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J Appl Physiol 114:1677-1688, 2013. First published 11 April 2013;
doi:10.1152/japplphysiol.00143.2013

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J Appl Physiol 114: 1677–1688, 2013.
First published April 11, 2013; doi:10.1152/japplphysiol.00143.2013.

Transcriptome analysis of neutrophils after endurance exercise reveals novel signaling mechanisms in the immune response to physiological stress

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[1]*Emerging Field Oxidative Stress and DNA Stability, and Research Platform Active Aging, Department of Nutritional Sciences, Faculty of Life Sciences, University of Vienna, Vienna, Austria;* [2]*Heart Foundation Research Centre, Griffith Health Institute, Griffith University, Gold Coast, Queensland, Australia;* [3]*Genomics & Systems Biology, Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia;* [4]*Harvard Medical School, Boston, Massachusetts;* [5]*School of Public Health and Centre for Health Practice Innovation, Griffith Health Institute, Griffith University, Gold Coast, Queensland, Australia;* [6]*School of Biomedical Sciences, Queensland University of Technology, Brisbane, Queensland, Australia; and* [7]*Liggins Institute, The University of Auckland, Auckland, New Zealand*

Submitted 31 January 2013; accepted in final form 5 April 2013

Neubauer O, Sabapathy S, Lazarus R, Jowett JB, Desbrow B, Peake JM, Cameron-Smith D, Haseler LJ, Wagner K-H, Bulmer AC. Transcriptome analysis of neutrophils after endurance exercise reveals novel signaling mechanisms in the immune response to physiological stress. *J Appl Physiol* 114: 1677–1688, 2013. First published April 11, 2013; doi:10.1152/japplphysiol.00143.2013.—Neutrophils serve as an intriguing model for the study of innate immune cellular activity induced by physiological stress. We measured changes in the transcriptome of circulating neutrophils following an experimental exercise trial (EXTRI) consisting of 1 h of intense cycling immediately followed by 1 h of intense running. Blood samples were taken at baseline, 3 h, 48 h, and 96 h post-EXTRI from eight healthy, endurance-trained, male subjects. RNA was extracted from isolated neutrophils. Differential gene expression was evaluated using Illumina microarrays and validated with quantitative PCR. Gene set enrichment analysis identified enriched molecular signatures chosen from the Molecular Signatures Database. Blood concentrations of muscle damage indexes, neutrophils, interleukin (IL)-6 and IL-10 were increased ($P < 0.05$) 3 h post-EXTRI. Upregulated groups of functionally related genes 3 h post-EXTRI included gene sets associated with the recognition of tissue damage, the IL-1 receptor, and Toll-like receptor (TLR) pathways (familywise error rate, P value < 0.05). The core enrichment for these pathways included TLRs, low-affinity immunoglobulin receptors, S100 calcium binding protein A12, and negative regulators of innate immunity, e.g., IL-1 receptor antagonist, and IL-1 receptor associated kinase-3. Plasma myoglobin changes correlated with neutrophil *TLR4* gene expression ($r = 0.74$; $P < 0.05$). Neutrophils had returned to their nonactivated state 48 h post-EXTRI, indicating that their initial proinflammatory response was transient and rapidly counterregulated. This study provides novel insight into the signaling mechanisms underlying the neutrophil responses to endurance exercise, suggesting that their transcriptional activity was particularly induced by damage-associated molecule patterns, hypothetically originating from the leakage of muscle components into the circulation.

neutrophil transcriptome; endurance exercise; systemic inflammatory response; muscle-derived damage-associated molecule patterns; counterregulation of inflammation

THE MOBILIZATION AND FUNCTIONAL adaptation of polymorphonuclear leukocytes, particularly neutrophils, represents one of the most prominent effects of acute bouts of prolonged, intense exercise on the immune system (33, 48, 55). The initial increase and activation of circulating neutrophils is an evolutionary conserved response to physical stress, preparing the body for potential further immune and repair responses in the event of injury or infection (36). The rapid increase of neutrophils in the circulation is mediated by the systemic release of cytokines such as interleukin (IL)-6 (28, 33, 48), and stress hormones such as catecholamines (48), followed by a cortisol-induced mobilization of neutrophils from the bone marrow, in proportion to the duration and the intensity of exercise (28, 33, 55). Moreover, neutrophils are mobilized in response to exercise-induced muscle damage (28, 35, 48), which is rapidly followed by their infiltration into damaged muscles (44). Although the proteolytic and free radical-mediated removal of cell debris by neutrophils may elicit secondary tissue damage, their phagocytic activity and communication with macrophages appear to be essential for the repair and the regeneration of injured muscle tissue (49, 52).

Due to their close association with both muscular and systemic inflammatory responses to exercise, neutrophils are convenient cells to study innate immune cellular activity induced by stimuli, such as tissue injury and/or systemic stress factors. Neutrophils are crucial immune mediators that restrain or promote inflammatory responses and initiate and maintain immune responses involving adaptive immunity (27, 51). Formerly thought of as short lived (less than 1 day), recent observations (38) suggest an average circulatory life span for (nonactivated) neutrophils of 5.4 days, which might have important implications for their immune-modulatory function. Furthermore, previous microarray studies demonstrated that neutrophils respond to inflammatory stimuli by initiating a highly coordinated transcriptional program, which regulates their differentiation, activation, inflammatory/immune responses (45, 51, 58), and apoptosis (21, 22). Detailed understanding of these complex processes requires systems biology approaches, including the analysis of transcriptional activity (51, 59). So far, the data on global neutrophil gene expression responses after exercise is extremely scarce (40, 41). Radom-Aizik et al. (40) demonstrated that 30 min of high-intensity

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cycling induced gene expression alterations associated with apoptosis, growth, tissue repair, and pro- and anti-inflammatory responses in healthy, but untrained subjects. Crucially, however, the molecular mechanisms upon which neutrophils are transcriptionally activated and primed in response to endurance exercise involving muscle damage remain incompletely understood (35).

With the present study, we sought to extend upon the results of Radom-Aizik et al. (40) by exploring the time course-dependent changes in the transcriptome of neutrophils after an experimental exercise trial (EXTRI), consisting of 1 h of cycling, immediately followed by 1 h of running. The rationale for the selected exercise protocol, similar to increasingly popular short- or middle-distance triathlon (6, 24) or duathlon events (47), was that the underlying physiological and biochemical demands would induce both a greater degree of muscle damage and a more pronounced systemic inflammatory response than a brief bout of cycling alone. The major aim of the present study was to explore the signaling pathways involved in the inflammatory response and subsequent counter-regulatory mechanisms of circulating neutrophils in healthy, endurance-trained individuals in response to endurance exercise involving a muscle-damaging component. We hypothesized that this endurance exercise protocol would induce characteristic transcriptional alterations in circulating neutrophils reflecting *1*) their activation and priming in response to an acute bout of intense, prolonged exercise involving muscle damage; and *2*) compensatory mechanisms to negatively regulate the systemic inflammatory response. As another extension upon the study of Radom-Aizik et al. (who only sampled blood immediately after exercise) (36), we aimed to evaluate the changes in the neutrophil transcriptome throughout recovery from exercise (i.e., 3, 48, and 96 h thereafter) to monitor both acute and potential sustained inflammatory responses through to its resolution.

MATERIALS AND METHODS

Subjects. Eight healthy, male, endurance-trained individuals volunteered to participate and provided written, informed consent. Participants provided information on their training history and underwent a preparticipation health screening to ensure they were nonsmokers, engaged in regular endurance training, and had no history of cardiopulmonary or metabolic disease. Throughout the study period, the study participants were required to abstain from any nutritional supplementation and medication. Subject characteristics are summarized in Table 1. The study was conducted according the guidelines of the Declaration of Helsinki and was approved by the Griffith University Human Research Ethics Committee (ethics no. MSC/16/10/HREC).

Table 1. *Characteristics of the eight study participants*

	Mean ± SD
Age, yr	25.0 ± 4.1
Body mass, kg	78.6 ± 7.4
Body height, cm	184.6 ± 4.6
Weekly net endurance exercise time, h/wk	6.6 ± 1.1
Relative $\dot{V}O_{2peak}$, $ml \cdot kg^{-1} \cdot min^{-1}$	56.3 ± 6.7
Peak power output, W	401 ± 41
Gas exchange threshold, %$\dot{V}O_{2peak}$	60.1 ± 4.5

$\dot{V}O_{2peak}$, peak oxygen consumption.

