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Microarray analysis after strenuous exercise in peripheral blood mononuclear cells of endurance horses

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Summary

It is known that moderate physical activity may have beneficial effects on health, whereas strenuous effort induces a state resembling inflammation. The molecular mechanisms underlying the cellular response to exercise remain unclear, although it is clear that the immune system plays a key role. It has been hypothesized that the physio-pathological condition that develops in athletes subjected to heavy training is caused by derangement of cellular immune regulation. The purpose of the present study was to obtain information on endurance horse gene transcription under strenuous conditions and to identify candidate genes causing immune system derangement. We performed a wide gene expression scan, using microarray technology, on peripheral blood mononuclear cells of ten horses chosen from high-level participants in national and international endurance races. The use of three different timepoints revealed changes in gene expression when post-effort samples (T1, taken immediately after the race; and T2, taken 24 h after the race) were compared with basal sample (T0, at rest). Statistical analysis showed no differences in gene expression between T0 and T2 samples, indicating complete restoration of homeostasis by 24 h after racing, whereas T1 showed strong modulation of expression, affecting 132 genes (97 upregulated, 35 downregulated). Ingenuity pathway analysis revealed that the main mechanisms and biofunctions involved were significantly associated with immunological and inflammatory responses. Real-time PCR was performed on 26 gene products to validate the array data.

Keywords gene expression, inflammation, peripheral blood mononuclear cells (PBMCs), qRT-PCR, stress.

Introduction

The horse is a natural athlete and good athletic performance is economically valuable. For historical and cultural reasons, selection of the best racehorses is not always based on objective parameters. However, all breeders and researchers agree that a winner is healthy and of high genetic value.

Knowledge of the molecular mechanism of exercise-induced stress is of fundamental importance in planning an

appropriate training schedule to obtain better performance and to preserve the health of the animal. Exercise induces marked transient physiological changes in the body, including increased blood flow, increased body temperature, changes in hormone levels, synthesis of heat-shock proteins, oxidative stress, and the production of interleukins and receptors together with many other substances related to inflammatory processes. Some of these physiological effectors have been discussed with reference to their effects on the blood immune cell system, the study of which is termed immunocompetence (Gleeson 2007).

It is known that leucocytes are involved not only in eradication of pathogens but also play roles in wound repair, muscle growth, and other key developmental processes (Sabroe & Whyte 2007; Tidball & Wehling-Henricks 2007). In addition, it is now known that substantial changes in the gene expression profile pattern of circulating leucocytes occur rapidly during exercise in both humans

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and horses (Cappelli *et al.* 2007; Barrey *et al.* 2006; Cappelli *et al.* 2009; Fehrenbach 2007). The impact of exercise on the immune response is increasingly seen as a novel mechanism by which physical activity modulates health, growth, and disease risk, owing to the immunomodulatory effects of exercise, training, and fatigue.

Regular moderate exercise is associated with a reduced incidence of infection compared with a completely sedentary state. However, prolonged bouts of strenuous exercise cause a temporary depression of various aspects of immune function, inducing an inflammation-like condition, depending on the intensity and duration of the exercise bout. Owing to the importance of this topic in medical and sports sciences, many studies have focused on the effects of exercise on white blood cells in peripheral blood, and the recent introduction of microarrays has promoted extensive research activity, particularly in this tissue, including studies on cytokine and receptor expression, apoptosis, and control of cell proliferation (Connolly *et al.* 2004; Zieker *et al.* 2005; Fehrenbach 2007; Buttner *et al.* 2007). Although strenuous exercise in horses has also been associated with increased susceptibility to infection (Folsom *et al.* 2001), little is known regarding the early immune responses involved in this phenomenon. It has been hypothesized that the physio-pathological condition that develops in horses subjected to heavy training (i.e. the overtraining syndrome) is caused by derangement of cellular immune regulation (Lakier Smith 2003).

Thus, the purpose of the present study was to analyse transcriptome modulation of the acute response during horse endurance races to identify candidate genes involved in immune system derangement. Similar to a human marathon runner, an endurance horse invests an extreme effort in racing, and peripheral blood mononuclear cells (PBMCs) may be the most appropriate cell type for investigation of immune and physiological changes connected to exhausting exercise.

Methods

Blood collection and RNA extraction

Animals were treated according to standard procedures governing animal welfare, with the permission of the owners, team veterinarians, and official racing veterinary commissions.

Blood samples were taken from the jugular veins of horses chosen from among high-level performers in national and international endurance races (90–120 km). Samples were obtained at three different time-points: before (T0), at the end of the race (T1) and 24 h after the race (T2). Only horses that passed FISE (Italian Equestrian Sports Federation) compulsory medical checks (pre-, during, and post-race) were included in this study. Ten endurance horses, comparable in terms of type and intensity of training, were

recruited. Horse age varied 5 from to 13 years, with a mean of 6.8 ± 2.44 . Veterinary examination included analysis of cardio-respiratory function, physical integrity, and metabolic condition, together with collection of a blood sample for a complete count.

