Modification of Gene Expression and Protein Profiles by Exhaustive Exercise in Pathogen-stimulated and Un-stimulated Peripheral Blood Cells

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2. **Gutachter:** Professor Dr. Veit Wank

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List of publications

The thesis is based on the following publications which are referred to in the text by their Roman numerals (I–VI):

I. **Asghar Abbasi**, Elvira Fehrenbach, Melanie Hauth, Michael Walter, Jens Hudemann, Veit Wank, Andreas M Niess, Hinnak Northoff. Changes in Spontaneous and LPS-induced ex vivo Cytokine Production and mRNA expression in Male and Female Athletes Following Prolonged Exhaustive Exercise. Exerc Immunol Rev. 2013;19:8-28.

II. **Asghar Abbasi**, Melanie Hauth, Michael Walter, Jens Hudemann, Veit Wank, Andreas M. Niess, Hinnak Northoff. Exhaustive exercise modifies different gene expression profiles and pathways in LPS-stimulated and un-stimulated whole blood cultures. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2013.10.023

III. Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, Fleshner M, Green C, Pedersen BK, Hoffman-Goetz L, Rogers CJ, Northoff H, **Abbasi A**, Simon P. Position statement. Part one: Immune function and exercise. Exerc Immunol Rev. 2011;17:6-63.

IV. Tonevitsky AG, Maltseva DV, **Abbasi A**, Samatov TR, Sakharov DA, Shkurnikov MU, Lebedev AE, Galatenko VV, Grigoriev AI, Northoff H. Dynamically regulated miRNA-mRNA networks revealed by exercise. BMC Physiol. 2013 Jun 7;13:9. doi: 10.1186/1472-6793-13-9.

V. Makarova J , Maltseva DV, Galatenko VV, **Abbasi A**, Maximenko DG , Grigoriev AI, Tonevitsky AG, and Northoff H. Exercise Immunology Meets MiRNAs. Exerc Immunol Rev. 2014

VI. Northoff H, Symons S, Zieker D, Schaible EV, Schäfer K, Thoma S, Löffler M, **Abbasi A**, Simon P, Niess AM, Fehrenbach E. Gender- and menstrual phase dependent regulation of inflammatory gene expression in response to aerobic exercise. Exerc Immunol Rev. 2008;14:86-103.

Abbreviation

URTI	upper respiratory tract infection
LPS	lypopolysaccharide
IFN-γ	Interfron gama
IFNβ1	interferon beta 1
TLRs	Toll like receptors
TLR4	Toll-like receptor 4
NF-KB	Nuclear factor Kappa B
RT-PCR	Real-time polymerase chain reaction
qRT-PCR	Quantitative Real-Time PCR
LDR	LPS-Dependent release
ELISA	Enzyme Linked Immunosorbent Assay
mRNA	messenger RNA
miRNA	microRNA
TNIP3	TNFAIP3-Interacting Protein 3
PRR	Pathogen recognition receptor
PAMPs	Pathogen-Associated Molecular Patterns
IL-1β	interleukin 1 beta
IL-1ra	interleukin 1 receptor antagonist
IL-2	interleukin 2
IL-4	interleukin 4
IL-6	interleukin 6
IL-8	interleukin 8
IL-10	interleukin 10
IL-12p40	interleukin p40
IL-12p70	interleukin p70
IL-12B1	interleukin 12 beta 1
IL-18RAP	interleukin 18 receptor accessory protein
PTGDR	Prostaglandin D2 Receptor
TNF-α	tumor necrosis factor alpha
TGF-B1	Transforming growth factor beta-1
MCP-1	Monocyte chemoattractant protein-1
GM-CSF	Granulocyte macrophage colony-stimulating factor
PBMCs	Peripheral blood mononuclear cell
GO	Gene ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
ARG-1	Arginase-1
ORMs	Orosomucoides
SOCS3	suppressor of cytokine signaling 3
SAP30	Sin3A-Associated Protein, 30kDa
DUSP-1	DUSP1 dual specificity phosphatase 1
GJB6	gap junction protein, beta 6
IRAKs	IRAK4 interleukin-1 receptor-associated kinase 4
PLAU	PLAU plasminogen activator, urokinase

CXCL10	Chemokine (C-X-C Motif) Ligand 10
SPON2	Spondin 2
FCRL6	Fc receptor-like 6
ACTH	Adrenocorticotropic hormone
OLIG2	oligodendrocyte lineage transcription factor 2
TMEM106B	transmembrane protein 106B
NR4A2	nuclear receptor subfamily 4, group A, member 2
DNMT1	DNA (cytosine-5-)-methyltransferase 1

Summary

It is well know that in contrast to moderate physical activity, an acute bout of prolonged, exhaustive exercise such as marathon or half-marathon running can cause adverse effects on immunity as reflected by transient immunosuppression following the event. We used microarray technology as well as other approaches to study the response of selected and non-selected immune-related genes and proteins following an exercise program. The capacity of whole blood cultures to produce cytokines in response to endotoxin (LPS) was studied (Paper I). Further, the early steps of the immune reaction to pathogen contact were evaluated in details using whole blood culture and gene expression profiling approach in athletes before, 30 min after, 3 h after and 24 h after a half-marathon run (Paper II). Gender and menstrual phase dependent differences in cytokine and gene expression profiles of 12 male subjects (M) and 9 women with regular menstrual cycles was also studied in response to an aerobic exercise at 93% of the individual anaerobic threshold (Paper VI), and the effect of exercise on the miRNA response of eight highly trained athletes before and after moderate exercise was investigated using microarray technology (papers IV).

A strong and significant reduction in LPS-dependent release (LDR) of TNF-α and slight reduction in LDR of IL-6 was observed in both male and female athletes following exhaustive exercise. There was a significant enhancement in the concentration and gene expression of IL-10 at 30min post exercise in both sexes. IL-10 was higher in men than in women and not influenced by LPS (Paper I). The results of the microarray study showed that expression of several genes with prominent anti-inflammatory function was strongly up-regulated by exhaustive exercise in both stimulated and un-stimulated cultures, but some genes such as TNIP3 (a prominent inhibitor of the LPS/TLR signaling cascade) were strongly up-regulated in LPS-stimulated cultures only (Paper II). The study of the miRNA response revealed four dynamically regulated miRNA- RNA networks following exercise. miRNAs- 24-2-5p, 27a-5p, and 181a-5p were up-regulated immediately after exercise, but tended to down-regulate at recovery. miRNA-21-5p demonstrated different expression profiles over time (Paper IV). Results of gender studies showed that women in luteal phase exhibit different responses of gene regulation as compared to women in follicular phase and men. Several pro-inflammatory genes including PTGDR, IL-18RAP and IL-12B1 were significantly up-regulated in women in luteal phase of their menstruation, while these genes were down-regulated in the follicular phase of the same women and in men. Conversely, women in luteal phase showed a strong trend towards down-regulation of anti-inflammatory genes (e.g. IL-6, IL-1ra).

For conclusion, the results of our studies demonstrate that exercise has a distinct impact on the early cytokine response to pathogen and this response has a dramatic anti-inflammatory bias. In addition, Microarray analysis could reveal a set of exercise reactive genes which can only be detected in presence of pathogen stimulation (e.g. TNIP3 and IFNβ1). Moreover, it is clear, from these results, that women in luteal phase of their menstrual cycle show significantly less anti-inflammatory regulation than women in follicular phase or men. And finally, microRNAs are involved in exercise induced gene expression changes and dynamically regulated miRNA/mRNA networks could be demonstrated.

Acknowledgements

Herewith, I would like to thank all those kind people who made this doctoral thesis possible and an unforgettable experience for me, to only some of whom it is possible to give particular mention here.