Prescribed training period and preliminary testing. All subjects had been engaged in regular endurance training (including cycling and running) for a least 1 yr before their participation in the study. After study enrollment, subjects undertook a prescribed 6-wk training program [including cycle to run transition training (24)] in preparation for commencing the 2-h EXTRI, as described below. Subsequent to this training program, and after abstaining from any intense exercise for 48 h, participants performed an incremental exercise test to volitional fatigue to determine their physiological characteristics [including peak oxygen uptake ($\dot{V}O_{2\,peak}$)] on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, the Netherlands). During this test, gas exchange was measured using a mouthpiece that was interfaced with a calibrated metabolic cart (MedGraphics Ultima CardiO2, Medical Graphics, St Paul, MN). Gas exchange and ventilator variables were measured breath by breath and then averaged over 30-s intervals. Cardiac rhythm and heart rate were monitored using a five-lead (limb leads + V5) ECG (X-Scribe, Mortara Instrument, Milwaukee, WI) integrated with the metabolic cart. Peak exercise variables were determined as the highest value recorded during a complete 30-s work stage during the test. The gas exchange threshold was determined using the simplified V-slope method (42).

Study design. Blood samples were taken 1 wk before the EXTRI (pre-EXTRI; baseline), and then 3, 48, and 96 h thereafter (post-EXTRI). We chose these sampling time points as we aimed to elucidate early inflammatory and apoptotic responses 3 h post-EXTRI. Early neutrophil gene expression changes after inflammatory stimuli are known to occur within hours (58). Although many of the early-response mRNA expression patterns are similar at 2, 3, and 4 h after stimulation (45), the time period between 3 and 6 h after neutrophil activation represents a critical measurement window to analyze molecular processes associated with the induction of the neutrophil apoptosis-differentiation program (22). At 48 and 96 h post-EXTRI, we aimed to monitor potential sustained inflammatory responses and the resolution of the systemic inflammation (28). To avoid circadian effects, all samples were collected between 1300 and 1400. Subjects were required to abstain from any intense exercise for 48 h before each sampling time point. Furthermore, before each sampling, the subjects were examined by a physician to ensure that they were free of any symptoms of acute illness.

Standardization of diet. For the 24 h preceding each sampling, including the day of the EXTRI, the subjects were instructed to abstain from alcohol and caffeine consumption and were provided with a prepackaged standardized diet that was individualized for food preferences and body mass (BM) using a computerized dietary assessment program (Foodworks Version 9.0, Xyris Software). The standardized diet was designed to provide ~9 g of carbohydrate (CHO)/kg BM, ~1.4 g of protein/kg BM, and ~1.4 g of fat/kg BM. Dietary compliance was then assessed in accordance with current dietary standardization recommendations (15).

Experimental EXTRI. To induce both muscle damage and a systemic inflammatory response, the subjects completed a laboratory-based, highly controlled EXTRI consisting of 1 h of continuous ergometer cycling, immediately followed by 1 h of continuous treadmill running, both performed at a predetermined intensity. The rationale for the selected exercise protocol was twofold. First, the physiological and biomechanical demands of this performance-oriented exercise protocol were expected to elicit significant muscular and systemic stress, similar to that of short- or middle-distance triathlon (6, 24) or duathlon events (47). Comparable bouts of intense, prolonged exercise involving running after cycling have been reported to induce both muscle damage (6, 47) and a systemic inflammatory response, including neutrophil activation (47). Second, this endurance exercise protocol has important practical implications due to the increasing popularity of triathlon and similar events, in particular also among nonprofessional and recreational athletes (6, 24).

The cycling bout was performed on a Lode Excalibur Sport cycling ergometer (Groningen, the Netherlands) at 105% of the power output

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attained at the gas exchange threshold, as assessed by the initial incremental exercise test. The running bout was performed on a motor-driven treadmill (set at 1% grade, Trackmaster TMX 425, Full Vision, Newton, KS). Study participants were encouraged to select a running speed that approximated a 10-km time trial pace. During the EXTRI, gas exchange was measured at 15-min intervals, and cardiac rhythm monitored continuously, as described for the incremental exercise test. Global ratings of perceived exertion (RPE) using the Borg 6–20 scale were also collected at these times. A lower limit of treadmill speed was imposed to ensure that the oxygen consumption values during the treadmill running were not below that achieved during the preceding cycling bout.

On the morning of the EXTRI, as part of their prepackaged diet, study participants consumed a standardized breakfast (containing ~2 g of CHO/kg BM) ~2 h before reporting to the laboratory. During the EXTRI, the subjects ingested 3 ml/kg BW of a beverage (Gatorade) containing 6% (or 60 g/l) CHO, and ~2.3 mmol/l of sodium (but no antioxidants) every 15 min (equivalent to 50 g of CHO/h). After the EXTRI, the participants were weighed and required to consume the same beverage in a volume equivalent to 150% of their pre- to post-EXTRI loss of BM for rehydration.

Blood samples and hematological profile. Blood samples (~20 ml) were drawn from an antecubital vein. The blood samples were drawn into EDTA and heparin vacutainers and immediately placed on ice. Whole blood was used for assessment of the hematological profile using an automated hematology analyzer (Beckham Coulter, Fullerton, CA). The EDTA blood samples were carefully layered in tubes containing Histopaque-1077 (Sigma-Aldrich, Castle Hill, NSW, Australia), and centrifuged at 400 *g* for 30 min at 22°C. Heparinized blood samples were also centrifuged at 400 *g* for 30 min at 22°C. Plasma aliquots were immediately stored at −80°C. Exercise-induced changes in plasma volume were calculated (7). All results for parameters assessed in the plasma are reported adjusted for changes in plasma volume, except for cortisol, as for factors released in an endocrine manner, it is important to consider their actual circulating concentration (28).

Plasma markers of muscle damage, complement factors, high-sensitive C-reactive protein, and heat shock protein 70. Plasma myoglobin, high sensitive C-reactive protein (hs-CRP), and complement proteins C3c and C4 were analyzed using commercially supplied kits based on immunoturbidimetric methods (Roche Diagnostics) on a Cobas Integra 400 chemistry analyzer (Roche Diagnostics). Creatine kinase (CK) activity was assessed spectrophotometrically in a coupled reaction by measuring the rate of NADPH formation (Roche Diagnostics) on the same machine. Plasma concentrations of heat shock protein (HSP) 70 [including the stress-inducible HSP72 (56)] were analyzed using the HSP70 high sensitive enzyme immunoassay kit (Enzo Life Sciences). All measurements were made in duplicate. The coefficients of variation for these assays were all $< 3\%$.

Plasma concentrations of cytokines and cortisol. Plasma concentrations of IL-1β and IL-1 receptor antagonist (IL-1ra) were detected using high-sensitivity ELISA and the platinum ELISA kit (both eBioscience, Vienna, Austria), respectively. Plasma concentrations of cortisol, IL-6, and IL-10 were assessed using the cortisol parameter assay kit and Quantikine high sensitive ELISA kits (R&D Systems, Minneapolis, MN). All samples and provided standards were analyzed in duplicate.

Neutrophil isolation, RNA extraction, and cDNA synthesis. Neutrophils were isolated by gradient separation using Histopaque-1077 (Sigma-Aldrich, Castle Hill, NSW, Australia), followed by lysis of the red blood cells after removing the top layer of plasma, the monocyte and lymphocyte interface, and the separation layer of Histopaque from that of the freshly collected EDTA-treated blood, as described previously (57). Briefly, the red blood cells were lysed by adding RNAse-free water (4°C), followed by shaking for 30 s. Isotonicity was restored by adding 10% phosphate-buffered saline (Invitrogen). The cells were centrifuged at 700 *g* (10 min, 4°C). The supernatant was removed until ~5 ml of neutrophil-rich solution remained, and another lysis was performed. The pellet of neutrophils remaining after the second lysis step was used for subsequent RNA extraction. Using eosin Y and thiazine-dye for staining, this technique obtained ≥95–98% polymorphonuclear purity in the pellet. The duration from blood sampling to stabilization of RNA never exceeded 90 min. Total RNA from neutrophils was extracted using the RNeasy Mini columns (Qiagen, Doncaster, VIC, Australia). RNA concentrations and estimation of purity were determined by measuring the absorbance of the eluate at 260 and 280 nm (Nanodrop, Thermo Scientific). One microgram of RNA was then reverse transcribed into cDNA using a first-strand cDNA synthesis kit (Thermo Scientific).

