	0.7	0.01
Integrin alpha \times (itgax)	-0.2	0.9	0.02
Selectin L (sell)	1.7	3.2	0.04
Glutathione S-transferase M3 (gstm3)	-0.3	0.8	0.04
ICAM 2 (icam2)	-0.9	0.5	0.04
Chemokine receptor 1	-1.3	0.4	0.05
Sphingomyelin phosphodiesterase 2	-0.4	0.8	0.09
CD 1C (cd1c)	-0.3	0.8	0.10
Endothelial differentiation sphingolipid			
G protein-coupled receptor 1	-0.3	0.8	0.15
MAPKAP K2 (mapkapK2)	0.5	1.4	0.15
CD 14	0.9	1.8	0.18
Thioredoxin (trx)	1.3	2.4	0.20
CD 19	-0.4	0.8	0.22
IL-2 receptor beta	-0.7	0.6	0.26
Chemokine ligand 4	-0.6	0.6	0.28
Thromboxane A synthase 1	0.9	1.8	0.30
CD 2	-1.2	0.4	0.33
IL-8 receptor alpha	1.0	2.0	0.35
Phospholipase C epsilon 1	-0.1	0.9	0.44
HSPB 1	0.8	1.8	0.47
CD 3E	-0.3	0.8	0.50
Protein C	-0.5	0.7	0.64
Thioredoxin 2	-0.3	0.8	0.69
Chemokine receptor 9	-0.7	0.6	0.88
CD 3E antigen	-0.5	0.7	0.91
IL-1 receptor antagonist (il-1ra)	0.7	1.7	0.93
Prostaglandin-endoperoxide synthase 1	-0.2	0.8	0.95
Adrenergic beta 2 receptor surface	-0.2	0.8	0.99

FC, fold change; Bonferroni Adj., Bonferroni adjusted. For further details, see *Differentially Expressed Genes* (METHODS).

We performed linear regression analysis to correlate the *x*-fold expression changes of cell type-specific surface molecules observed by microarray analysis with the observed individual cellular *x*-fold shifts of peripheral blood cells. As presented in Table 6, differential expression of all relevant specific cell surface markers significantly correlated with the respective *x*-fold change of cell counts.

DISCUSSION

During and some hours after exhaustive endurance exercise like marathon, one-half marathon, or triathlon, a number of peripheral immunological parameters have been demonstrated to change significantly (38). These include induction of cytokines and hormones as well as induction of tolerance to pathogenic stimuli and changes in natural killer (NK) cell activity and in adhesion markers in addition to changes in numbers of cells. Changes observed may serve to supply energy for the working muscles or help to curb undue inflam-

Table 5. Evaluation of differential expression between *t*₀ and *t*₁ by real-time PCR

Gene	P Value
CD1c (cd1c)	0.03
CD244 (cd244)	0.27
CD81 (cd81)	0.02
Glutathione S-transferase M3 (gstm3)	0.02
ICAM2 (icam2)	0.57
IL-1 receptor antagonist (il-1ra)	0.0002
Integrin alpha × (itgax)	0.03
Selectin L (sell)	0.004
Mapkap K2 (mapkapK2)	0.02
Thioredoxin (trx)	0.03

Significance (paired 2-sample *t*-test) was evaluated by additional real-time PCR measurements (see METHODS).

matory reactions to muscular stress, and may also be involved in staleness or in the transient immunosuppression that can occur after exhaustive endurance exercise (63). Usually, most of the changes return back to normal after 24 h (38).

Using an “inflammation-centered” cDNA chip, we intended to find evidence and new indications to further clarify the area. We did not expect to find all exercise-induced alterations of immunological parameters that are described in the literature to come out as significant changes in our experiments, since these changes may occur at different times or even outside peripheral blood (e.g., IL-6). Despite applying very stringent statistics for microarray analysis to avoid false positives, we found interesting candidate genes that showed significant regulation. Using real-time PCR, we confirmed most of the candidate genes. These and some further genes of special interest shall be discussed in detail below, trying to find out possible relevance in the given setting.