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I also would like to express my very sincere gratitude to Prof. Dr. AM Niess for his continuous guidance, his support in numerous ways in the organization of exercise program and his deep knowledge in the preparation and completion of my PhD thesis and research.

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1. Introduction

1-1. Introduction and statement of problem

A growing number of published reports on exercise immunology provide evidence that in contrast to moderate physical activity, an acute bout of prolonged (>1.5 h), exhaustive exercise such as marathon or half-marathon running can cause adverse effects on immunity as reflected by transient immunosuppression and inflammation-like reactions following the event (Abbasi et al. 2013). While the beneficial effects of moderate exercise were reported with some delay, reports on increased risks of upper respiratory tract infection (URTI) following acute exhaustive bouts of exercise have been accumulating since 2 decades (Nieman et al., 1990; Nieman, 2007).

The time of decreased host protection following exhaustive exercise may last 3-72 hours and has been called ``open window`` (Fig-1) and it is now widely accepted that in this time bacteria and viruses may gain a foothold to establish an infection (see review Nieman, 2007).

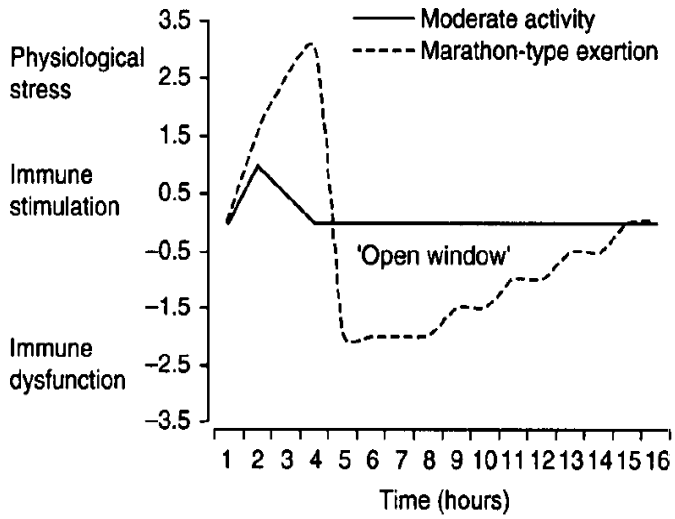

Figure 1. The ``open window theory`` established by DC. Nieman (see review Nieman DC 2007a).

Demonstrated immune parameters which were affected by exhaustive exercise comprise changes in peripheral cell numbers; decreases in granulocyte burst activity, NK cytotoxic activity and lymphocyte proliferation. Certain cytokines appear in plasma (IL-6, IL-8, IL-1, IL-10), but stimulated in vitro production of some cytokines is decreased (IL-1, TNF-α, IFN-

γ) (Stewart et al., 2005; Kakanis et al., 2010; Woods et al., 2000; Shephard and Shek, 1999; Ostrowski et al., 1999; Weinstock et al., 1997). Body temperature changes, increased blood flow and dehydration, and changes in stress hormones including adrenaline and glucocorticoids have been discussed as underlying mechanisms (Nieman, 1995). In particular, corticoids are known for their broad immunosuppressive effects and have been shown to be elevated in response to prolonged exhaustive endurance exercise (Keast et al., 1988). Even so, at present, the sequence of biological reactions leading to transient post-exercise immunosuppression is not really clear. One theory focuses on the observation that the normal cytokine response in vitro is strongly suppressed following exhaustive exercise. In special IFN-γ was > 90% suppressed after a marathon (Northoff et al., 1994). In fact, these experiments have shown that many possibly important effects of exhaustive exercise could neither be detected in native plasma nor in un-stimulated blood cultures, but required in vitro stimulation to become visible. LPS is a very prominent and suitable stimulant since it mimics presence of gram negative bacteria. It will rapidly engage pattern recognition receptors (in this case TLR-4) leading to activation of NF-κB transcription factor and release of a host of pro-inflammatory cytokines including IFNs and chemokines (Beutler, 2000).

Although different aspects of the immune system including macrophage activation, Natural killer cell number and activation, lymphocyte proliferation, and cytokine production have been investigated to elucidate which exercise induced changes do occur, the molecular mechanisms by which exercise exerts its negative or positive effects on the immune system are poorly understood. Several studies have examined the effect of exercise on expression of selected individual genes (mostly cytokines and heat shock proteins, which seem to be key players of inflammation and immune reactions) in peripheral blood using RT-PCR (Fehrenbach et al. 2003; Nieman et al. 2006, Nieman 2007).

Today, the microarray technology makes it possible to evaluate large numbers of genes and to assess the pattern of gene regulation simultaneously in one tissue sample. Accordingly it is now a widely used tool for comprehensive analysis of gene expression and has also been used in several exercise related studies (Connolly et al. 2004; Zieker et al. 2005; Büttner et al. 2007; Radom-Aizik et al. 2009a,b, 2008, 2013, 2014; Königsrainer et al. 2010, 2012). However all of the existing studies focus directly on the effect of exercise on organ tissues and cells of the peripheral blood and studies investigating the effect of exhaustive exercise on

the early steps of the immune reaction to pathogen contact using high throughput analysis were lacking before our work.

Therefore, we decided to use LPS stimulation of whole blood cultures as a model for an in vivo infection at different time points in relation to exercise, and to analyze the effects of exercise, the effects of LPS stimulation, and the effects of their combined action using gene expression microarray technology. We therefore took blood from athletes at different time points before and after exercise and performed short term whole blood culture in presence or absence of pathogen stimulation (LPS) and compared the protein and gene expression response by microarray technology. This should lead to the identification of new pathways, candidate genes or interaction patterns of exercise and pathogen effects, ultimately helping to further understand the mechanisms underlying exercise-induced immunosuppression and thus the ``open window for infection`` phenomenon.

We also included sex-specific aspects in our studies. For some good reasons we were encouraged to consider this as a second aim of our studies. First of all, the majority of exercise studies have been done in male athletes/individuals. Therefore, it is still unclear to what extent gender influences immunological responses to exercise. Sex-specific differences in the immune response to exercise have clear implications for understanding sex-specific adaptations to exercise for athletic performance and overall health. Secondly, most of the studies which have investigated sex-specific differences in immune response under exercise conditions have not considered the sex hormones fluctuations /menstrual phases in women.

Further, only few studies have investigated the sex-specific changes in gene expression profiling using microarray technology (Northoff et al. 2008, Liu et al. 2010, 2013), and finally, there is no study available investigating sex and menstrual phase dependent gene regulation of endotoxin stimulated blood culture in response to physical exercise. Therefore, to our knowledge, our studies are the first to meet these aims using high throughput technology.

2-1. Brief summaries of the publication

Paper I is an original article that has been published in Journal *Exercise Immunology Review 2013.* It uses standard individual measurement technology to evaluate the capacity of whole blood to produce certain selected cytokines upon short-time (1h) exposure to endotoxin (LPS) following a half-marathon run in well-trained male and female athletes. Cytokine

concentration was measured by ELISA technology, and LPS-dependent release (LDR) was calculated by comparison with controls. The gene expression of selected cytokines was measured by qRT-PCR. The results of this paper showed strong and significant reduction in LDR of TNF-α and slight reduction in LDR of IL-6. LDR of IL-8 was enhanced post exercise in men and TGF-β1 in women. Men showed significantly higher LDR of IL-1ra at rest and 30min post exercise as compared with women and the protein pattern was roughly paralleled by mRNA. There was a significant enhancement in the concentration and parallel mRNA expression of the anti-inflammatory cytokine IL-10 at 30min post exercise in both sexes. IL-10 was higher in men than in women and not influenced by LPS. The main conclusion in this paper was that changes in cytokine release could only in part be attributed to changes in mRNA, and that women in their luteal phase showed less pronounced anti-inflammatory responses than men.