Microarray gene expression measurement and statistical analysis. RNA was reverse transcribed using a T7-promoter-oligo(dT) primer followed by the Ambion labeling procedure. Relative transcript abundance in the biotin-labeled cRNA samples was measured using Illumina HT12 version 3 microarrays and processed using the Illumina (San Diego, CA) iScan platform. Data were exported after background correction in GenomeStudio software. Analysis was performed in the R (version 2.15.1) statistical environment. Background-corrected raw data were quantile normalized using the Bioconductor Lumi package (version 2.10.0), and array quality was assessed using Array Quality Metrics. The 47.323 transcript probes on the array were nondifferentially filtered to remove 18.732 transcript probes that were below the threshold of reliable detection, according to the default Illumina scanner software. Differential expression between the baseline values and each post-EXTRI time point was tested using the Bayesian moderated *t*-statistic available in Limma, in a paired design to account for within-subject correlation. All reported probe *P* values were adjusted to control the false discovery rate to 0.05 within each comparison, using the Benjamini-Hochberg method in Limma. These *P* values are presented for a number of comparisons without additional adjustment.

Identification of coordinated changes in the expression of groups of functionally related genes by gene set enrichment analysis. Gene set enrichment analysis (GSEA) (25, 46) was used to detect coordinated changes in the expression of groups of functionally related genes. GSEA is a statistical method for identifying systemic gene expression changes that are characteristic of a biological process (25, 46). The GSEA algorithm takes all analyzed genes ranked in order of evidence (the signed moderated *t*-statistic from Limma) of most highly upregulated to most downregulated, and tests whether prespecified sets of genes are enriched at the top (i.e., extremely upregulated) or at the bottom (i.e., extremely downregulated) of this ranked list. Gene sets are defined as groups of genes that are related to a biological process, a cellular component, or a molecular function, as annotated according to gene ontology terms (46). These gene sets were obtained from the Molecular Signatures Database (http:/www.broadinstitute.org/gsea/) and included curated gene sets from Reactome, BioCarta, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases. To determine whether any of these gene sets showed significant differences between pre-EXTRI and one of the post-EXTRI time points (i.e., whether a group of functionally related genes was significantly up- or downregulated), the GSEA permutation familywise error rate (FWER *P* value) was used to determine statistical significance. Gene sets were considered as significantly enriched at or below an FWER *P* value of 0.05. Other results identified by GSEA included the core enrichment of gene sets that consisted of a subset of genes that contributed most to the enrichment results.

Quantitative PCR validation of gene expression. Six candidate genes were selected for confirmation of differential gene expression with quantitative real-time, reverse transcriptase polymerase chain reaction (qPCR) using TaqMan gene expression assays (Applied Biosystems, Life Technologies) on an Applied Biosystems StepOnePlus instrument (Life Technologies). These genes were selected based on their potential relevance to the exercise-induced physiological changes. Predesigned primers for the TaqMan assays were used to

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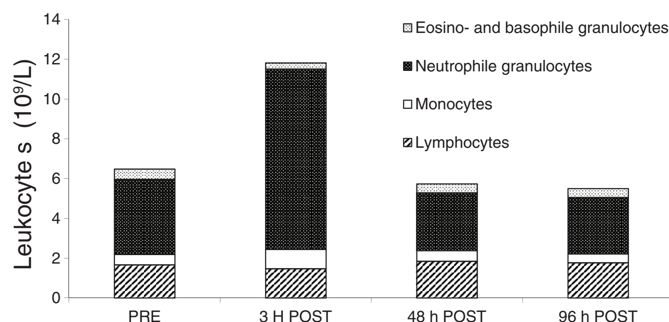

Fig. 1. Time course of blood concentrations of leukocytes and subpopulations before (pre) the experimental exercise trial (EXTRI) and 3 h, 48 h, and 96 h thereafter (post). Presented are mean concentrations for 8 study participants. For details, see RESULTS.

analyze mRNA levels of the following target genes (official gene symbol and assay ID in parenthesis): IL-1 receptor 1 (*IL1R1*, Hs00991002_m1), IL-1ra (*IL1RN*, Hs00893626_m1), IL-1 receptor associated kinase-3 (*IRAK3*, Hs00936103_m1), Toll-like receptor 4 (*TLR4*, Hs00152939_m1), granulysin (*GNLY*, Hs00246266_m1), and chemokine (C-C motif) ligand 5 (*CCL5*, Hs00174575_m1). The reaction was prepared according to the Taqman Fast Advanced Master Mix protocol in a total volume of 10 μl with target gene primers and probe, and endogenous control gene primers and probe in the same reaction (Applied Biosystems, Life Technologies). All samples were analyzed in triplicate. qPCR data were normalized as described by Vandesomplete et al. (54). Briefly, β-actin (Hs99999903_m1), and β2-microglobulin (Hs99999907_m1) were evaluated among several potential control genes and validated as the most stably expressed endogenous control genes by using geNorm analysis (54). qPCR data were then normalized to these two endogenous control genes.

Non-microarray statistical analysis. All statistical analyses, except for the microarray data (described above), were performed using PASW Statistics 17 (IBM SPSS). All non-microarray data were normally distributed as determined by the Kolmogorov-Smirnov test. The main effect of time for all dependent variables was assessed by using repeated-measures ANOVA, followed by paired *t*-tests, to assess the changes of all variables between the baseline values and each post-EXTRI time point. In accordance with the paired-design statistical analysis of the microarray data, paired *t*-tests also determined the significance in the change in the differential expression of the six target genes measured by qPCR. Pearson correlations were used to examine significant relationships between variables. Statistical significance was set at a $P < 0.05$.

RESULTS

Experimental EXTRI. All subjects successfully completed the 2-h EXTRI. The mean power output during the cycling phase was 207 ± 37 W (mean ± SD), and treadmill speed during the running phase was 11.8 ± 0.8 km/h. During the cycling phase, the mean O_2 consumption was 40.9 ± 7.5 ml·kg^{-1}·min^{-1} (73.7 ± 11.0% $\dot{V}O_{2\,peak}$), and the mean heart rate was 87.3 ± 10.3% of maximum (as determined during the incremental exercise test). The mean O_2 consumption during the running phase was 48.1 ± 5.2 ml·kg^{-1}·min^{-1} (87.3 ± 8.1% $\dot{V}O_{2\,peak}$, as determined during the incremental exercise test), while the mean heart rate was 95.0 ± 4.4% of maximum (as determined during the incremental exercise test). RPE (6–20 Borg scale) increased significantly ($P < 0.01$) from initial stages of exercise (RPE at 5 min; cycling: 12 ± 1; running: 14 ± 1) through the end of each exercise phase (cycling: 15 ± 2; running: 17 ± 1).

Total leukocyte counts and leukocyte subpopulations in the circulation. Total circulating leukocyte counts and neutrophil counts increased significantly ($P < 0.05$) by 89 and 147%, respectively, 3 h after the completion of the EXTRI. Counts for total leukocytes and all subpopulations had returned to baseline values by 48 h post-EXTRI. All changes in leukocyte subpopulations are summarized in Fig. 1.

Plasma concentrations of muscle damage markers, cortisol, complement factors, and hs-CRP. The time course of muscle damage markers is shown in Fig. 2. Compared with baseline (pre-EXTRI), plasma concentrations of myoglobin increased significantly ($P < 0.05$) by 443% 3 h post-EXTRI, returned to baseline values at 48 h post-EXTRI, and moderately increased again 96 h after completing the EXTRI (+41%; $P < 0.05$). Plasma CK activity progressively increased after the EXTRI and was significantly elevated 3 h post-EXTRI (+153%; $P < 0.01$). No significant changes in plasma concentrations of cortisol, HSP70, hs-CRP, and complement factors C3C and C4 were observed throughout the study period (Table 2).

Plasma cytokine concentrations. The time course of plasma cytokine concentrations is presented in Table 2. Three hours post-EXTRI, plasma concentrations of IL-1ra, IL-6, and IL-10 increased by 310% ($P = 0.072$), 327% ($P < 0.01$), and 36% ($P < 0.05$), respectively. Plasma concentrations of all analyzed cytokines had returned to baseline values by 48 h post-EXTRI. No statistically significant changes were observed for plasma IL-1β concentrations.