Immediately after collection of blood, PBMCs were isolated by the Ficoll-Hypaque method (GE Healthcare). Total RNA was extracted using the Aurum Total RNA Fatty and Fibrous Tissue kit (Bio-Rad) according to the manufacturer's instructions. Genomic DNA was eliminated using DNase supplied with the kit. Extracted RNA was quantified using the Quant-It RNA assay (Invitrogen) in a VersaFluor fluorometer (Bio-Rad), and RNA quality was verified by microchannel electrophoresis (RNA 6000 Nano LabChip; Bioanalyzer). Successful removal of DNA contaminants was demonstrated by absence of PCR amplification of the *MCI1R* gene [GenBank accession number X98012; primers as described by Rieder *et al.* (2001)].

Production of the equine oligonucleotide microarray

Gene expression analysis was performed using an equine/murine oligonucleotide microarray including 384 equine transcripts, of which 50 were probes for the mitochondrial and 334 were probes for the nuclear genome (GEO Platform a.n.; GPL8349; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL8349>). The 50 probes of the equine mitochondrial genome were designed to measure expression of the 13 genes (two different probes per gene) coding for protein units of the respiratory chain, as well as 22 tRNAs and 2 rRNAs. This oligonucleotide microarray was synthesized by the method employed to construct open-access human and mouse long oligonucleotide microarrays (Barrey *et al.* 2009). Microarrays were spotted by a microarray service (LEFG; CEA, Evry, France) by printing equine probes suspended in a spotting buffer of 50% (v/v) dimethyl sulfoxide (DMSO) in TE onto hydrogel slides, using a Microgrid-II robot (Schott Nexterion).

Gene expression analysis using the oligonucleotide microarray

The race (T1) and the 24-h (T2) samples were hybridized against the basal sample (T0). The hybridization protocol has been described previously (Barrey *et al.* 2009). Fluorescence intensities of Cy3 (532 nm) and Cy5 (635 nm) were separately scanned using a GenePix 4000B laser scanner.

TIFF images (10 μm /pixel) were analysed using specific software (GenePix Pro 6.0 software) that automatically locates spots, with a manual control, and quantifies fluorescence intensity. For each microarray, fluorescence intensities of all spots corresponding to any particular gene constituted the data output. This information was imported into genomic data analysis software (Gene Spring) for

normalization prior to filtering and statistical analysis. For each sample, duplicate analysis (dye-swap) was performed and average fluorescence results were used. Next, all results were normalized by individual spot, and by microarray, using the Lowess regression method, to obtain a linear relationship between each sample and control. Finally, a gene expression ratio was obtained for each sample and gene:

$$\text{Ratio} = \text{Expression of T1 or T2} / \text{Expression at T0}$$

For statistical analysis, a log transformation ratio was used to normalize distribution. A list of significant genes was obtained by filtering genes with a cut-off t-test P value of <0.05 , with Benjamini and Hochberg multiple testing correction, to reduce or eliminate false discovery (Benjamini & Hochberg 1995).

Reference gene selection and primer design

Housekeeping genes encoding succinate dehydrogenase complex subunit 1 (*SDHA*), and hypoxanthine phosphoribosyl transferase (*HPRT*), selected from a group of nine potential genes, were utilized as controls to accurately estimate gene expression during exercise-induced stress (Cappelli *et al.* 2008). Primers were designed based on available sequences using Primer3 software (Rozen & Skaltsky 2000). Mfold (Zuker 2003) was employed to check chosen sequences for absence of template secondary structure. Whenever possible, primers were located in different exons or at an exon–exon junction. Primer sequences, amplicon lengths, and amplification efficiencies are shown in Table S1.

Reverse transcription qPCR

Total RNA (500 ng) was retro-transcribed using a Superscript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. PCR amplifying the horse β -actin gene (forward primer 5'-GAGCAAGAGGGGCAT-CCTGA-3', reverse primer 5'-GGTCATCTTCTCGCGTTGG-3'; Gene ID: 100033878) was performed on each cDNA sample to confirm successful retro-transcription. RT-qPCR, with SYBR Green chemistry, was performed as described elsewhere (Cappelli *et al.* 2009). Raw C_q (quantification cycle) data from the MX3000P instrument were exported to a common exchange data file and analysed using GeneEx Pro (Kubista *et al.* 2006) to normalize data to those of the two housekeeping genes. During pre-processing, data were corrected for PCR efficiency and averaged over three repeats. The selected reference genes *HPRT* and *SDHA* were subsequently used to normalize C_q values, the relative levels of which were calculated using the maximum C_q numbers. Software R (R Development Core Team 2007) was used to perform analysis of variance (ANOVA) employing a non-linear mixed-effects model.

Data mining

Biological interpretation of the data was performed using Ingenuity pathway analysis (IPA) (<http://www.ingenuity.com>). This identified the most significant biological functions affected by changes in gene expression. Genes described in prior reports that were significantly associated with known biological functions were considered for analysis. For each gene, Fischer's exact test was used to calculate a p value determining the probability that any biological function assigned to a particular dataset resulted from chance alone.