Paper II was published in the journal *Brain Behavior Immunity-Special issue 2014*. It uses probes from the same run (half-marathon) as paper I. Its purpose was to conduct a broad scale investigation into the effect of exercise on the early steps of the immune reaction to pathogen contact on the gene expression level and compare them to exercise effects in absence of pathogen. An important aim was to avoid artifacts from preparation procedures. We therefore used short time whole blood culture ± LPS and developed a new methodology to adapt the work up procedures to analysis by microarray. It was the first (exercise related) paper to publish microarray data on pathogen stimulated cultures, and its strategy was rewarded: some genes such as TNIP3 (prominent inhibitor of the LPS/TLR signaling cascade) were strongly up-regulated in LPS-stimulated cultures only. The data in this paper confirm that there is an anti-inflammatory bias in the reaction to exercise and also prompt the authors to hypothesize that the reaction to exercise may be more of a primary, preemptive, protective anti-inflammatory reaction rather than a counteraction to exercise-induced inflammatory stimuli.

Paper III which has been published in journal ``*Exerc Immunol Rev. 2011*`` is a huge paper to which many experts in exercise immunology have contributed. It is a position statement paper that focuses on the scientific basis of what is known, accepted and deemed to be important about the influence of exercise on immune function. This paper has different sets of authors and each author or group has its or their own part for contribution. Our main contribution to this paper was the chapter ``Omics in exercise``. Here we discuss how often and how effectively exercise studies, especially exercise immunology studies, have used Omics

technologies, on the basis of existing data. The use of each Omics technology including transcriptomics, metabolomics, and proteomics in different tissues and different types of exercise was considered in detail. All authors had equal contribution in this review article.

Paper IV, which has been published in journal ``*BMC Physiol. 2013*``, is an original article investigating the effect of moderate exercise on the expression of mRNAs and miRNAs and the dynamics of miRNA-mRNA regulatory networks in circulating leukocytes. Microarray technology was used to monitor the changes in transcriptome of the whole blood of eight highly trained athletes before and after 30 min of moderate exercise followed by 30 min and 60 min of recovery period. This study revealed four dynamically regulated miRNA- RNA networks following exercise and was the first study to monitor miRNAs and mRNAs in parallel into the recovery period. Controversies and future directions are also discussed in this paper, which was in cooperation with our Russian partner group.

Paper V was newly published in the Journal ``*Exercise Immunology Review 2014*``. This paper is a review article evaluating the role of miRNA in exercise immunology, with the focus on existing data. The biological roles of miRNAs in immune system, their expression and function in circulating leukocytes and muscles in response to physical exercise, and their possible role in the beneficial effect of exercise in different diseases are discussed in this paper. This review has also been written with the cooperation of our Russian partner group, and also contained some new original data on miRNAs in exercise.

Paper VI is an original article published in journal ``*Exercise Immunology Review 2008*``. This paper investigates the role of gender and menstrual phase cycles in the reaction of the immune system to exercise. We report immune-related gene expression patterns in response to an aerobic exercise at 93% of the individual anaerobic threshold of 12 male subjects (M) and 9 women with regular menstrual cycles and no use of oral contraceptives who ran both at day 10 (follicular phase, F) and at day 25 (luteal phase, L) of their cycle. Self-produced microarrays were used to analyze and compare the differentially expressed genes between males and females, and between two different phases of the female cycle. According to this paper women in luteal phase showed a distinctly different pattern of gene regulation in response to exercise, compared with women in follicular phase or males. The overall direction of gene expression changes of women in luteal phase is clearly pro-inflammatory.

2. Theoretical background

2-1. The human immune system

2-1-1. Introduction and overview of the immune system

Immunity refers to protection against many diseases. The immune system is the collection of cells, tissues and molecules that functions to defend us against infectious microbes of different kinds and cancer. The coordinated reaction of the immune system against infections (and other foreign substances) is known as the immune response. Abnormalities of the immune system that result in defective immune responses make individuals susceptible to infections by viruses, bacteria, fungi and parasites (Abbas AK and Lichtman AH, 2011). The immune system is particularly important in defending the body against pathogenic (diseases-causing) microorganisms including bacteria, protozoa, viruses and fungi (Gleeson and Bosch-2013). In humans, the importance of the immune system becomes clinically apparent when it is defective. Thus, inherited and acquired immunodeficiency states are characterized by increased susceptibility to infections. The immune system also plays an important role in defending us against cancer by identifying and destroying tumor cells (Gleeson and Bosch-2013). However, immune responses are also capable of causing damage. Many common diseases are caused by uncontrolled or excessive immune responses (examples include rheumatic fever, asthma and glomerulonephritis, inflammatory bowel disease, autoimmune thyroiditis, and multiple sclerosis) (Abbas AK and Lichtman AH, 2011).

2-1-2. The components of the immune system

The cells of the immune system originate in the bone marrow, where many of them also mature. They then migrate to patrol the tissues, circulating in the blood and in the vessels of the lymphatic system. All the cellular elements of blood, including red and white blood cells, derive ultimately from the same progenitor or precursor cells, the hematopoietic stem cells in the bone marrow (Janeway et al. 2005). Granulocytes, monocytes/macrophages and lymphocytes are the major components of white blood cells. Granulocytes and monocytes/macrophages are derived from myeloid progenitors and lymphocytes are derived from lymphoid progenitors (Figure 2).

Monocytes are the largest type of leukocytes in the blood, and normally constitute 5-15% of the leukocytes. Monocytes which migrate from the bloodstream to other tissues differentiate into resident macrophages and dendritic cells. Three main functions of

Monocytes/macrophages are phagocytosis and intracellular killing, antigen presentation to lymphocytes, and cytokine production. Monocytes can perform phagocytosis using intermediate opsonisation proteins, such as antibody and or complement that coat the pathogen, as well as by binding to the microbe directly via pattern-recognition receptors that recognize pathogens. These receptors are called toll-like receptors (TLRs). Monocytes digest the phagocytosed materials and present selected peptides thereof on molecules of the major histocapability complex (MHC II).

The granulocytes (also called polymorphonuclear leukocytes) are so called because they have densely staining granules in their cytoplasm. They are short lived, are produced in large numbers and migrate to sites of infection or inflammation (Janeway et al. 2005). The **neutrophils**, which are avidly phagocytic, are the most abundant and most important cellular component of the innate immune system: severe deficiencies in neutrophil function or number lead to overwhelming, fatal bacterial infection (Janeway et al. 2005). **Eosinophils** seem to be important in defense against parasitic infections. The function of **basophils** is probably similar and complementary to that of eosinophils (Janeway et al. 2005).

Lymphocytes account for 12-15% of blood leukocytes and comprise three major subgroups: 1- Natural killer cells (NK-cells) which are engaged by group-specific antigens or change of MHC I molecules on virus infected or cancer cells, and kill suspicious cells using cytotoxic enzymes like *perforin*. NK-cells are part of the innate immune system. 2- T cells, which show antigen-specific clonal expansion and function as helper cells (Th), regulatory cells (Tr), or cytotoxic effector cells. 3- B cells which expand specifically and develop into antibody producing plasma cells. Th and Tr determine the extent of the specific immune response by providing or regulating the production of cytokines. Both, T and B cells form long-lived memory cells which constitute our immunological memory and are thus the basis for immunization. T and B cells have a large repertoire of diverse receptors/antibodies. Thereby, one cell is more or less specific for one epitope (Janeway et al. 2005).