Microarray analysis. After adjusting to maintain a false discovery rate of 0.05, microarray analysis revealed that a total of 1.540 transcripts were differentially expressed 3 h post-EXTRI compared with pre-EXTRI (586 genes upregulated, and 954 genes downregulated). No transcripts were significantly differentially expressed from pre-EXTRI to 48 h or 96

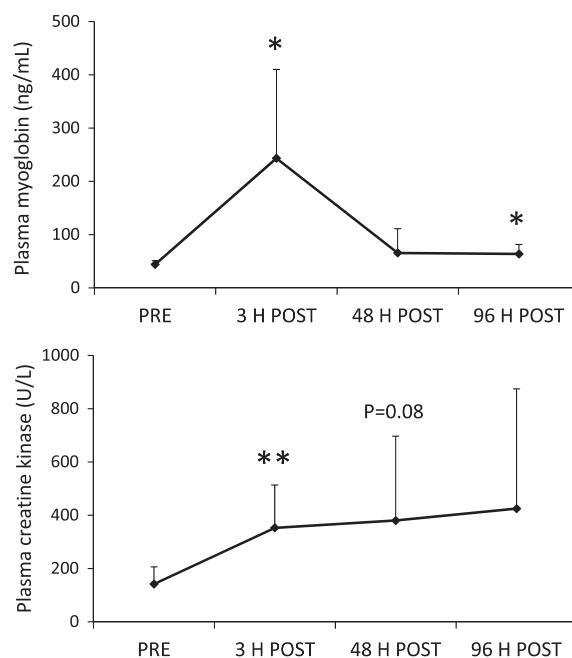

Fig. 2. Time course of plasma myoglobin concentrations and plasma creatine kinase activity pre-EXTRI and 3 h, 48 h, and 96 h post. Data are mean concentrations ± SD for 8 study participants. *Change was significantly different from baseline, $P < 0.05$. **Change was significantly different from baseline, $P < 0.01$. For observed trends, the *P* value is indicated.

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Table 2. *Plasma concentrations of cortisol, hsCRP, complement factors, HSP70, IL-1β, IL-1ra, IL-6, and IL-10 at baseline and 3, 48 and 96 h after the experimental EXTRI*

	Pre	3 h Post	48 h Post	96 h Post	Time Effect (*P* value)
Cortisol, nmol/l	271 ± 34	264 ± 41	248 ± 59	249 ± 46	0.623
hsCRP, mg/l	0.57 ± 0.54	1.90 ± 3.20	2.54 ± 3.95	1.45 ± 1.70	0.191
C3C, g/l	0.88 ± 0.22	0.95 ± 0.15	0.96 ± 0.15	1.05 ± 0.14	0.147
C4, g/l	0.18 ± 0.06	0.18 ± 0.08	0.20 ± 0.05	0.22 ± 0.06	0.140
HSP70, ng/ml	0.55 ± 0.26	0.55 ± 0.33	0.46 ± 0.27	0.77 ± 0.68	0.167
IL-1β, pg/ml	0.20 ± 0.07	0.23 ± 0.07	0.26 ± 0.08	0.27 ± 0.13	0.427
IL-1ra, pg/ml	187 ± 65	771 ± 788†	153 ± 22	167 ± 50	0.064
IL-6, pg/ml	0.73 ± 0.47	3.12 ± 1.41‡	0.61 ± 0.35	0.57 ± 0.28	0.001
IL-10, pg/ml	0.39 ± 0.06	0.53 ± 0.16*	0.42 ± 0.07	0.42 ± 0.08	0.025

Values are means ± SD; $n = 8$. hsCRP, high-sensitive C-reactive protein; HSP70, heat shock protein 70; IL-1β, interleukin (IL)-1 beta; IL-1ra, IL-1 receptor antagonist; Pre, baseline; Post, after the experimental exercise trial (EXTRI). *Significantly different from Pre values, $P < 0.05$. ‡Significantly different from Pre values, $P < 0.01$. †Trend toward a difference from Pre values ($P = 0.072$).

h post-EXTRI. The microarray data discussed in this study have been deposited in the NCBI Gene Expression Omnibus website (GEO; http://www.ncbi.nlm.nih.gov/geo) and are accessible through GEO Series accession number GSE43856.

Enrichment of groups of functionally related genes determined by GSEA. GSEA identified 40 enriched gene sets 3 h post-EXTRI, with a FWER *P* value < 0.05 (7 gene sets were enriched for highly upregulated, and 33 for highly downregulated genes). These gene sets are summarized in Table 3. No gene sets were enriched at 48 h post-EXTRI. The following three gene sets were downregulated at 96 h post-EXTRI: "reactome tRNA aminoacylation", "reactome translation of ZAP70 to immunological synapse", and "KEGG aminoacyl tRNA biosynthesis" (for all, FWER *P* value < 0.009).

qPCR validation of the expression of selected genes. To validate changes in transcript abundance within the microarray analysis, qPCR was used to assess the differential expression of six representative genes, including *IL1R1*, *IL1RN*, *IRAK3*, *TLR4*, *GNLY*, and *CCL5*. At 3 h post-EXTRI, *IL1R1*, *IL1RN*, *IRAK3*, and *TLR4* increased 5.2-fold ($P = 0.059$), 5.8-fold ($P = 0.117$), 2.0-fold ($P < 0.05$), and 2.9-fold ($P < 0.05$), respectively. Expression of *CCL5* decreased 3 h post-EXTRI (0.3-fold, $P < 0.05$). No significant change in expression was observed for *GNLY*. None of the selected genes were significantly different from pre-EXTRI at 48 or 96 h post-EXTRI. The time course of the expression changes for *TLR4*, *IRAK3*, and *IL1RN* compared with the microarray data are shown in Fig. 3. These results indicated a robust consistency between qPCR and microarray data, as depicted by the correlation between these two data sets (Fig. 4).

Associations with neutrophil TLR4 expression and circulatory neutrophil counts. Correlations between the fold-change of neutrophil *TLR4* gene expression from pre- to 3 h post-EXTRI, and pre- to 3-h post-EXTRI changes in the plasma concentrations of myoglobin ($r = 0.74$, $P < 0.05$) existed (Fig. 5). Furthermore, the pre- to 3-h post-EXTRI change in neutrophil counts, and the pre- to 3-h post-EXTRI change in plasma concentrations of IL-6 was correlated ($r = 0.83$, $P < 0.05$).

DISCUSSION

The present study demonstrates that intense endurance exercise induces a transient activation of proinflammatory IL-1 receptor (IL-1R) and TLR signaling pathways and groups of genes functionally related to the recognition of tissue damage in circulating neutrophils. The molecular signature of neutrophils 3 h after an experimental EXTRI, consisting of 2 h of cycling followed by running, reflects distinct transcriptional responses that indicate the activation and priming of neutrophils in response to intense, prolonged exercise involving moderate muscle damage. The present data suggest that the appearance of damaged muscle tissue fragments and/or muscle-derived proteins in the circulation could trigger receptor pathways similar to those involved in sterile inflammation (4), but with the difference that the exercise-induced inflammatory response was transient and tightly counterregulated. Furthermore, the present data reveal novel mechanisms, including the transcriptional activation of potent negative regulators of innate immune cellular activity (e.g., *IRAK3*), through which neutrophils may counterregulate the innate immune response to strenuous exercise. We speculate that this response might assist in preventing an excessive inflammatory response (5).

Indications of muscle damage and the systemic inflammatory response. The systemic inflammatory response to the bout of intense, prolonged exercise was characterized by increases in the circulatory total leukocyte count, plasma concentrations of the cytokine/myokine IL-6, and the anti-inflammatory cytokine IL-10, as demonstrated in previous studies (28, 29, 48). The pronounced leukocytosis was primarily due to an increase in circulating neutrophils (Table 2). Forty-eight hours after the EXTRI, total leukocyte counts, including all leukocyte subpopulations, had returned to baseline concentrations, suggesting the resolution of the systemic inflammatory response. Furthermore, our endurance exercise protocol, and, most likely, in particular the running phase, induced a moderate degree of muscle damage, as indicated by the increase of the muscle protein myoglobin and the muscle enzyme CK in the circulation, with similar values as reported after 1 h of high-intensity running (33) or a duathlon race (47). Prolonged running [involving frequent eccentric/lengthening contractions (31)], especially when performed at a high intensity (33), over a very long distance (28, 48), and/or immediately after cycling (6, 28, 47), is a known cause for moderate-to-severe muscle damage (31). The ultrastructural damage to the extracellular matrix of the muscle might increase the permeability of the sarcolemma membrane (31) and facilitate the leakage of muscle components across the myocellular membrane (as indicated by the efflux of myoglobin and CK) (35).