Results

After filtering the microarray data, we found 132 (97 upregulated, 35 downregulated) genes that showed significantly different (fold change from T0 of at least ± 1.5) expression levels when T0 and T1 samples were compared. In the comparison between T0 and T2, only 19 (four upregulated, 15 downregulated) such genes were found. All array data are plotted and shown in supplementary material (Fig. S1). We focused our bioinformatics comparisons on the T0 vs T1 samples. We used IPA to identify the biological functions that were most significantly affected by changes in gene expression. As input, we chose all genes that were significantly modulated ($P < 0.05$), without filtering by fold change to avoid loss of gene interconnection information (Table S2). This resulted in 262 eligible network molecules that were used in functional analysis and to generate network clusters. The modulated gene list was classified by cellular functional categories and canonical and signalling systems, revealing that the main pathways and biofunctions affected were significantly related to immunological and inflammatory responses (Table 1), with a relative abundance of molecules involved in cell-to-cell cross-talk (i.e. cytokines). Furthermore, within the category 'Inflammatory Response', 129 molecules fell into the following subcategories: inflammatory response (59); modulation of levels of phagocytes and neutrophils (76); chemotaxis, migration, and activation of leucocytes (73); immune response (36); and immune cell trafficking (99). These results are supported by examination of the genes most affected in expression. Table 2 lists the genes with the greatest up- or downregulation.

Among individual IPA networks, the three with the highest number of involved genes are shown in Figs. 1–3. These are Network #1 Cell Death, Cellular Development, and Hematological System Development and Function (score 20, 35 molecules); Network #2 Inflammatory Response, Inflammatory Disease, and Antigen Presentation (score 8, 18 molecules); and Network #3 Inflammatory Response, Cell-To-Cell Signalling and Interaction, and Free Radical Scavenging (score 3, 20 molecules). These networks have not been established in the horse, but instead are inferred in the horse.

Table 1 Main functions identified by Ingenuity pathway analysis ($P > 0.0001$).

Top biological functions	
Diseases and disorders	#Molecules
Inflammatory response	129
Immunological disease	36
Connective tissue disorders	35
Inflammatory disease	37
Skeletal and muscular disorders	35
Molecular and cellular functions	
Cell death	49
Cellular compromise	19
Cellular function and maintenance	36
Antigen presentation	72
Cellular movement	63
Physiological system development and function	
Haematological system development and function	160
Hematopoiesis	86
Immune cell trafficking	99
Tissue development	41
Tissue morphology	57
Top canonical pathways	
	Ratio
CCR5 signalling in macrophages	27/63 (0.429)
Hepatic fibrosis/hepatic stellate cell activation	40/103 (0.388)
T-cell receptor signalling	36/94 (0.383)
MIF regulation of innate immunity	14/31 (0.452)
April mediated signalling	16/37 (0.432)

To validate array data, 30 samples (from ten horses at three timepoints) were analysed by RT-qPCR for the expression of 26 genes. The selected list included genes identified as being maximally either up- or downregulated in array analysis, or critically involved in the networks described (Table 3).

Discussion

We chose for our experimental design an equine/murine oligonucleotide microarray validated according to the methods previously described (Muller *et al.* 2004; E Mucher *et al.* 2006) and performed a wide gene expression scan on PBMCs from ten endurance horses.

The analysis of three different time-points revealed changes in gene expression when post-effort samples T1 and T2 were compared with resting samples, T0. Twenty-six of the maximally up- or downregulated genes were tested by RT-qPCR, and modulation in both intensity and direction for almost all genes (20 of 23 amplified) revealed good concordance of the two techniques.

The present work provides a snapshot of the acute response to strenuous exercise in horses. Many of the genes affected by exercise clearly belong to pathways with crucial roles in both the immune response and inflammation. Acute exercise is considered a stress factor inducing severe inflammation (Gleeson 2007) that involves many tissues, including the liver, muscles, and joints, but after chronic exercise training, the acute-phase reaction induced by a

Table 2 Top 20 array genes ranked by fold change up and downregulated from T1.