2-1-3. Innate immunity versus adaptive immunity

T and B cells form the acquired arm of the immune system, also called ``specific`` or ``adaptive``. Clonal expansion of the antigen contact and formation of memory cells are the basis for a powerful and long-lasting specific reaction. It does however need some time to bring its full potential into action.

The first line of our defense is therefore the innate immune system which has receptors for group wise recognition of microbes (pathogen recognition receptors, PRR). Endotoxin (LPS) which is present on the wall of gram negative bacteria is one of those danger signaling molecules (PAMPs), which are recognized by PRRs, in this case Toll-like receptors (TLRs). The cells of the innate immune system are granulocytes, monocytes/macrophages and NK cells. They are numerous enough to go into effective immediate action. Granulocytes perform bulk phagocytosis. Monocytes/macrophages are also phagocytic, but in addition they present peptides derived from the phagocytosed material on their MHC II molecules to lymphocytes to start the adaptive response. They also supply an array of cytokines which govern the immune response. Granulocytes and monocytes are also known as ``inflammatory cells``(Janeway et al. 2005).

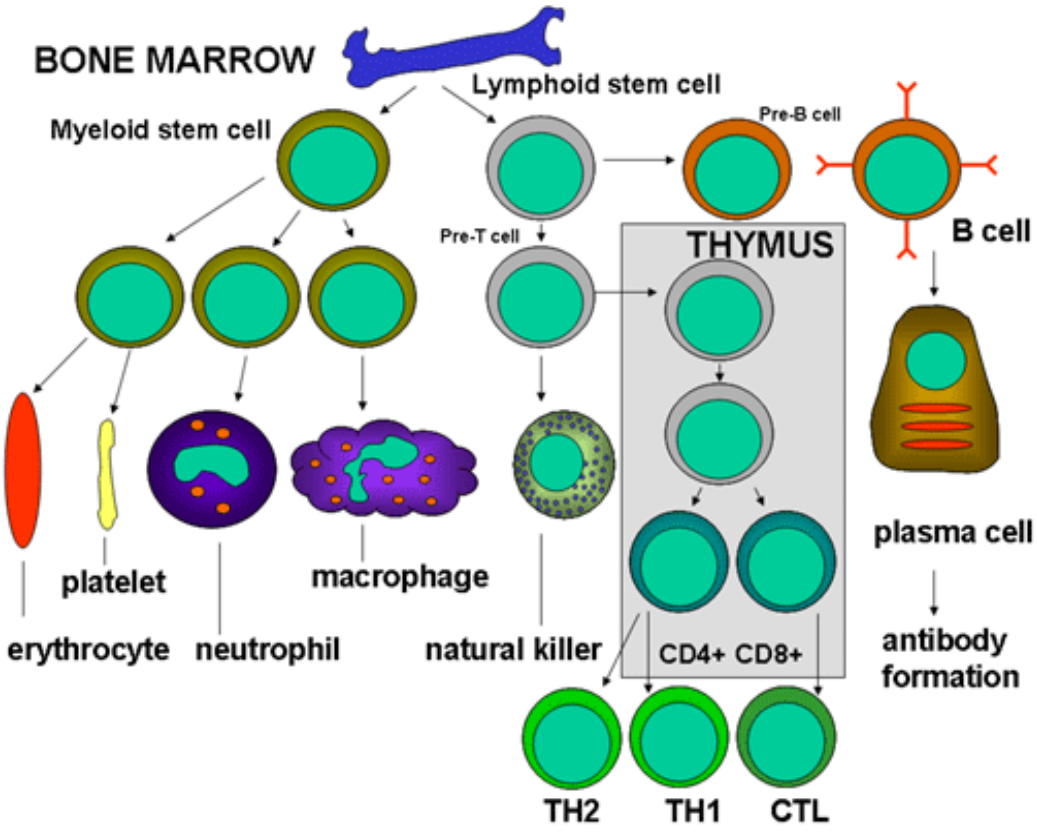

Figure 2. The cellular components of blood, including the lymphocytes of the adaptive immune system, arise from hematopoietic stem cells in the bone marrow

2-1-4. Recognition of pathogens by Toll-like receptors

Toll-like receptors (TLRs) are the most numerous PRRs. The name giving Toll receptor was originally identified in Drosophila and has a role in the antifungal response of flies (Akira and Takeda, 2004; Janeway and Medzhitov, 2002).

To date, a family of 12 mamalian TLRs (TLR1-12) is known. They are mainly expressed by monocytes, macrophages and dendritic cells (Medzhitov, 2001). PAMPs recognized by TLRs include endotoxin (lipopolysaccharide, LPS), lipoproteins, peptidoglycan, lipoteichoic acid, and bacterial DNA (Beutler and Rietschel 2003—exercise immunology book). LPS, is probably the most powerful microbial stimulant of innate immune responses (O`Niell and Brint, 2005). By inducing rapid release of inflammatory cytokines, LPS can mobilize both innate and specific immune responses at great distances from the site of infection, but massive systemic presence of LPS will cause shock and death. LPS is mostly recognized by TLR4 (Figure 3). Effective binding of LPS to TLR4 requires LPS binding protein (LBP), which forms a high-affinity complex with the lipid A moiety of LPS, for transfer on CD14. This enables LPS to be transferred to the LPS receptor complex composed of TLR4 and MD2 (Figure 3).

Downstream signaling of the TLR4 receptor complex in response to LPS (summarized in Fig. 3) is largely mediated via the recruitment of adapter proteins, including myeloid differentiation factor 88 (MyD88), MyD88 adapter-like protein (MAL), TIR-containing adapter molecule (TRIF, also known as TICAM-1), and TRIF-related adapter molecule (TRAM). Thereby, MAL-dependent recruitment of MyD88 orchestrates production of inflammatory cytokines in response to LPS-treatment (Kawai et al. 1999, Kenny and O'Neill 2008). This requires caspase 1 dependent processing of MAL (Miggin et al. 2007) and involves the activation of the IκB/NFκB pathway as well as of the p38MAPK and the c-jun N terminal kinase (JNK) members of the mitogen activated protein kinase (MAPK) family. In addition to the early MyD88-dependent signals TLR4 triggers a delayed MyD88-independent (Kawai et al. 2001), TRIF-dependent signal transduction (Yamamoto et al. 2002) via TRAF family member associated NF-κB-activator binding kinase (TBK)1-mediated activation of the IFN response factor (IRF)3 and late activation of NF-κB. This delayed part of TLR4 signaling requires dynamin-dependent internalization of TLR4 and subsequent recruitment of TRAM which initiates TRIF-dependent pathways enabling TLR4 to trigger the release of type I interferons (Kagan et al. 2008).