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Table 3. *Differentially regulated gene sets 3 h after the experimental EXTRI as identified by gene set enrichment analysis*

Gene Set Name	Size	ES	NES	FWER *P* Value
Positively regulated gene sets 3 h post-EXTRI				
Reactome RNA polymerase I promoter opening	34	0.752	2.579	0.000
Reactome packaging of telomere ends	32	0.716	2.389	0.000
KEGG Systemic Lupus Erythematosus	96	0.539	2.243	0.001
Response to other organism	63	0.564	2.195	0.004
KRCTCNNNNMANAGC_unknown	31	0.664	2.17	0.009
Biocarta IL-1 receptor pathway	27	0.661	2.116	0.023
KEGG Toll-like receptor signaling pathway	79	0.517	2.103	0.029
Negatively regulated gene sets 3 h post-EXTRI				
Reactome translation	120	−0.585	−2.43	0.000
Reactome tRNA aminoacylation	38	−0.73	−2.401	0.000
Reactome GTP hydrolysis and joining of the 60-s ribosomal subunit	106	−0.587	−2.385	0.000
Structural constituent of ribosome	79	−0.613	−2.377	0.000
KEGG aminoacyl tRNA biosynthesis	40	−0.698	−2.359	0.000
Reactome formation of a pool of free 40-s subunits	95	−0.592	−2.349	0.000
Nucleolus	103	−0.58	−2.335	0.000
Reactome peptide chain elongation	84	−0.577	−2.294	0.001
Reactome translation initiation complex formation	56	−0.626	−2.274	0.001
KEGG ribosome	85	−0.585	−2.273	0.001
Reactome formation of the ternary complex and the 43S complex	49	−0.647	−2.267	0.001
Reactome cytosolic tRNA aminoacylation	21	−0.776	−2.26	0.001
Reactome gene expression	403	−0.473	−2.248	0.001
Reactome influenza viral RNA transcription and replication	99	−0.556	−2.242	0.001
Reactome viral mRNA translation	84	−0.566	−2.213	0.001
Reactome PD1 signaling	22	−0.748	−2.207	0.002
Translation factor activity nucleic acid binding	35	−0.683	−2.207	0.002
Reactome metabolism of RNA	90	−0.551	−2.161	0.006
Reactome influenza life cycle	134	−0.516	−2.161	0.006
Ribonucleoprotein complex biogenesis and assembly	71	−0.564	−2.153	0.009
tRNA metabolic process	16	−0.782	−2.146	0.010
Mitochondrial inner membrane	60	−0.583	−2.144	0.010
Reactome insulin synthesis and secretion	121	−0.514	−2.134	0.012
Reactome mRNA splicing minor pathway	39	−0.631	−2.131	0.014
Interphase of mitotic cell cycle	50	−0.598	−2.127	0.014
Reactome processing of capped intron containing pre mRNA	131	−0.509	−2.112	0.020
Reactome regulation of gene expression in beta cells	89	−0.53	−2.11	0.020
Mitochondrial part	132	−0.503	−2.11	0.020
Mitochondrial matrix	46	−0.605	−2.106	0.022
Translation regulator activity	37	−0.632	−2.095	0.029
Reactome RNA polymersase III transcription	31	−0.658	−2.092	0.030
Reactome regulation of beta cell development	96	−0.514	−2.067	0.047
Reactome RNA polymerase III transcription initiation	26	−0.672	−2.064	0.048

Size, no. of genes in the gene set after filtering out those genes not in the expression data set; ES, enrichment score for the gene set (i.e., the degree to which this gene set is overrepresented at the top or bottom of the ranked list of genes in the expression data set); NES, normalized ES (i.e., the ES for the gene set after it has been normalized across analyzed gene sets); FWER *P* value, familywise error rate (i.e., a conservative estimated probability that the NES represents a false positive finding); KEGG, Kyoto Encyclopedia of Genes and Genomes. For more details refer to MATERIALS AND METHODS.

Transcriptional changes associated with the activation of innate immunity. Along with the increase in circulating neutrophils, 40 groups of functionally related genes (i.e., gene sets) were differently expressed 3 h post-EXTRI (Table 3). The transcriptomic changes confirm the current concept that the acute neutrophil response to inflammatory stimuli, such as physical stress and/or tissue injury, is regulated at a transcriptional level (40, 51). Furthermore, and perhaps most importantly, the positively regulated gene sets included the "biocarta IL-1R pathway", the "KEGG TLR signaling pathway", and annotated gene sets associated with the recognition of tissue damage. The transcriptional activation of these proinflammatory pathways indicates the acute activation of innate immune cellular activity following strenuous physical activity. This observation is in accordance with the paradigm of the "acute phase response" to physical stress in which immune cells, particularly neutrophils, are "alarmed" (i.e., mobilized and activated) to initiate further inflammatory and immune responses to potential threats, such as invading pathogens or tissue injuries (5, 36, 41). The activation of these specific pathways, however, is a novel finding, providing important information on the molecular mechanisms underlying the neutrophil response to a single bout of intense, prolonged exercise involving moderate muscle damage.

Indications for muscle-derived damage-associated molecular patterns as stimulus for the transcriptional activation of TLR signaling in neutrophils. IL-1R and TLR signaling are closely related to the innate immune response (8). Due to a highly homogenous cytoplasmic receptor domain, the ligation of IL-1 to *IL1R1*-like receptors (30), and microbial motifs or endogenous factors such as HSPs to TLRs (4, 17), induce similar signal transduction cascades (8, 30). IL-1R and TLR signaling activate common downstream pathways, such as the nuclear factor-κB (NF-κB) and the mitogen-activated protein

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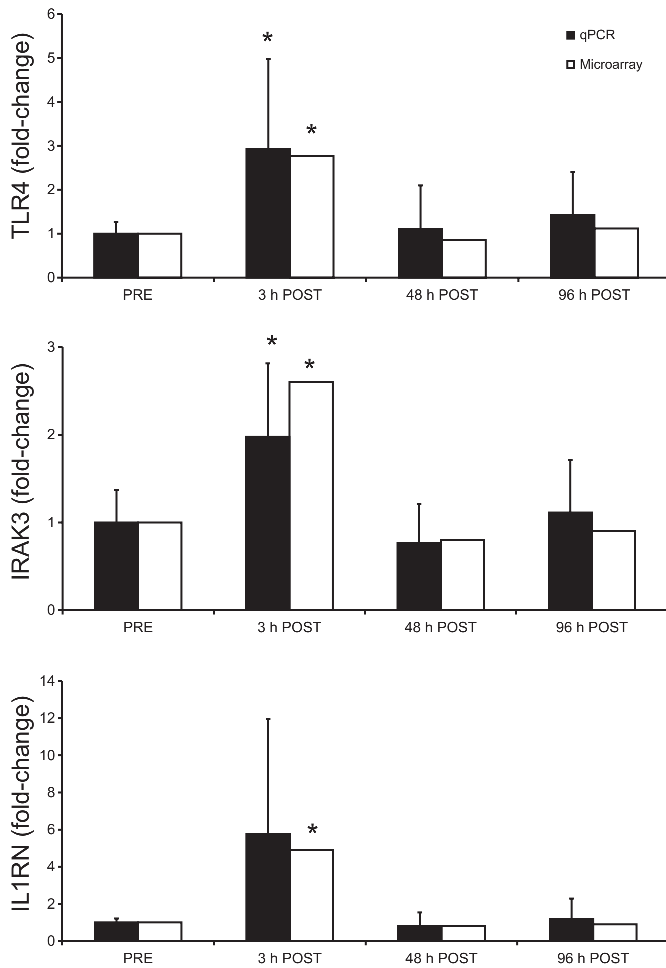

Fig. 3. Fold changes in the gene expression of Toll-like receptor 4 (*TLR4*), interleukin (IL)-1 receptor-associated kinase 1 (*IRAK3*), and IL-1 receptor antagonist (*IL1RN*) from pre- to 3 h, 48 h, and 96 h post-EXTRI. Presented are quantitative (q) PCR (mean fold changes ± SD) and microarray data (mean fold changes) for 8 study participants. *Change was significantly different from baseline, $P < 0.05$.

kinase pathway, resulting in the upregulation of proinflammatory cytokines and chemokines (4, 8). Furthermore, there is increasing evidence that TLRs not only detect structures found in pathogenic microorganisms (referred to as pathogen-associated molecular patterns), but also nonmicrobial, host-derived molecules that are hidden from the recognition by the host immune system under normal physiological conditions (4, 17). In the case of cellular stress or injury, these intracellular factors can be released into the extracellular environment and trigger a "sterile inflammatory response" (4). Collectively, these endogenous "danger signals" have been termed damage-associated molecular patterns (DAMPs) (4).