Fold change	<i>P</i> -value	Entrez Gene ID (<i>Homo sapiens</i>)	Symbol	Gene name	Description
7.924	2.20E-10	7850	<i>IL1R2</i>	Interleukin 1 receptor, type II	Transmembrane receptor
6.435	8.41E-12	4312	<i>MMP1</i>	Matrix metalloproteinase 1 (interstitial collagenase)	Peptidase
2.911	6.41E-08	3606	<i>IL18</i>	Interleukin 18 (interferon-gamma-inducing factor)	Cytokine
2.646	1.11E-09	85439	<i>STON2</i>	Stonin 2	Other
2.64	1.13E-07	1051	<i>CEBPB</i>	CCAAT/enhancer binding protein (C/EBP), beta	Transcription regulator
2.63	1.58E-06	2920	<i>CXCL2</i>	Chemokine (C-X-C motif) ligand 2	Cytokine
2.446	4.04E-08	10468	<i>FST</i>	Follistatin	Other
2.259	6.34E-06	3576	<i>IL8</i>	Interleukin 8	Cytokine
1.981	1.51E-06	9021	<i>SOCS3</i>	Suppressor of cytokine signalling 3	Other
1.927	3.50E-12	4311	<i>MME</i>	Membrane metallo-endopeptidase	Peptidase
-1.489	1.64E-07	140499	<i>UBE2J2</i>	Ubiquitin-conjugating enzyme E2, J2 (UBC6 homolog, yeast)	Enzyme
-1.494	1.33E-06	1231	<i>CCR2</i>	Chemokine (C-C motif) receptor 2	G-protein coupled receptor
-1.529	3.65E-06	29810	<i>BAG3</i>	BCL2-associated athanogene 3	Other
-1.537	6.49E-07	2205	<i>FCER1A</i>	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	Transmembrane receptor
-1.544	3.22E-04	26401	<i>MAP3K1</i>	Mitogen-activated protein kinase kinase 1	Kinase
-1.556	3.36E-04	6775	<i>STAT4</i>	Signal transducer and activator of transcription 4	Transcription regulator
-1.567	5.05E-08	6352	<i>CCL5</i>	Chemokine (C-C motif) ligand 5	Cytokine
-1.684	6.51E-05	13714	<i>ELK4</i>	ELK4, ETS-domain protein (SRF accessory protein 1)	Transcription regulator
-1.745	1.19E-06	15975	<i>IFNAR1</i>	Interferon (alpha, beta and omega) receptor 1	Transmembrane receptor
-20.803	4.64E-02	20463	<i>COX7A2L</i>	Cytochrome c oxidase subunit VIIa polypeptide 2 like	Enzyme

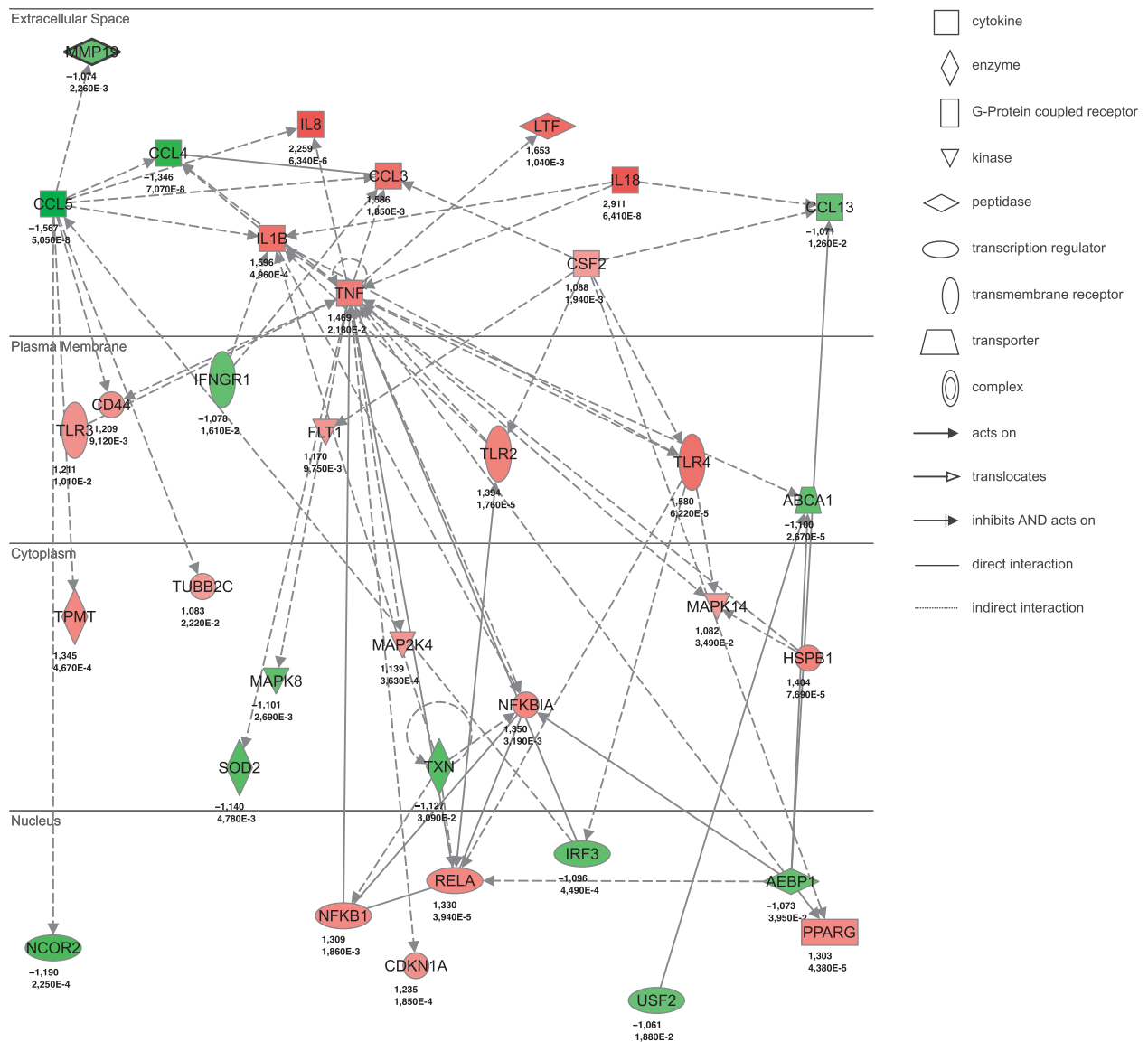


Figure 1 Ingenuity pathway analysis Network 1. Differentially regulated genes in PBMC involved in Cell Death, Cellular Development, and Hematological System Development and Function. The network is displayed graphically as nodes (gene/gene products) and edges (the biological relationships between nodes). Node colour intensity indicates the level of expression. Red, upregulated; green, downregulated, with respect to T1 vs T0. The fold change and *P* values are indicated under each node. The shape of a node indicates the functional class of the gene product, as shown in the legend.