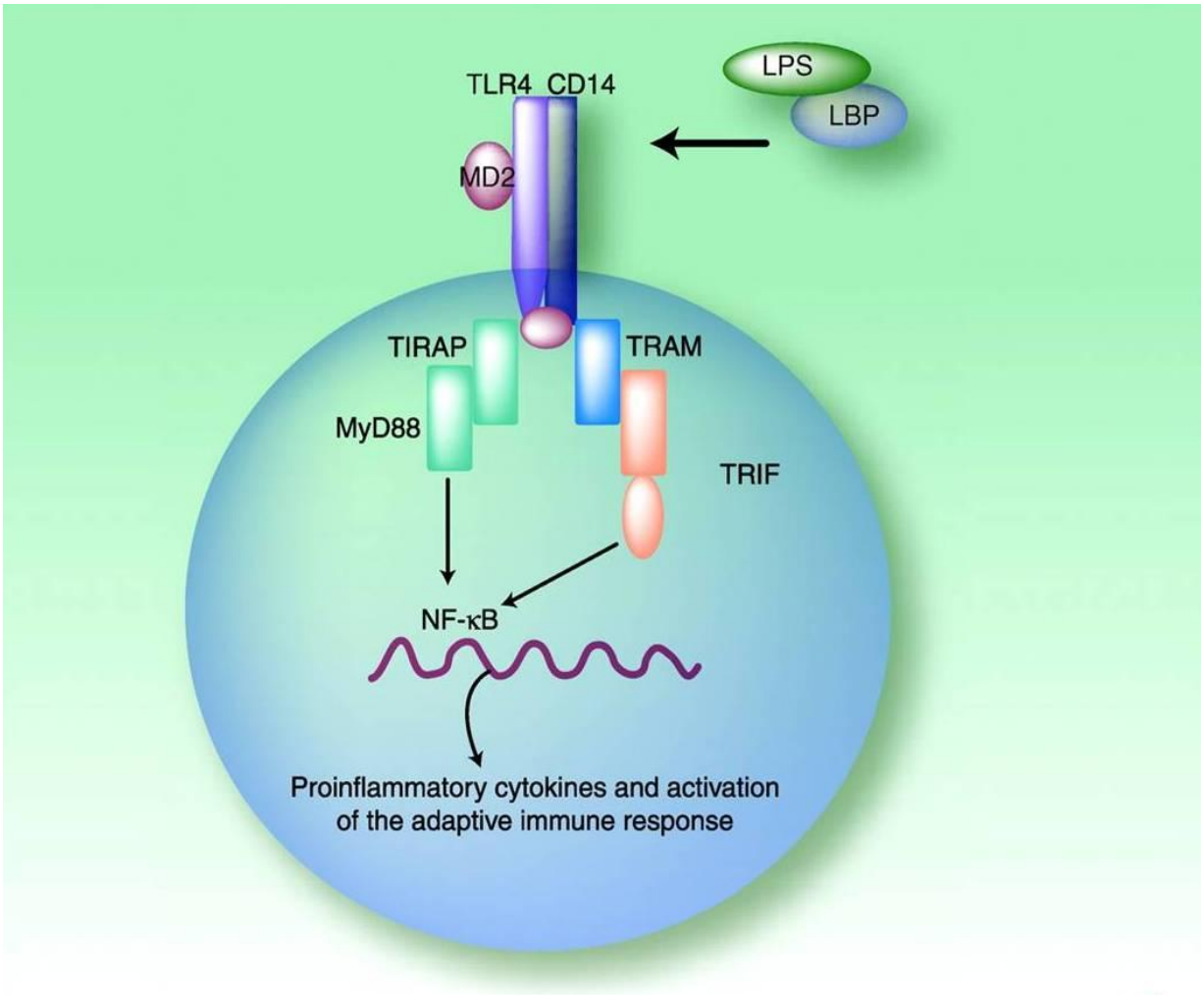

Figure 3. LPS signals the TLR4 pathway on an APC (i.e., monocyte or dendritic cell). Adapted by Paulos C M et al. 2007. Publisher Ltd: Clin Cancer Res 2007;13:5280-5289. Image taken from MacMillan Publisher Ltd: Nature Immunology 4; 1144–1150; Copyright 2003

2-2. Exercise and immune system

2-2-1. Exercise and infection risk

Acute upper respiratory tract infection (URTI, such as coughs and colds, influenza, sinusitis, tonsillitis, other throat infections and middle ear infections) are among the most common illnesses experienced at all ages. These infections are also one of the main reported causes of illness in athletes (Gleeson M, Bishop N, ad Walsh N 2013). For example, according to Alonso`s report (2012), among the 126 reported illnesses from the athletes competing at the world Athletics Championship in Daegu (South Korea-2011), 40% of illnesses affected the upper respiratory tract with confirmed infection in almost 20% of cases (Alonso et al. 2012).

A growing number of evidence reported that physical exercise has an effect on respiratory infection incidence, depending on the amount, intensity and duration of exercise (Matthewes et al. 2002; Nieman et al. 2011). The relationship between exercise intensity/volume and susceptibility to URTI has been summed up in the J-shaped model by Nieman et al (1994) (figure 4). This model suggests that, although the risk of upper respiratory tract infection may decrease below that of a sedentary individual when engaging in moderate exercise training, the URTI risk is elevated during periods of heavy training and in the 1–2-week period following participation in competitive endurance races (Nieman et al,1994).

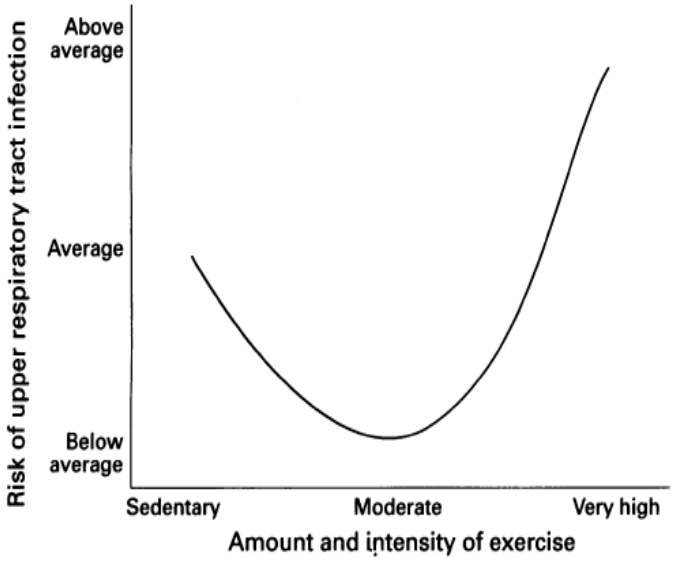

Figure 4. J- shaped model of the relationship between various amounts of exercise and risk of upper respiratory tract infection. (Nieman, 1994)

Two early studies which have investigated the incidence of self-reported URTI following marathon-type events have suggested that participating in competitive endurance exercise is associated with an increased risk of URTI during the 7-14 days after exercise (Peters and Bateman 1982; Nieman et al. 1990). In addition, significant negative relationship between running time and post-exercise illness was shown, with far higher prevalence of URTI symptoms in runners who completed the race in less than 4 hours (Peters and Bateman 1982). Nieman et al (1990) reported similar findings for runners who participated in the 1987 Los Angeles Marathon. Almost 13% of the runners reported URTI symptoms during the week

after marathon compared with only 2,2% of the control group. It is important to note that the relative risk of an episode of URTI is increased following heavy/exhaustive exercise but still the majority of athletes do not experience an episode of URTI after prolonged strenuous activity (Gleeson M, Bishop N, ad Walsh N 2013). The duration of exercise may be an additional determining factor for post-exercise susceptibility for respiratory infection because performing 5-km, 10-km, and 21-km races did not increase the reporting of URTI symptoms in the following week (Nieman et al. 1989). Reported infections following race could be due to the reactivation of recent infection. This was shown in the runners who reported URTI symptoms in the 3 weeks before the 2000 Stockholm marathon and post-race episodes after same marathon (Ekblom et al. 2006).

In addition to the incidence of URTI in response to a single bout of heavy exercise, several studies have also reported episodes of URTI symptoms for athletes who were training intensively (Niman et al. 1990; Gleeson et al. 1999, Bury et al. 1998). 40% of Los Angeles Marathon runners reported experiencing at least one episode of URTI symptoms during the 2 months prior to the marathon (Nieman et al. 1990). After controlling for confounding factors, it was shown that those who ran more than 96 km (60 miles) per week in training were twice as likely to suffer illness compared with those who trained less than 32km per week. Other investigators have also found higher incidence of URTI for athletes who trained intensively as compared to moderately exercising athletes (Gleeson et al. 1999; Bury et al. 1998). There are however also rare reports which could not find a relationship between training volume and URTI (Ekblom et al. 2006).