On the basis of several indications revealed by our data, we hypothesize that tissue fragments and/or endogenous molecules released into the circulation by stressed and/or injured cells were recognized by neutrophils, which, in turn, might have led to the upregulation of genes involved in TLR signaling (including *TLR1*, *TLR4*, *TLR6*, and *TLR8*) (Table 4). Infectious microorganisms can be ruled out as a stimulus for the transcriptional activation of the TLR pathway. It is unlikely that the 2-h EXTRI (performed in temperate conditions) induced a gut ischemia-associated leakage of endotoxins into the circulation as observed after a long-distance triathlon performed in extreme heat conditions (16). A far more likely explanation is that DAMPs, hypothetically originating from damaged skeletal muscle tissue, were associated with the activation of the TLR signaling pathway in circulatory neutrophils. This hypothesis is supported by the observed correlation between plasma myoglobin and neutrophil *TLR4* gene expression 3 h post-EXTRI (Fig. 5). Potential additional sterile inflammatory signals that are known to interact with TLRs and to increase in the circulation after strenuous exercise include endogenous nucleic acids (4, 17), such as circulating cell-free

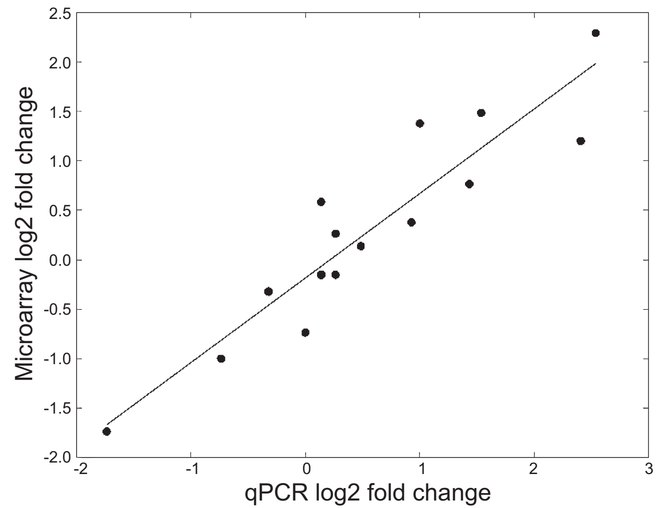

Fig. 4. Correlation between qPCR and microarray data after $\log_2$ transformation on the mean differential expression (n = 8) of IL-1 receptor 1 (*IL1R1*), *IL1RN*, *IRAK3*, *TLR4*, granulysin (*GNLY*), and chemokine (C-C motif) ligand 5 (*CCL5*) at 3, 48, and 96 h post-EXTRI, compared with pre-EXTRI. Note that there are multiple data points with overlapping coordinates.

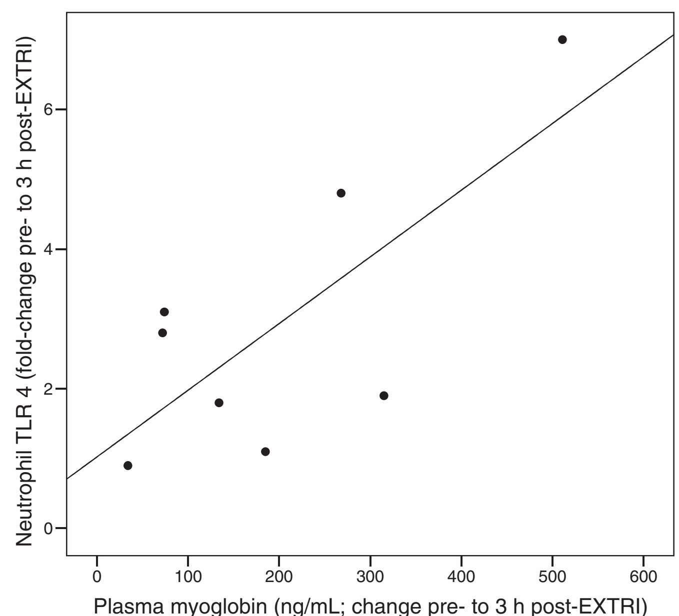

Fig. 5. Correlation between the fold change of neutrophil *TLR4* gene expression from pre- to 3 h post-EXTRI, and pre- to 3 h post-EXTRI changes in the plasma concentrations of myoglobin ($r = 0.74$; $P < 0.05$).

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Table 4. *Upregulated gene sets related to IL-1R, TLR signaling, and damage-associated molecule patterns 3 h following the experimental EXTRI, and the time course of expression changes of representative genes contributing to the core enrichment of these gene sets*

Selected Upregulated Gene Sets 3 h Post-EXTRI			Representative Genes Contributing to the Core Enrichment		3 h Post-EXTRI		48 h Post-EXTRI		96 h Post-EXTRI	
Size	ES	FWER *P* value	Gene symbol	Gene name	Adjusted *P* value	FC	Adjusted *P* value	FC	Adjusted *P* value	FC
				KEGG Systemic lupus erythematosus						
96	0.539	0.001	*HIST1H2AC*	Histone cluster 1, H2ac	0.005	7.3	1.000	0.7	0.999	1.0
			HIST1H2BK	Histone cluster 1, H2bk	0.005	3.7	1.000	1.0	0.999	1.0
			HIST2H4A	Histone cluster 2, H4a	0.008	2.6	1.000	1.1	0.998	1.2
			FCGR2A	Fc fragment of IgG, low affinity IIa, receptor (CD32)	0.008	3.8	1.000	1.1	0.998	1.1
			FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	0.031	2.5	1.000	1.0	0.998	1.5
			FCGR3B	Fc fragment of IgG, low affinity IIIb, receptor (CD16b)	0.070	6.9	1.000	0.7	0.998	1.4
			FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	0.161	2.3	1.000	0.9	0.998	1.6
			IL10	interleukin 10	0.307	1.4	1.000	1.0	0.999	1.0
			CTSG	Cathepsin G	0.351	1.3	1.000	1.0	0.999	1.1
				Response to other organism						
63	0.564	0.004	*S100A12*	S100 calcium binding protein A12	0.006	6.8	1.000	0.7	0.998	1.2
			TLR6	Toll-like receptor 6	0.008	4.1	1.000	1.0	0.998	1.1
			CAMP	Cathelicidin antimicrobial peptide	0.012	4.4	1.000	1.0	0.998	1.3
			TLR8	Toll-like receptor 8	0.034	3.3	1.000	0.6	0.998	1.5
			IFNGR2	Interferon gamma receptor 2	0.022	2.5	1.000	0.8	0.998	1.3
			IFNAR1	Interferon (alpha, beta and omega) receptor 1	0.027	2.5	1.000	0.7	0.998	1.3
			CXCR4	Chemokine (C-X-C motif) receptor 4	0.039	3.8	1.000	1.0	0.998	1.0
			BNIP3L	BCL2/adenovirus E1B 19 kDa interacting protein 3-like	0.049	2.0	1.000	0.8	0.998	1.2
			BCL3	B-cell CLL/lymphoma 3						
			NLRC4	NLR family, CARD domain containing 4	0.049	3.1	1.000	0.8	0.998	0.9
				Biocarta IL-1 receptor pathway						
27	2.116	0.023	*IL1RN*	Interleukin 1 receptor antagonist	0.005	4.8	1.000	1.0	0.998	1.5
			MAPK14	Mitogen-activated protein kinase 14	0.010	2.9	1.000	1.2	0.998	1.3
			IRAK3	Interleukin-1 receptor-associated kinase 3	0.018	2.6	1.000	0.8	0.998	0.9
			IL1B	Interleukin 1, beta	0.024	2.9	1.000	0.7	0.998	1.6
			MAP2K3	Mitogen-activated protein kinase kinase 3	0.031	1.9	1.000	1.1	0.998	1.0
			IL1R1	Interleukin 1 receptor, type I	0.086	2.2	1.000	1.5	0.998	1.7
			IL1RAP	Interleukin 1 receptor accessory protein	0.195	1.9	1.000	1.1	0.998	1.6
			NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	0.341	1.5	1.000	1.1	0.998	1.1
				KEGG Toll-like receptor signaling pathway						
79	0.517	0.029	*TLR6*	Toll-like receptor 6	0.008	4.1	1.000	1.0	0.998	1.1
			MAPK14	Mitogen-activated protein kinase 14	0.010	2.9	1.000	1.2	0.998	1.3
			TLR8	Toll-like receptor 8	0.034	3.3	1.000	0.6	0.998	1.5
			FADD	Fas (TNFRSF6)-associated via death domain	0.015	2.2	1.000	1.0	0.999	1.0
			TLR1	Toll-like receptor 1	0.016	5.2	1.000	1.0	0.998	2.0
			LY96	Lymphocyte antigen 96	0.018	4.7	1.000	0.7	0.998	2.2
			TLR4	Toll-like receptor 4	0.022	2.8	1.000	0.9	0.998	1.1
			IL1B	Interleukin 1, beta	0.024	2.9	1.000	0.7	0.998	1.6
			IFNAR1	Interferon (alpha, beta and omega) receptor 1	0.027	2.5	1.000	0.7	0.998	1.3
			MAP2K3	Mitogen-activated protein kinase kinase 3	0.031	1.9	1.000	1.1	0.998	1.0
			CASP8	Caspase 8, apoptosis-related cysteine peptidase	0.072	1.6	1.000	1.3	0.999	1.0
			TLR2	Toll-like receptor 2	0.147	1.8	1.000	0.8	0.998	1.3
			FOS	FBJ murine osteosarcoma viral oncogene homolog	0.300	2.1	1.000	0.7	0.998	0.8