The present data reveal up- and downregulated transcripts in peripheral blood cells of one-half marathon runners immediately after the run compared with preexercise levels. Most of these significantly changed mRNAs encode proteins hitherto unknown to be involved in exercise physiology. Nevertheless, these transcripts neatly fit into the context of exercise stress, including oxidative stress and protective proteins [thioredoxin (TRX), GSTM3, IL-1ra], cellular contact mechanisms (CD81, adhesion proteins), signal transduction, and cellular protection

Table 6. Fold changes of gene expression, fold changes of cell shift, and their correlation

Marker	Cell Type	FC Cell Count	FC Expression
CD62L	granulocytes	4.32 ± 0.88	3.40 ± 1.45
CD14	monocytes	1.69 ± 0.72	1.88 ± 0.52
CD1c	lymphocytes	0.55 ± 0.14	0.81 ± 0.07
CD2	lymphocytes	0.55 ± 0.14	0.47 ± 0.18
CD3e	lymphocytes	0.55 ± 0.14	0.83 ± 0.08
CD19	lymphocytes	0.55 ± 0.14	0.78 ± 0.09

$$y = 0.37 + 0.72x \quad R^2 = 0.97 \quad P \text{ value} = 0.0003$$

Top: mean values ± SD for FCs of gene expression and the cell shift FCs for every marker and cell type. *Bottom*: expression (*y*) and cell shift (*x*) FCs were correlated for the 8 different markers, and a high correlation was revealed.

[mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP-K2), TRX]. Some of these genes were also shown to be significantly changed in peripheral blood mononuclear cells (PBMC) by Connolly et al. (10) using Affymetrix arrays.

Gene Expression Changes in Concordance with Cellular Shifts

Being aware of the exercise-induced changes of cell counts that may influence mRNA expression levels of whole blood cells, as measured here, we start by discussing those genes in which changes in the microarray-analyzed gene expression were closely paralleled by relevant cellular shifts. This concerns mainly genes for cell surface markers that are differently expressed in individual cell populations of peripheral blood. Sonna et al. (55) already stated that cell type-specific microarray-detected gene expression changes after heat injury may reflect cellular shifts rather than changes within a cell.

In our study it pertains to L-selectin, which was significantly changed, but also to some additional genes like CD1c, CD2, CD3e, CD14, and CD19, which show a strong trend but did not reach significance (see Table 4).

L-Selectin (CD62L) mRNA was upregulated significantly in this study. This pattern corresponds to the situation known

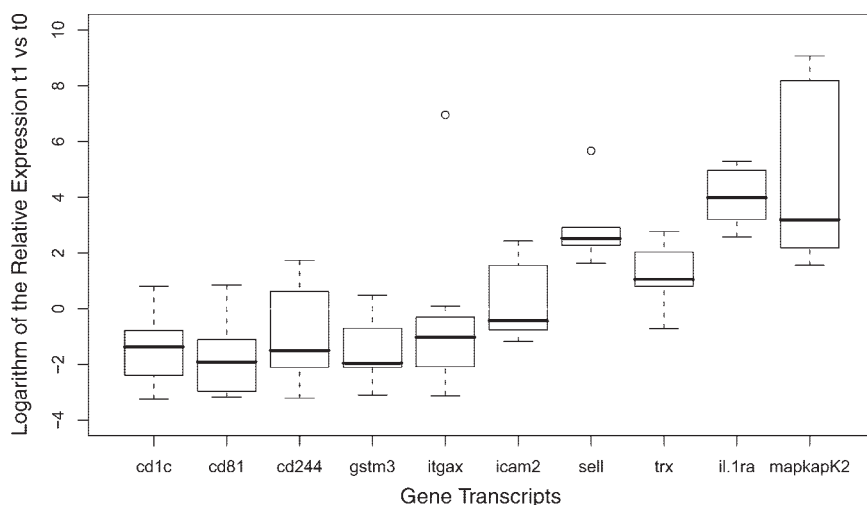


Fig. 2. Box plots of the real-time PCR measurements as \log_2 values of the fold change between time points *t*₁ (immediately after exercise) and *t*₀ (before exercise). The box whisker plots visualize the minimum (end of the *bottom* whisker), the first quartile (*bottom* border of the box), the median (line through the box), the third quartile (*top* border of the box), and the maximum (end of the *top* whisker) of the distribution. The separately drawn points are outliers according to the standard definition used inside R (36). Points are regarded as outliers if the minimum of their distance to the first and the third quartile is greater than 1.5 times the interquartile range (IQR = third quartile – second quartile).

from the literature (30), where it was shown by flow cytometric analysis that, during recovery after exercise, the expression of CD62L on granulocytes was significantly increased. However, in this study a 3.4-fold increase in gene expression may well be attributable to the increase in granulocyte numbers (4.3-fold). CD62L is strongly expressed on granulocytes.