2-2-2. Exercise and distribution of leukocytes

Over the past 30 years, a variety of studies have demonstrated that physical exercise considerably influences the circulating leukocytes subpopulations (Mackinnon et al. 1994; Nieman 1994; Pedersen BK, Nieman DC 1998). As exercise induces leukocytosis (an elevated number of white cells in blood), it was initially assumed that physical exercise induces an inflammation like reaction, however according to Simpson`s report (Gleeson M, Bishop N, ad Walsh N 2013) it is now obvious that the exercise-induced leukocytosis is a transient phenomenon, with number and composition of leukocyte subsets usually returning to basal values within 6-24 hours after exercise. In general, neutrophil concentrations increase during and post-exercise, whereas lymphocyte concentrations increase mildly during exercise

and fall below pre-values after long-duration physical exercise. Monocytes do not change much during exercise, but increase their number post-exercise.

Neutrophils account for the greatest part of the exercise-induced leukocytosis. Intensive exercise may increase the neutrophil count two fold, while prolonged exhaustive exercise may increase neutrophil numbers 3-4 fold after exercise. The number of neutrophils reaches peak values during the recovery of exercise, although the magnitude and kinetics of neutrophil reaction are affected by exercise intensity and duration (Robson et al. 1999).

Blood monocytes have been shown to increase in response to many different types of exercise including prolonged bouts of endurance exercise, short bouts of high intensity exercise and acute bouts of resistance exercise (Booth et al. 2010; Lancaster et al, 2005; Simpson et al. 2009; Steppich et al. 2000; Simonson and Jackson 2004). The composition of their subsets is also changed. Briefly, the small subset of the monocytes carrying CD16 (inflammatory CD14+/CD16++ or intermediate CD14++/CD16+) show higher relative increases after exercise than the classical majority type (CD14++/CD16–) (Booth et al. 2010).

The effect of exercise on lymphocyte numbers is substantial and biphasic. During and immediately after exercise, numbers increase before they rapidly fall below the pre-exercise values during early recovery. They gradually return to basal levels in the following hours (Booth et al. 2010; Shek et al. 1995). Increased lymphocyte concentration during exercise is most likely due to the recruitment of all lymphocyte subgroups (T cells, B cells, and NK cells) to the vascular compartments. Within the T cell compartment, the relative changes in the CD4+ (cytotoxic) T cell subset are greater than in the CD4+ (helper) subset.

2-2-3. Effect of exercise on innate immune function

As explained before, the innate immunity is our first line of defense against infectious pathogens. The innate immunity includes monocytes and macrophages, neutrophils, dendritic cells, NK cells and soluble mediators like complement, interferons and anti-microbial peptides (defenses). The effect of exercise on innate immunity has been well studied. To understand the mechanisms by which exercise influences the innate immune system it is necessary to determine whether this occurs by altering the number of cells, cell function or both. The effect of exercise on total number of innate immune cells is described in earlier chapter and here we summarize the effects of exercise on some innate immune functions.

2-2-3-1. Exercise and chemotactic response of neutrophils

Chemotaxis is the movement of inflammatory cells (e.g. neutrophils and monocytes) to the infection site. The migration of neutrophils occurs in response to certain stimuli including C5a – complement fragment, IL-8, platelet activating factor (PAF), leukotriene B4 (LTB4), or fragments of collagen or fibrin. Several studies have investigated the effect of acute exercise on neutrophil chemotactic function, but the results are controversial. While some studies report an increased neutrophil chemotactic activity in response to acute exercise (Ortega 2009; Giraldo et al. 2009; Ortega et al. 1993), other reports indicate either suppressive effects or no effect at all (Wolach et al. 2005; Saxton et al. 2003; Gavrieli et al. 2008). It has been shown that neutrophil adherence at rest is lower or unaltered in trained vs untrained individuals, respectively (Lewicki et al. 1987; Ortega et al. 1993).

Regarding the effect of chronic exercise on neutrophil chemotactic activity a similar picture evolves. Chronic regular exercise does not appear to change neutrophil function. The number of available studies is small and controversial (Syu et al. 2012; Bote et al. 2013).

2-2-3-2. Acute exercise and phagocytosis

As mentioned before the ability to engulf and ingest foreign material by inflammatory cells is called phagocytosis. Neutrophils and monocytes/macrophages are the major phagocytic cells of innate immune system. The majority of studies have shown that an acute bout of exercise enhances neutrophil phagocytic activity (Hack et al, 1992, Lewicki et al. 1987; Ortega et al. 1993; Giraldo et al. 2009; Garcia et al. 2011; Nieman et al,1998), although few others have reported no change (Syu et al. 2012; Gabriel et al. 1994).

In the case of monocyte phagocytic activity this is more complicated. Although brief exercise reduced phagocytic activity of monocytes (Bieger et al. 1980), an enhancement has been reported following long-duration exercise (2.5 hours at 75% VO2max) (Nieman et al,1998). The phagocytic function of macrophages seems to be dependent on the exercise intensity (Gleeson et al. 2013). Whereas moderate exercise increases macrophages phagocytic activity, acute exercise to exhaustion shows no effect (Ortega, 2003).

2-2-3-3. Monocyte TLRs expression and function

Toll-like receptors (TLRs) are trans-membrane proteins that play an important role in the detection and recognition of microbial pathogens and subsequent production of cytokines

(Medzhitov et al. 2001). The study of TLR response to exercise is a new research area in exercise immunology that is becoming more and more interesting as of lately. Recent studies have demonstrated that both acute exhaustive and chronic exercises reduce monocyte expression of TLRs (Lancaster et al. 2005b; Flynn et al. 2003; McFarlin et al. 2004; Stewart et al. 2005, Coen et al. 2010; Radom-Aizik et al. 2014). In one of the initial studies Lancaster et al (2005b) found a decrease in monocyte (CD14+) TLR expression (TLR1-2-4, but not TLR-9) following an acute bout of exhaustive exercise lasting for 1.5h. Oliveira and Gleeson (2010) investigating the effects of prolonged exercise reported a decrease in monocyte cell surface expression of TLR1-4 in trained male cyclist (Oliveira and Gleeson 2010). In a newly published work by Radom-Aizik et al (2014) monocyte TLR4 mRNA expression was downregulated in healthy men who performed a brief exercise (ten 2min bouts of cycle ergometer exercise). With regard to the effect of exercise training, Stewart et al (2005) reported a decreased CD14+ cell surface expression of TLR4 in young and old physically inactive subjects following 12 weeks of endurance and resistance exercise (Stewart et al. 2005). Flynn et al (2003) showed a similar reduction in TLR4/CD14 expression in resistance exercise-trained women who were in traditional hormone replacement therapy (Flynn et al 2003). Therefore, apart from very few contrasting results (Fernandez-Gonzalo 2012; Carpenter et al. 2012), a large body of studies shows a decrease of TLR4 through exercise, suggesting that TLRs may play a role in exercise-induced immunosuppression.

Taken together, the effect of exercise on TLRs expression and function may represent a mechanism through which physical exercise regulates both innate and adaptive immunity, since the production of cytokines through stimulation of TLRs is an important event in activation of adaptive immune response.

2-2-3-4. Exercise and Leukocyte cytokine production

The different cells of the immune system communicate by intercellular mediators called cytokines (see above). Cytokines regulate survival, growth, differentiation and effector functions of immune cells (Townsend et al. 2000). Unlike hormones, cytokines are not stored in glands as preformed molecules, but are rapidly synthesized and secreted by different cells mostly after stimulation. Cytokines are pleiotropic in their biological activities and play pivotal roles in a variety of responses, including the immune response, hematopoiesis, neurogenesis, embryogenesis, and oncogenesis. They frequently affect the action of other cytokines in an additive, synergistic or antagonistic manner.