Size, number of genes in the gene set after filtering out those genes not in the expression dataset; FC, fold change.

DNA (2, 3), free fatty acids (4, 13), and HSPs (14), in particular the stress-inducible isoform of the HSP70 family, HSP72 (1, 12). Considering previous studies showing increases in plasma/serum HSP70/72 concentrations immediately after intense, prolonged exercise (10, 34, 56) and a decrease thereafter (34, 56), we might have missed detecting a significant plasma HSP70 response at 3 h post-EXTRI, similar as with cortisol (23, 56). This, however, does not preclude that a potential earlier increase in plasma HSP70 could have contributed to the transcriptional activation of neutrophil TLRs in the present study. In addition to HSPs, increased plasma concentrations of cytokines and stress hormones are thought to mediate exercise-induced changes in TLR cell surface expression on monocytes (13, 14, 23). Furthermore, the upregulation of genes related to IL-1R signaling [including IL-1ra (*IL1RN*), mitogen-activated protein kinase 14, and IL-1β (*IL1B*)] (Table 4) might have also been induced by TLR ligands (8) and NF-κB activation (50). Plasma concentrations of IL-1, as a

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known stimulus for increased transcription of IL-1 mRNA (8), do not change significantly following exercise (29, 31), which was confirmed in the present study.

Further gene sets upregulated in response to DAMPs. The upregulation of functionally related gene groups associated with the immune response to a potential internal or invasive threat (i.e., the gene set referred to as "response to other organism"), and the autoimmune disease lupus erythematosus 3 h post-EXTRI supports the concept that innate immunity was activated, particularly in response to DAMPs. Representative genes that contributed most to the coordinated changes in the expression of the gene set, "response to other organism," included S100 calcium binding protein A12 (*S100A12*), cathelicidin antimicrobial peptide (*CAMP*, also known as LL37), and interferon-γ receptor 2 (*IFNGR2*) (Table 4). S100 proteins are classified as a novel group of DAMP molecules that act as danger signals, are recognized by TLRs (11), and can also be released from skeletal muscle tissue (26). S100A12 proteins are mainly secreted by activated granulocytes in the bloodstream or in inflamed tissue, inducing the recruitment of (more) granulocytes and monocytes to the inflammatory site (11). CAMP is a specific granular peptide with chemotactic and antimicrobial capabilities (27) that is also responsible for the recognition of endogenous nucleic acids (17).

Systemic lupus erythematosus is characterized by circulating immunoglobulin G (IgG) auto-antibodies that are specific for self-antigens such as cell-free DNA (3, 4). Exercise-induced muscle damage is also known to affect neutrophil receptors for IgG molecules and complement proteins that coat the surface of tissue fragments (33). Consequently, the transient appearance of host tissue fragments in the circulation originating from cell/muscle damage could be responsible for upregulation of genes encoding low-affinity IgG receptors such as FCGR2A (CD32), and FCGR3A (CD16a), which contributed to the core enrichment of the gene set "KEGG systemic lupus erythematosus" (Table 4). Peake et al. (33) reported a decrease in CD16 expression on the surface of neutrophils after running, which was, hypothetically, related to the potentially cortisol-induced, delayed release of a new population of immature neutrophils that had shed CD16 when they were mobilized from the bone marrow (33, 48). Based on these previous and the present findings, we hypothesize that newly recruited neutrophils began to replace CD16 receptors on a transcriptional level in response to exercise-induced muscle damage.

Collectively, the observed molecular signature of neutrophils may reflect a distinct transcriptional profile, particularly, although not exclusively, in response to the muscle-damaging component of the EXTRI (i.e., intense running immediately after intense cycling). In addition to the ultrastructural damage of muscle tissue, metabolic, hormonal, thermal, and oxidative stress are characteristic responses to prolonged, strenuous exercise, all of which can lead to the release of cytokines, acute phase proteins, and to the regulation of certain lines of the cellular immune system, particularly neutrophils (28). However, the conclusion that, in particular, DAMP molecules derived from damaged muscle tissue were responsible for the activation and priming of neutrophils is also supported when comparing our results with those of the study of Radom-Aizik et al. (40), which involved only high-intensity cycling. Cycling involves minimal eccentric contractions and thus far less muscle-damaging work than intense running (31). Although additional factors, such as the different training status of the study participants and the use of different microarray technologies, have to be considered (40), a comparison between the findings of Radom-Aizik et al. and the present data reveal obvious differences. For example, neither TLRs, IL-1R-associated genes, low-affinity IgG receptors, nor S100 proteins were upregulated in neutrophils after cycling (40), suggesting that the responses as observed in the present study were related to muscle-damaging component of the EXTRI.

Transcriptional changes in neutrophils indicating counterregulatory mechanisms. The acute, systemic inflammatory response to strenuous, prolonged exercise in trained individuals is counterregulated by potent compensatory mechanisms (28, 37, 55). The latter includes the increase of anti-inflammatory cytokines, such as IL-10 (28) and IL-1ra (29), which was confirmed in the present study. Various mechanisms tightly regulate innate immune function to avoid overshooting inflammation (e.g., an overproduction of proinflammatory cytokines and chemokines or a persistent activation of proinflammatory pathways) (19). Notably, potent negative regulators of these pathways were strongly upregulated. Among the genes that contributed to the core enrichment of the biocarta IL-1R pathway were IL-1ra (*IL1RN*, the translated protein is IL-1ra) and *IRAK3*. *IL1RN* increased more than fourfold, and *IRAK3* more than twofold at 3 h post-EXTRI (Fig. 3). Both genes were represented at the top of the ranked list of genes among the IL-1R pathway members (Table 4). The anti-inflammatory cytokine IL-1ra binds to IL-1R, and it blocks the activity of IL-1 (8). Neutrophils are capable of producing and secreting IL-1ra at inflamed tissue sites and in the circulation (32, 43) and might have contributed to the clear trend toward increased IL-1ra plasma concentrations 3 h post-EXTRI. *IRAK3* is an inhibitor of IL-1R and TLR signaling (18, 19). Induced through a negative feedback loop upon TLR stimulation, *IRAK3* inhibits IL-1R and TLR signaling, possibly by interrupting its downstream signaling mediated by the myeloid differentiation primary response gene 88 (18, 19). To the best of our knowledge, this study is the first to demonstrate the upregulation of the *IRAK3* gene in neutrophils in response to exercise. As shown in immunological studies (18, 19), *IRAK3* may also contribute to the stabilization of the innate immune response to strenuous exercise (Fig. 6). Moreover, the subset of genes that contributed to the core enrichment of the TLR signaling pathway included genes with potential inhibitory functions, such as FAS-associated protein with death domain (FADD) and caspase-8. The activation of the FADD/caspase-8 axis through TLR2 signaling initiates apoptosis (51) and may, in turn, contribute to the resolution of inflammation (21). Furthermore, FADD can inhibit TLR- and IL-1R-induced NF-κB signaling (53). The present results indicate that the upregulation of genes, such as *IL1RN*, *IRAK3*, and *FADD*, could contribute to the negative regulation of the proinflammatory IL-1R and TLR pathways, as proposed in Fig. 6.