single exercise session is modified, and the acute inflammatory response at the systemic level is attenuated, inducing changes that favour an anti-inflammatory milieu (Lira *et al.* 2009).

Blood cells are envisaged to be the ‘server’ of this network, playing decisional and causal roles leading to a pathological state or establishment of a new physiological homeostasis.

Thus, in the first IPA-generated network (Fig. 1), containing most of the mis-regulated molecules in the experiment, transient inflammation induction, which is immediately blocked by activation of feedback signals, is highlighted. Tumour necrosis factor (TNF), an acute-phase response molecule belonging to the pro-inflammatory

cytokine family, has a central role in this network, and indeed it is involved in a wide spectrum of cellular processes during inflammation, together with interleukins like IL18, IL8, IL1 β and CCL3 (chemokine C-C motif ligand 3), the genes encoding which were in the list of maximally upregulated genes by IPA analyses. Conversely, genes encoding other chemokines of the network, such as CCL5 and IFNGR1 (*interferon gamma receptor 1*), were downregulated in the array and also in RT-qPCR, together with STAT4 (*signal transducer and activator of transcription*), encoding a signal transducer through which IFN proteins stimulate cellular responses to cytokines (Trenerry *et al.* 2008). The general repression of inflammatory pathways

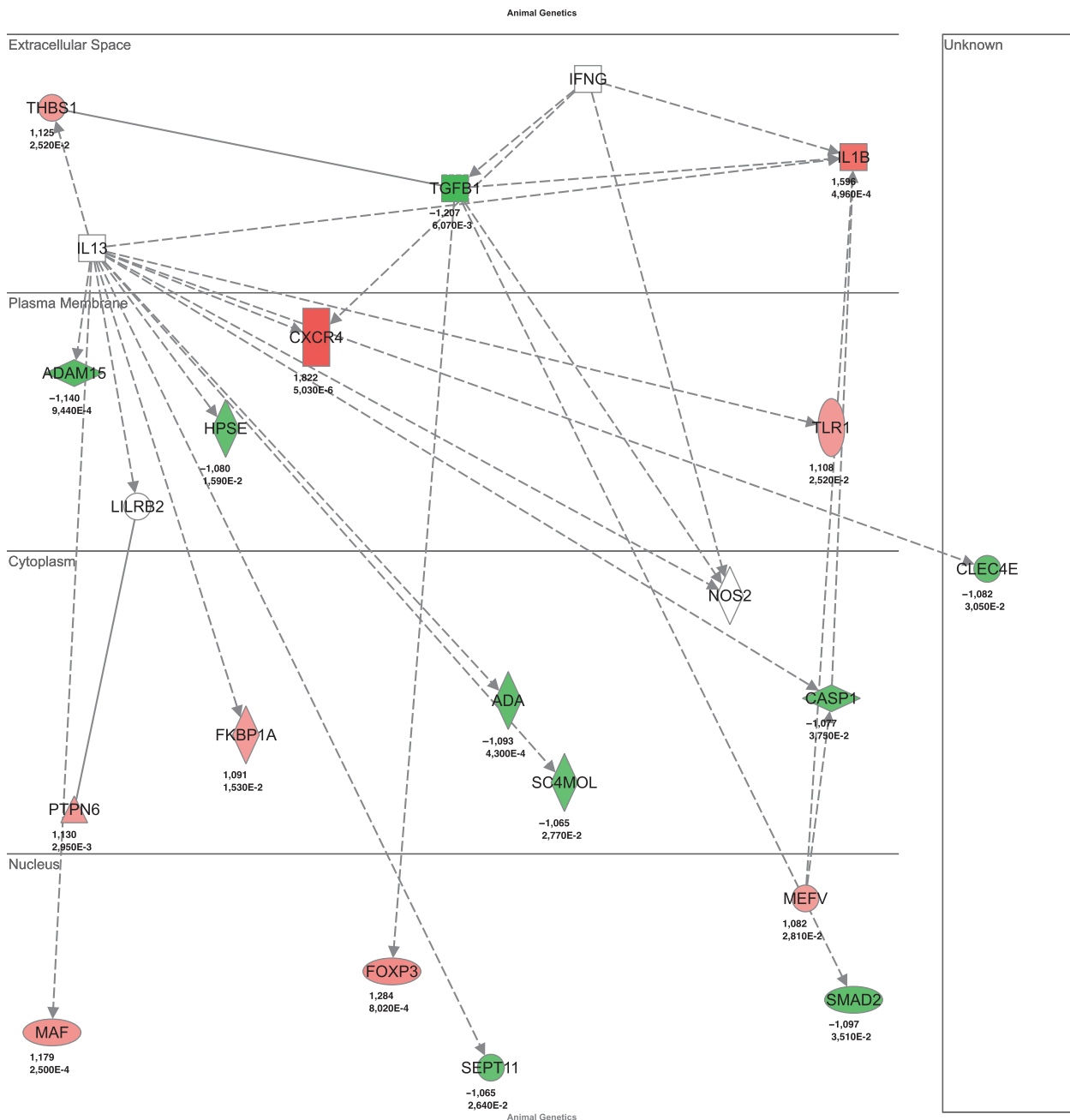


Figure 2 Ingenuity pathway analysis Network 2. Differentially regulated genes in PBMC involved in inflammatory response, inflammatory disease and antigen presentation. The network is displayed graphically as nodes (gene/gene products) and edges (the biological relationships between nodes). Node colour intensity indicates the level of expression of genes: Red, upregulated; green, downregulated, with respect to T1 vs T0. The fold change and P values are indicated under each node. The shape of a node indicates the functional class of the gene product, as shown in Fig. 2.

induced by cytokine production was confirmed by the fact that a significant negative regulator of *STAT* and cytokine signalling, *SOCS3*, was one of the most upregulated molecules, which demonstrates the trend towards restoration.

Moreover, the poor induction of *TNF* (1.46 AFC at T1) may be caused by the action of muscle *IL6*, a potent suppressor of *TNF α* (Gleeson 2007). *IL6* is abundant in circulation during exercise. We did not assess plasma or muscle *IL6* expression levels, but *IL6*-dependent DNA-

binding protein (*IL6DBP*), directly induced by *IL6*, was one of the most upregulated molecules in T1 samples, and it is known that exercise induces its transient increase in liver, contributing to signalling for *PEPCK* (*phosphoenolpyruvate carboxykinase 1*) gene transcription and increasing gluconeogenesis during exercise (Nizielski *et al.* 1996).

The second network (Fig. 2) shows upregulation of genes encoding receptors and proteins that play roles during activation of innate immunity (*TRL1*, *MEFV*), signal