Cytokines have been classified on the basis of their biological responses into pro- or anti-inflammatory cytokines, depending on their effects on immunocytes (Jawa et al. 2011). Cytokines act in networks or cascades. Major Cytokines include the ILs (Interleukins), Growth Hormones, IFNs (Interferons) and TNFs (Tumor Necrosis Factors-Alpha and Beta). Many of the cytokines act locally like autocrine hormones and their targets are cells of the same or similar type as the cytokine-producing cell.

Taken together, cytokines are important mediators governing the immune response, and their regulation or enhancement may yield valuable information pertinent to questions like transient post-exercise immunosuppression, beneficial anti-inflammatory (e.g., anti-atherosclerotic) effects of cytokines, and/or exercise- induced asthma (Abbasi et al. 2013a).

Since Northoff and colleagues discovered in the early 1990s (1991-1994) that IL-6 is a key player in the response of the immune system to exercise, several investigators started working on the effect of different types of exercise on circulating cytokines. Some reviews of studies performed on the reaction of cytokines to an acute bout of exhaustive exercise are available (Norhoff et al. 1994; Suzuki et al. 2002). It is established knowledge that prolonged exhaustive exercise increases the concentration of different cytokines including IL-6, IL-8, IL-10, IL-1ra, MCP-1 and IL-2R. TNF-α, IL-1β or IL-2 are not or only marginally elevated (Weinstock et al. 1997, Ostrowski et al. 1999, Drenth et al. 1998; Moldoveanu et al. 2000, Nieman et al. 2001, 2006; Peake et al. 2005, Suzuki et al. 2000, 2003; Sugama et al. 2013). Levels of the elevated cytokines are generally returned to resting values on the following day. IL-6 is usually the first cytokine appearing in the circulation following exercise (Figure 5) (Northoff et al. 1991; Drenth et al. 1995; Nieman et al. 2001; Steensberg et al. 2001). The circulating concentration of IL-6 can increase up to 100- fold in response exhaustive exercise, depending on the intensity and duration of exercise (Northoff et al, 1991, 1994; Suzuki et al. 2000, 2003).

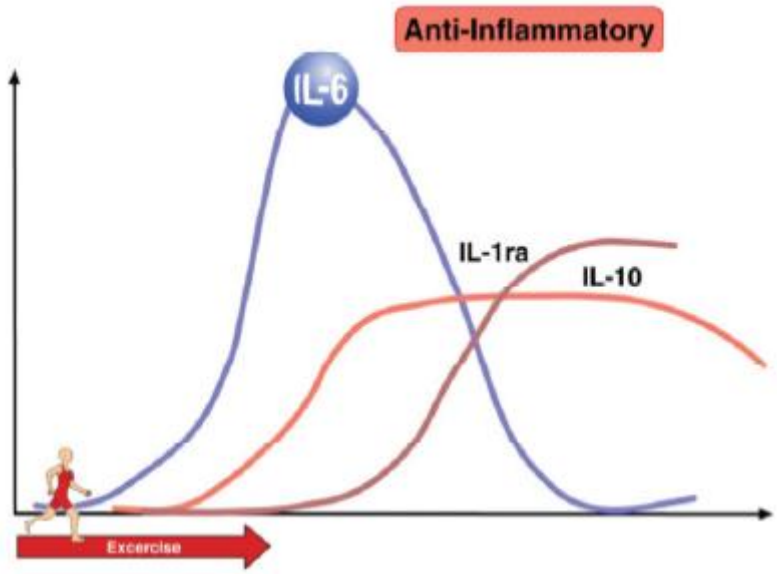

Figure 5. The release of cytokines in circulation during exercise.

Many cytokines which were elevated in plasma could also be detected in urine (IL-1β, IL-1ra, IL-6, IL-8, IL-10) and some (IL-2, IL-4, IL-12, IFN-γ) were found elevated in urine in absence of elevated plasma levels (Sugama et al. 2013).

There are no reports showing the increase in circulating levels of the strongly immunomodulatory cytokines IFN-γ and IL-2 following exercise. However in *in vitro* cultures following exercise, leukocytes showed spontaneous production of very low amounts of IFN-γ which may explain its appearance in urine. At the same time the capacity of such cultures (peripheral blood) respond to endotoxin stimulation by secretion of IFN-γ was dramatically reduced (Northoff et al, 1991, 1994, 1998; Weinstock et al. 1997; Steensberg et al. 2001).

With the exception of TNF-a, IL-2 and IFN-γ which are reported to be decreased after exercise, the findings of most of the studies about the changes in endotoxin stimulated cytokine concentration are inconsistent at present. The findings of our group and others demonstrate clearly that an acute bout of exhaustive exercise strongly suppresses LPS-stimulated secretion of TNF-α, IL-6 and IFN-γ at post-exercise (Weinstock et al. 1997; Drenth et al. 1995, 1998, Tanaka et al. 2010). LPS-stimulated concentration of IL-1ra has also been shown to increase in response to physical exercise (Drenth et al. 1998).

2-2-4. Gene expression profiling in blood cells in response to exercise

It has been well shown that physical exercise changes gene expression profiling of human leukocytes, depending on the type, intensity, and duration of exercise as well as training status of athletes (see review Fehrenbach 2007). Several genes and biological pathways have been documented as results of exercise effect on human leukocytes, although we are still far away from having a complete list of changed genes and pathways.

Microarrays, the widely used tools for the comprehensive analysis of gene expression, enable us to analyze hundreds to thousands of genes simultaneously. Using this technology, the gene expression fingerprints (Specific patterns of gene expression) and/or new candidate genes in a certain situation can be determined. Whole genome arrays may even help us to find genes that were unknown in association with exercise until that time. Therefore, the findings from microarray analysis facilitate characterizing and defining the complex stress response to acute and chronic exercise on the molecular level.

In humans, circulating leukocytes are a rich and easiest accessible source to evaluate stress parameters on the cellular and molecular level (Palmer et al. 2006). Gene expression profiling of peripheral blood cells in response to exercise, therefore, allows gaining new insights into mechanisms through which exercise changes immune function. Additionally, a growing number of publications has referred to the potential of using circulating leukocytes as a surrogate tissue for traditional tissues for diagnosis (Bosio and Gerstmayer 2008), suggesting that the use of leukocytes may potentially obviate the need of muscle biopsies (Zeibig et al. 2005).

Whole blood has been increasingly considered as a valuable source of RNA for gene expression profiling (Vartanian et al. 2009). The whole-blood model has been applied to measure the complete bactericidal activity of blood and allowed simultaneous analysis of bactercidial activity, neutrophil activation, cytokine production, and bacterial-antigen expression (Methods in Molecular Medicine). The use of whole blood cultures has several advantages in the context of our study, avoiding possible effects from in vitro manipulation (Fannin et al., 2005), allowing us to see collaborative effects, and enabling short and precise timing.

Several studies have investigated leukocyte gene expression profiling following different types of exercise using microarrays (Connolly et al. 2004; Zieker et al. 2005, Hilberg et al.