Transcriptional changes associated with apoptosis and the resolution of inflammation. The transcriptional downregulation of 33 gene sets 3 h post-EXTRI (including groups of genes functionally involved in transcription and translation) (Table 3), is likely explained by the induction of early stages of apoptosis 3 h after neutrophil activation (20). Concurrently, antiapoptotic responses were also observed, which is in accordance with previous findings showing the concomitant activa-

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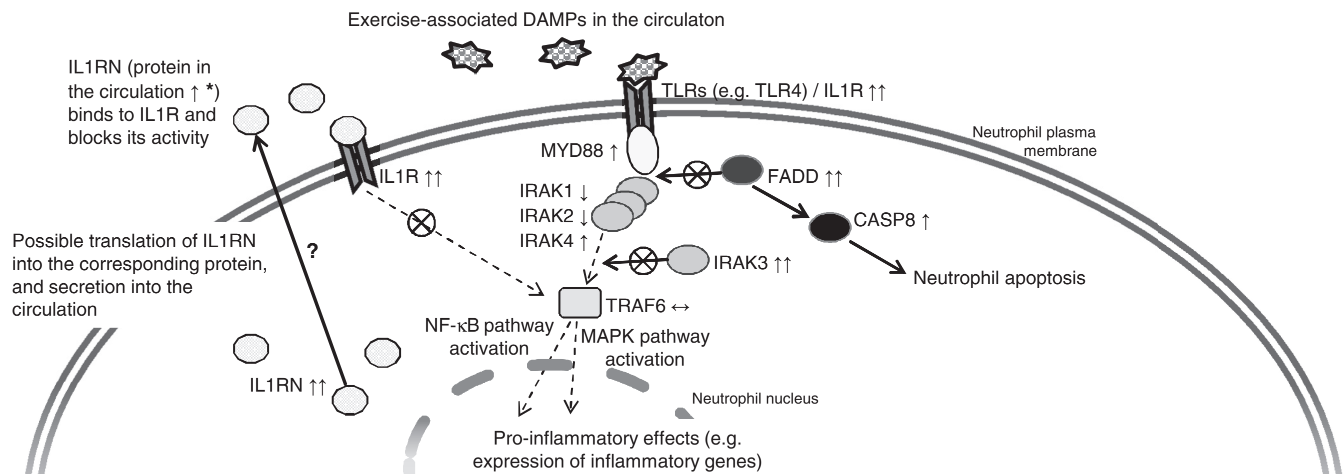

Fig. 6. Hypothesized model of mechanisms by which neutrophils appear to counterregulate proinflammatory TLR and IL-1 receptor (IL-1R) signaling in response to intense endurance exercise. *IRAK3* inhibits IL-1R and TLR signaling, possibly by interrupting its downstream signaling mediated by the interaction between *IRAK1* and *IRAK4*, and myeloid differentiation primary response gene 88 (*MYD88*) (18, 19). The activation of the axis of Fas (TNFRSF6)-associated via death domain (*FADD*) and caspase 8, apoptosis-related cysteine peptidase (*CASP8*) can initiate apoptosis (53), which may, in turn, contribute to the resolution of inflammation. The anti-inflammatory cytokine *IL1RN* (the translated protein is referred to as IL-1ra) binds to IL-1R and blocks the activity of IL-1. For details, the reader is referred to the text. DAMPs, damage-associated molecule patterns; NF-κB, nuclear factor-κB; *MAPK*, mitogen-activated protein kinase; *IRAK1* (-2, -3, -4), IL-1 receptor-associated kinase-1, (-2, -3, -4); *TRAF6*, tumor necrosis factor receptor-associated factor 6. Circle with X, inhibition; ↓, decreased gene expression 3 h post-EXTRI; ↑, increased gene expression 3 h post-EXTRI; ↑ ↑, more than twofold increase in gene expression 3 h post-EXTRI; ↔, no significant changes in gene expression; ↑* increase of ILRN protein (IL-1ra) concentration in the circulation 3 h post-EXTRI ($P = 0.072$). For further details on plasma concentrations of IL-1ra and fold changes of genes, see Tables 2 and 4, respectively.

tion of pro- and antiapoptotic genes in neutrophils in response to exercise (40) and other inflammatory stimuli (45). Together with the negative regulation of programmed cell death protein 1 signaling, several genes involved in the protection from oxidative stress (e.g., superoxide dismutase 2, increased nearly eightfold), and in the inhibition of apoptosis (e.g., myeloid cell leukemia sequence, BCL-2 related, increased more than threefold) were upregulated 3 h post-EXTRI. These findings are consistent with the concept that the activation of neutrophils is accompanied by responses for prolonging their functional longevity, including the inhibition of apoptosis or upregulation of cellular-protective mechanisms (9, 40, 45). The prolongation of the life span of activated neutrophils could enable them to migrate to infected or injured tissue areas (45), including muscle tissue. It should also be noted that systemic inflammation rapidly leads to a functionally heterogeneous neutrophil pool in the circulation (39). Hence, these data might reflect the transcriptional responses of different neutrophil subpopulations, e.g., mature and immature neutrophils, and neutrophil subsets initiating apoptosis while others were primed to infiltrate into skeletal muscle.

Importantly, none of the gene sets that were enriched 3 h post-EXTRI were differentially regulated 48 and 96 h post-EXTRI, nor were any other gene sets upregulated persistently. This suggests that the activated neutrophils may have infiltrated into the muscle tissue and/or had undergone apoptosis, thus facilitating the resolution of the systemic inflammatory response, while the circulating neutrophil population had apparently returned to its (inactivated) baseline state. Collectively, our data support the concept that the initial proinflammatory response of neutrophils to strenuous exercise is rapidly counterregulated in healthy, trained individuals.

Perspectives and significance. The data from the present study provide an advanced understanding on the molecular basis upon which neutrophils are transcriptionally activated and primed in response to a single bout of intense endurance exercise involving moderate muscle damage. Potential stimuli for coordinately changing the expression of functionally related gene groups, including those related to IL-1R and TLR signaling, and pathways involved in the sensing and reacting to tissue damage, include (but are not limited to) muscle components released into the circulation (e.g., myoglobin). We recognize the complexity of the regulatory mechanisms underlying the neutrophil immune responses (27, 51) and that the observed changes in the transcriptome of neutrophils are probably further regulated by micro-RNA involvement and on a protein expression level (37). Additional research is warranted to determine both the exact mechanisms behind and the functional consequences of the transcriptomic responses of neutrophils to strenuous endurance exercise involving muscle damage. However, these findings reflect an important advancement in the research field and appear to be crucial for establishing a novel group of specifically exercise-induced DAMPs. Moreover, the data from the present study suggest a novel mechanism by which neutrophils could counterregulate the acute innate immune response to strenuous exercise to avoid excessive inflammation. Notably, this mechanism may contribute to the shift from a proinflammatory to an anti-inflammatory environment after acute exercise. The potential role of neutrophils in regulating not only the initial systemic inflammation, but also immune homoeostasis in response to strenuous exercise, might have important implications for understanding the anti-inflammatory and health-promoting effects of regular exercise training (10, 33).

ACKNOWLEDGMENTS

The authors thank the study participants for participation, Jennifer Isautier, Glenn Stewart and Kavita Bisht for support in the laboratory and during the experimental exercise trials, and Dr. Laurie Kelly for medical support.

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GRANTS

O. Neubauer acknowledges the Austrian Science Fund for the award of an Erwin Schrödinger-Fellowship Abroad (project no. J 3066-B09).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: O.N., S.S., B.D., J.M.P., D.C.-S., L.J.H., K.-H.W., and A.C.B. conception and design of research; O.N., S.S., A.C.B., R.L., and J.B.J. performed experiments; O.N., R.L., and A.C.B. analyzed data and interpreted results of experiments; O.N. prepared figures and drafted manuscript; O.N., S.S., B.D., J.M.P., D.C.-S., L.J.H., and A.C.B. edited and revised manuscript; O.N., S.S., R.L., B.D., J.M.P., D.C.-S., L.J.H., K.-H.W., and A.C.B. approved final version of manuscript.

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