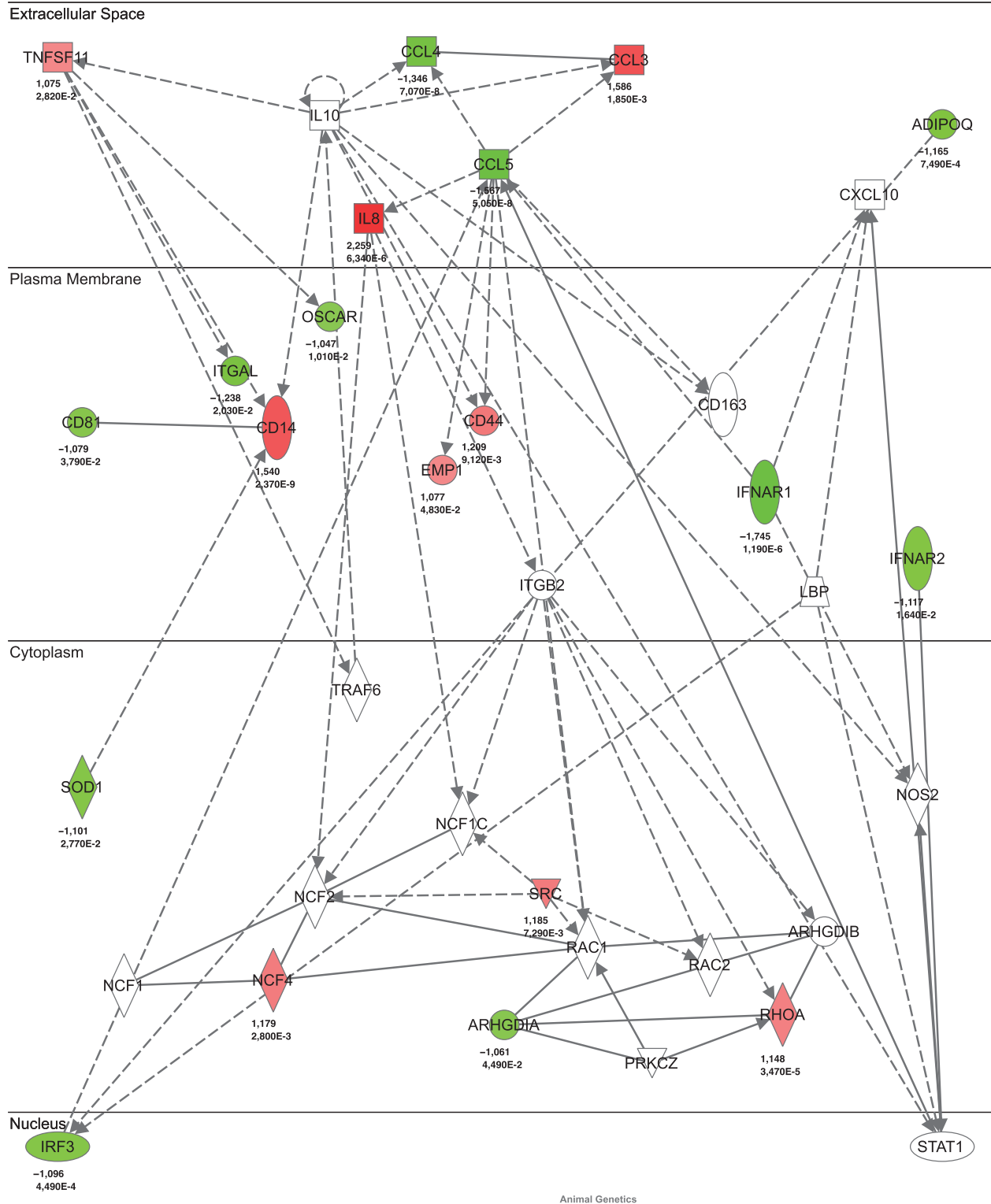


Figure 3 Ingenuity pathway analysis Network 3. Differentially regulated genes in PBMC involved in inflammatory response, cell-to-cell signalling and interaction, and free radical scavenging. The network is displayed graphically as nodes (gene/gene products) and edges (the biological relationships between nodes). Node colour intensity indicates the level of expression of genes: Red, upregulated; green, downregulated, with respect to T1 vs T0. The fold change and *P* values are indicated under each node. The shape of a node indicates the functional class of the gene product, as shown in Fig. 2.

Table 3 Expression values of the top molecules in the array and in the real-time experiments.

Gene	Description	Entrez Gene ID (<i>Homo sapiens</i>)	Race vs Basal			24 h vs Basal			Real time		
			Array		Real time	Array		Real time	FC		P value
			FC	P Value	FC	FC	P Value	FC	P value	FC	P value
<i>IL1R2</i>	Interleukin 1 receptor type II	7850	7.924	2.20E-10	4.978	0.00E+00	1.485	2.16E-02	2.282	3.00E-04	
<i>MMP1</i>	Matrix metalloproteinase 1 (interstitial collagenase)	4312	6.435	8.41E-12	2.722	0.00E+00	1.301	7.98E-03	1.152	3.69E-01	
<i>MMP13</i>	Matrix metalloproteinase 13 (collagenase 3)	4322	4.585	2.12E-10	N/A	N/A	1.226	1.14E-03	N/A	N/A	
<i>IL18</i>	Interleukin 18 (interferon-gamma-inducing factor)	3606	2.911	6.41E-08	2.102	0.00E+00	0.802	1.14E-01	-1.440	6.58E-02	
<i>STON2</i>	Stonin 2	85439	2.646	1.11E-09	1.222	6.27E-02	0.802	7.69E-03	-1.056	6.39E-01	
<i>CEBPB</i>	CCAAT/enhancer binding protein (C/EBP) beta	1051	2.64	1.13E-07	3.350	0.00E+00	1.048	6.78E-01	-1.018	9.39E-01	
<i>CXCL2</i>	Chemokine (C-X-C motif) ligand 2	2920	2.63	1.58E-06	1.988	0.00E+00	1.289	4.28E-02	1.244	1.67E-01	
<i>FST</i>	Follistatin	10468	2.446	4.04E-08	-1.234	3.66E-01	0.969	5.08E-01	1.241	2.53E-01	
<i>IL8</i>	Interleukin 8	3576	2.259	6.34E-06	2.118	0.00E+00	1.188	2.61E-01	1.312	1.46E-01	