CD1c, CD2, CD3e, and CD19 are markers of lymphocytes. All of these were downregulated in nearly the same range as the downshift of lymphocytes, which was ~45% in this study. In contrast to this, CD14 as a monocyte marker, which was upregulated by ~88%, nicely reflected changes in numbers of monocytes, which were increased by 69% after exercise.

The expression of CD244, another surface molecule, may also have been altered due to cellular shifts. CD244 is the NK cell receptor 2B4 and is expressed as coreceptor on human NK cells, on macrophages, and on a subset of CD8+ T cells (36). It is known that numbers of NK cells in peripheral blood increase immediately after exhaustive exercise but can show a fast and sharp drop with high individual variation (39). Further expression studies should show whether CD244 data parallel the kinetics of NK cell numbers.

The finding of a highly significant correlation of the fold changes of cell surface markers with the respective fold changes in cell counts underscores the accuracy of the performed methodology, but nevertheless pinpoints the problem to securely separate expression differences from the bias induced by cellular shifts (see Table 6). This is not only a particular problem of this study (10, 55) but may also be true for microarray studies of solid tissue, for instance, for the frequently performed comparison of tumor with benign tissue, where differences in the presence of immune competent cells may severely bias expression analysis.

Gene Expression Changes Not Attributable to Obvious Cellular Shifts

Cellular communication. Integrin- α (CD11c) and CD81 mRNA were both downregulated significantly in this study. This cannot be attributed to cellular shifts, since CD11c is strongly or moderately expressed in monocytes, macrophages, NK cells and granulocytes, while it is low in T and B cells (26), and CD81 is expressed on polymorphonuclears (PMN), monocytes, and B and T lymphocytes (59). The lymphocytes are the minor population and therefore can hardly explain the strong 50% overall downregulation of gene expression.

Regular moderate exercise leads to a decreased expression of adhesion proteins, especially β 2-integrins (26). It was suggested that the decreased expression of adhesion molecules immediately after exercise negatively influences adhesion and transmigration of the leukocytes into the vessel wall and the surrounding tissue. Shortly after exercise, this may contribute to a reduced defense against infectious agents (37).

To our knowledge, the downregulation of the CD81 gene expression has not yet been demonstrated in context with exhaustive exercise. The protein encoded by this gene is a member of the transmembrane 4 super family (tetraspanins) involved in signal transduction events in the regulation of cell development, activation, growth, and motility. The tetraspanins are common components of exosomes. The CD81 protein may play an important role in the reaction or adaptation to strenuous exercise. As mentioned above, reduced interaction

with integrins may contribute to downregulation of undue proinflammatory stimuli resulting from acute muscular (cellular) stress.

Integrins also play a role in the cell contact area during interaction between T cells and antigen presenting cells (APC), which is termed immunological synapse (IS) (33). During formation of IS, the tetraspanin CD81 is redistributed to the contact area of the cells in an antigen-dependent manner and is involved in the antigen-specific B cell-mediated immune response (33). Furthermore, the presence of CD81 at the IS may play an essential role during TH1/TH2 polarization (11). Downregulated CD81 protein may partly contribute to the attenuated cell-mediated immunity and a change of the TH1/TH2 balance in favor of TH2 after exhaustive exercise, rendering the athlete more susceptible to infections. However, CD81 mRNA and corresponding protein changes in individual leukocyte subpopulations still warrant further analyses.

Signal transduction and cellular protection. In contrast to the cellular communication genes, signal transduction and cellular protection cannot be clearly related to individual subpopulations of peripheral blood. In this study, MAPKAP-K2, TRX, GSTM3, and IL-1ra were changed and confirmed as significantly altered by real-time PCR.

The finding that the gene expression level of MAPKAP-K2 is significantly upregulated in athletes after exhausting endurance exercise has already been shown for the muscle (65).

MAPKAP-K2 is a downstream substrate in the pathway of the MAPK family. The MAPKAP-K2 protein phosphorylates the small heat shock protein 27 (HSP27, HSPB1) (16, 49, 65). It is also known that MAPKAP-K2 is involved in IL-6 synthesis by stabilizing IL-6 mRNA (34). A recent study showed that MAPKAP-K2 is an immediate early gene that is posttranslationally regulated by the mRNA stabilizing protein HuR, which may explain the early and isolated increase in peripheral blood as reported here (32).

We recently showed that inducible nitric oxide synthase (iNOS), IL-6, and HSP27 are upregulated after exercise at mRNA and protein levels (15, 16, 44). It remains to be shown which role the upregulation of MAPKAP-K2 mRNA as demonstrated in this study may play for the posttranscriptional modification of these and possibly other substrates in exercise. Interestingly, hsp27 mRNA (HSPB1) is 1.8-fold upregulated in our study and is included in our list of differentially expressed genes (see Table 4).

The finding of increased TRX mRNA after the one-half marathon is new but does not come entirely unexpected. The TRX protein is a 12-kDa oxidoreductase enzyme containing a dithiol-disulfide active site and is released from various types of human cells, excluding neutrophils (28), in response to oxidative stress (25). It possesses a variety of biological functions including the ability to modulate transcription factor activity (5, 23). The multifunctional redox-active TRX scavenges free radicals, modulates chemotaxis and suppresses leukocyte infiltration into sites of inflammation, and protects cells against TNF-induced cytotoxicity and general oxidative stress (12, 24). The TRX enzyme was found to be elevated in patients with oxidative stress profiles (29, 47). Similarly, heavy exercise is associated with oxidative stress (1, 31, 41, 42). Therefore, the regulation and maintenance of the cellular redox status by intracellular redox-regulating molecules such as TRX become important to maintain tissue homeostasis also after

intensive exercise. The TRX enzyme may represent an important anti-oxidative protection mechanism in peripheral blood cells after exercise stress. It is important to mention, however, that ROS generated through cellular metabolism do have positive functions as cellular second messengers through the regulation of numerous signal transduction pathways. The level of ROS needs to be carefully balanced to fulfill their positive tasks without producing undue damage. An upregulation of TRX has already been shown to enable the maintenance of such low and protective ROS levels (19).

Interestingly, another part of the oxidative stress response is GSTM3, which we demonstrate for the first time to be downregulated on gene expression level after endurance exercise. Glutathione transferases (GSTs) form a group of multigene isoenzymes involved in the cellular detoxification of both xenobiotic and endobiotic compounds. More than a dozen genes are known to encode GSTs. In this context, GSTs bind to or catalyze the inactivation of a variety of carcinogens (8). GST gene expression studies in humans have shown that polymorphisms in members of the GST gene family are associated with an increased risk of lung cancer (51). Levels of GST isoform expression have been shown to vary between different tissue and cell types. Within the peripheral blood, expression of GSTM3 mRNA has so far only been demonstrated in patients with acute lymphoblastic leukemia, where its expression was related to good prognosis (27). The role of GSTM3 in exercise immunology remains to be determined.

An additional gene that may also be viewed as part of the cellular protection mechanism is IL-1ra. The main assignment of IL-1ra is the regulation and the control of the effects caused by IL-1 β by a competitive inhibition of the receptor. An overbalance of IL-1 β at the receptor leads to inflammation and tissue destruction. This anti-inflammatory cytokine is known to be increased in serum by prolonged exercise (46, 52, 57). Upregulation of IL-1ra mRNA in our study confirms that IL-1ra as opposed to IL-6 is generated in peripheral blood cells. Changes of IL-1ra mRNA in PBMC after exercise have already been shown (10).

The upregulation of IL-1ra may play an important role for the balance between the proinflammatory and the anti-inflammatory cytokines in the immune system.

In conclusion, we have investigated gene expression in peripheral blood of eight marathon runners using a cDNA microarray focused on inflammatory markers. A number of genes regulated in response to exercise have been identified. We presently have no information about corresponding protein changes. Some of these genes may have been altered as a consequence of cellular shifts. Furthermore, three genes have been found of particular interest that have not yet been described in relation to exercise. These are downregulated CD81, which may attenuate inflammatory reaction; upregulated TRX, which may play an important part in anti-oxidative stress reaction; and downregulated GSTM3, another gene involved in balancing the redox system.

It is beyond the scope of this study to carry out additional investigations on up- or downstream genes. Moreover, correlations of gene transcript abundance with corresponding protein levels will have to be done in the future.

Altogether, the reliability of our microarray results is confirmed by the good reproducibility in real-time PCR measurements. The nice reflection of cellular shifts in some of the

transcript changes and the confirmation of several previously known effects strongly support the notion that such microarrays may serve as a suitable screening tool for exercise monitoring. Microarray analysis of separated cell subsets, excluding cell shift-associated changes, should be performed in the future.

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