<i>SODCS3</i>	Suppressor of cytokine signalling 3	9021	1.981	1.51E-06	2.596	0.00E+00	1.100	1.37E-01	1.170	4.76E-01	
<i>MME</i>	Membrane metallo-endopeptidase	4311	1.927	3.50E-12	N/A	N/A	1.022	3.81E-01	N/A	N/A	
<i>IL1β</i>	Interleukin 1, beta	3553	1.596	4.96E-04	2.099	1.00E-04	1.150	1.61E-01	1.497	3.26E-02	
<i>CCL3</i>	Chemokine (C-C motif) ligand 3	6348	1.586	1.85E-03	1.436	5.30E-03	0.914	4.24E-01	-1.032	8.26E-01	
<i>TLR4</i>	Toll-like receptor 4	7099	1.58	6.22E-05	1.528	0.00E+00	0.935	4.38E-01	-1.154	1.45E-01	
<i>GLUL</i>	Glutamate-ammonia ligase (glutamine synthetase)	14645	1.577	2.15E-05	2.197	0.00E+00	1.023	6.79E-01	1.030	7.78E-01	
<i>UBE2J2</i>	Ubiquitin-conjugating enzyme E2, J2 (UBC6 homolog, yeast)	140499	-1.489	1.64E-07	N/A	N/A	1.070	5.13E-02	N/A	N/A	
<i>CCR2</i>	Chemokine (C-C motif) receptor 2	1231	-1.494	1.33E-06	2.293	0.00E+00	0.788	4.54E-04	1.158	4.64E-01	
<i>LCK</i>	Lymphocyte-specific protein-tyrosine kinase	18618	-1.518	3.55E-03	-1.775	2.00E-04	0.890	1.32E-01	1.076	4.38E-01	
<i>BAG3</i>	BCL2-associated athanogene 3	29810	-1.529	3.65E-06	-2.072	3.00E-04	1.020	7.68E-01	1.044	7.05E-01	
<i>FCER1A</i>	Fc fragment of IgE high affinity I receptor for; alpha polypeptide	2205	-1.537	6.49E-07	-1.731	1.00E-04	0.747	9.87E-04	-1.284	1.43E-02	
<i>MAP3K1</i>	Mitogen-activated protein kinase kinase kinase 1	26401	-1.544	3.22E-04	-1.149	3.71E-01	0.888	5.66E-02	1.113	4.33E-01	
<i>STAT4</i>	Signal transducer and activator of transcription 4	6775	-1.556	3.36E-04	-1.458	3.22E-02	1.020	7.79E-01	1.402	8.20E-03	
<i>CCL5</i>	Chemokine (C-C motif) ligand 5	6352	-1.567	5.05E-08	-1.286	1.24E-01	0.790	3.19E-04	-1.053	7.21E-01	
<i>ELK4</i>	ELK4, ETS-domain protein (SRF accessory protein 1)	13714	-1.684	6.51E-05	-1.150	2.43E-01	0.913	1.44E-01	1.040	7.16E-01	
<i>IFNAR1</i>	Interferon (alpha, beta and omega) receptor 1	15975	-1.745	1.19E-06	1.911	2.00E-04	0.812	1.62E-03	1.217	2.82E-01	
<i>COX2A2L</i>	Cytochrome c oxidase subunit VIIa polypeptide 2 like	20463	-20.803	4.64E-02	-1.471	7.41E-02	0.183	N/A	1.181	2.98E-01	

FC, fold change expressed in log2 base.

transduction (*CXCR4*, *TRL1* and *FKBP1A*) and transcriptional regulation (*MAF*), together with the major pro-inflammatory cytokine *IL1 β* , up-regulated in microarray and RT-qPCR. *CXCR4* and Toll-like receptors (TLRs) were top molecules upregulated in IPA analyses and evident in Networks #1/#2 (Fig. 1: *TRL2*, *TRL4* Fig. 2: *TRL1*, *CXCR4*) and, together with *IL1 β* play an important role in mediating whole-body inflammation in antigen-presenting cells and in production of inflammatory cytokines and proteins (Gleeson *et al.* 2006). In addition, this second network highlights the pathway of 'controlled' inflammation.

The final network (Fig. 3) shows *IL8* to be the main gene of the cluster. *IL8* is involved in immune cell communication and glucocorticoid receptor signalling, together with other cytokines (*CCL5*, *CCL3*); all three genes are strongly up-regulated in the microarray and RT-qPCR. *IL8*, together with *MMP1* and *MMP13* (*matrix metalloproteinase 1* and *13*), is in the list of maximally upregulated array molecules, indicating that lymphocyte trafficking and recruitment of progenitor stem cells from bone marrow (Cappelli *et al.* 2009) is probably activated during the response to acute exercise.

Of the maximally downregulated genes, *CCR2* (encoding chemokine C-C motif receptor 2), the monocyte chemoattractant protein-1, involved in inflammatory processes, BCL2-associated athanogenes (*BAG3*) encoding cytoprotective proteins that bind to and negatively regulate Hsp70 family molecular chaperones (Homma *et al.* 2006), and the EST-domain protein (encoded by *ELK4*) transcription factor involved in regulation of gene expression were prominent. Moreover, we detected downregulation of the kinase signalling pathway through suppression of mitogen-activated kinase kinase 1 (encoded by *MAP3K1*) and lymphocyte-specific protein-tyrosine kinase (encoded by *LCK*).

The negative modulation of these genes and the suppression of chemokine receptor gene expression (*CCL5* and *CCR2*), together with upregulation of the interleukin 1 receptor type II gene (*IL1R2*, the most highly upregulated gene in T1) that inhibits *IL1* activity by acting as a decoy target for *IL1* (Colotta *et al.* 1993), indicate again that after an increase in inflammation signalling during racing, a rapid restoration of homeostasis occurs over a few hours.

In our view, 'basal homeostasis', which was deeply disrupted by major exercise, was restored in <24 h in the well-trained horses included in the present study, probably because the repetition of various exercises during training may increase adaptive responses. However, limited recovery from exercise, reduced substrate availability, reduction in protein synthesis, and the influence of other stressors would cause fatigue, decrease adaptation and may result in loss of function in cases of overreaching or overtraining (Steinacker *et al.* 2004).

In conclusion, this work presents a transcription snapshot of acute response to exercise in top endurance horses, and identifies the genes and the pathways involved in the rela-

tionship between acute inflammation, prolonged exercise and homeostasis restoration.

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Conflicts of interest

The authors have declared no potential conflicts.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Primer pairs used in the real-time experiments.

Table S2 List of probes of the array significant modulated in T1 and T2.

Figure S1 ‘Volcano plot’ of the horse array data for the T0 vs T1 (a) and T0 vs T2 comparisons (b).

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