2005; Bütner et al. 2007; Radom-Aizik et al. 2008, 2009a,b, 2013, 2014; Königsrainer et al. 2010, 2012; Neubauer et al. 2013; Carlson et al 2011). Among them, no study has investigated exhaustive endurance exercise, and only 6 studies have used whole blood while others have used PBMCs, neutrophils and NK cells as a source for gene expression profiling. These studies revealed several interesting candidate genes and biological processes that might be important in the exercise response. Immune response/inflammatory, stress and apoptosis, signal transduction, cell growth and development, and metabolism were the most significantly reported biological pathways changed with exercise in human leukocytes. Inflammatory and heat shock protein genes were mainly affected by exercise, indicating that exercise-induced inflammation and hyperthermia might explain some of the observed changes in leukocyte gene expression (Fehrenbach 2007).

The progress in bioinformatics helped us to find the biological pathways which change in human leukocytes through exercise. GO, KEGG and Ingenuity pathway analyses have also revealed several pathways in circulating leukocytes in response to physical exercise. These were activation of natural killer cell signaling, hematopoietic cell lineage, T cell receptor signaling, cancer, cytokine–cytokine receptor interaction, antigen processing and presentation, Type1 diabetes mellitus, JAK-STAT signaling, apoptosis and TLR signaling pathways (Radom-Aizik et al., 2009a,b; Carlson et al., 2011; Radom-Aizik et al., 2013; Königsrainer et al., 2012).

The studies which have investigated gene expression profiles of circulating leukocytes in response to physical exercise have revealed several candidate genes involved in different biological pathways. It is difficult to integrate the results of all these studies since they have used different exercise programs as well as different tissues for gene expression analysis. However, among the studies those have used whole blood as a source for gene expression profiling there was a significant change in the expression of inflammation related genes such as IL-1ra, IL-1a, alox5, IL-8RA, CD14, stress related genes such as HSPA1A, HSPB1, HSPH1 and apoptosis related genes like BCL2A1 (Zieker et al. 2005; Hilberg et al. 2005; Büttner et al. 2007; Sakharov et al. 2012). Others using PBMCs and leukocyte subpopulations such as neutrophils and NK cells have also found significant changes in several interesting candidate genes grouped to inflammation [TNF (Radom-Aizik et al. 2014), IL-6 (Northoff et al. 2008), IL-1ra (Connolly et al. 2004; Neubauer et al. 2013), HIF-1α (Connolly et al. 2004), IL-18RAP (Connolly et al. 2004; Northoff et al. 2008; Radom-Aizik et al. 2008), SOCS3

(Radom-Aizik et al. 2014), TLR4 (Neubauer et al. 2013; Radom-Aizik et al. 2014), IRAK3 (Neubauer et al. 2013)], stress response and heat shock proteins [HSP70 (Connolly et al. 2004; Radom-Aizik et al. 2008; 2009a; 2014), DUSP-1,-2, -5 (Connolly et al. 2004; Radom-Aizik et al. 2014; Carlson et al. 2011), SPON2 (Connolly et al. 2004; Radom-Aizik et al. 2008)], growth factor and transcription [G protein-coupled receptors (Connolly et al. 2004), EGR1, EGR2 (Connolly et al. 2004; Radom-Aizik et al. 2009b, 2014), EREG (Connolly et al. 2004; Radom-Aizik et al. 2014; Carlson et al. 2011)], apoptosis [Granzymes and perforin 1 (Radom-Aizik et al. 2008; 2009a, b; Connolly et al. 2004), Caspase 3 (Radom-Aizik et al. 2009a), FASLG (Radom-Aizik et al. 2009a)], asthma [PTGDR (Northoff et al. 2008; Connolly et al. 2004; Radom-Aizik et al. 2009b), ALOX5 (Northoff et al. 2008), IL-12RB1 (Northoff et al. 2008)].

Taken together, due to the methodological differences such as different microarray platforms, RNA preparation methods, sampling, and cell populations, comparing the real exercise-related results in microarray studies is difficult. For example, except our own work (Northoff et al. 2008) no study has reported the significant change of IL-6 mRNA by exercise in human leukocytes using array technology.

In conclusion, microarray analysis is relevant to distinguish exercise-dependent gene expression profiles in circulating leukocytes. Using a whole genome array provides new insights into the molecular mechanisms of exercise-induced perturbation in immune system as well as exercise-induced adaptation.

3- Discussion

3-1. Exhaustive exercise changed cytokine protein and mRNA response

Cytokine protein secretion was monitored in 1h ex vivo cultures in presence or absence of LPS before and after a half-marathon race. Peripheral cells when cultured for 1 hour in absence of exogenous stimulus are normally quiescent and do not produce any detectable cytokines. The results obtained in our supernatants of un-stimulated cultures therefore mainly reflect cytokine plasma concentrations generated during the run plus eventual ongoing exercise induced production during culture. In our un-stimulated (control) cultures, exhaustive exercise significantly changed the concentrations of cytokines IL-8, IL-6, IL-1ra, IL-10, MCP-1, and TGF-β1. This is in agreement the findings of previous studies which have

reported similar increases in cytokine concentrations in plasma following different types of exercise (Nieman DC et al, 2006,2007b, 2007c; Peake et al. 2005; Suzuki et al. 2000). There was no induction of TNF-α by exercise in un-stimulated cultures, and IL-1β and GM-CSF were undetectable in both sexes. Only few of the secreted cytokines, namely IL-1ra, IL-10 and in a borderline way IL-8 showed parallel changes in protein and mRNA alteration in the same cultures, suggesting that the circulating leukocytes are not the major producers of most of the cytokines in supernatants of un-stimulated cultures. The strong elevation of IL-10 mRNA seems however high enough to conclude that circulating leukocytes make at least a substantial contribution to the elevated plasma IL-10 concentrations. The opposite is the case for IL-6 and TGF-β1. While the concentrations of IL-6 and TGF-b were significantly higher in un-stimulated cultures of both sexes following exercise, no change was reported for their mRNA following exercise, confirming the notion that elevated cytokines in the supernatants following exercise can be due to their production outside of the blood. It has been previously shown that some cytokines such as IL-6 and IL-8 are produced massively in skeletal muscles during and following physical exercise (Pedersen et al. 2007). It is now widely accepted that contracting skeletal muscles are the main source of elevated IL-6 levels in the circulation.

The main aim of our study was to investigate the early steps of the pathogen response in relation to previous exercise and we chose to use the whole blood culture system in order to create a situation as close as possible to the natural one. Doing this, we needed to control for plasma concentrations (see above) and for cellular shifts. Therefore, LPS-dependent release (LDR) of the cytokines under investigation was designed as mean values of the delta between cytokine concentration in stimulated and un-stimulated cultures, normalized to monocyte numbers (except IFN-γ, see paper Abbasi et al. 2013a).

As expected, LPS induced remarkable amounts of cytokines during the short (1h) incubation period, and these were strongly modified through previous exercise. LPS-inducible production of TNF-α was significantly and strongly suppressed shortly after exercise which is in agreement with the results of other studies (Drenth et al. 1995, 1998; Weinstock et al. 1997). Although there was some parallel down-regulation of TNF-α mRNA in the same cultures, the extent of this was so mild that it is highly questionable if this can explain the dramatic drop in protein release. Post-transcriptional modification is therefore likely to be part of the regulation.

The LPS-dependent release of IL-1ra showed different patterns of reaction between sexes. At rest and during the first 3 hours post exercise men produced substantially higher amounts of LPS-induced IL-1ra. After 24h, LDR of IL-1ra was below resting values in men or back to initial values in women. IL-1ra protein reaction was roughly paralleled by mRNA changes. Up-regulation of IL-1ra mRNA following exercise has been reported previously by other studies (Drenth et al. 1998; Nieman et al. 2007b, 2007c; Zieker et al. 2005). In contrast to TNF-α, LDR of IL-6 was only mildly reduced after prolonged-exhaustive exercise, with women showing a faster kinetics. This is in accordance with the finding of Weinstock et al (1997), who found a moderate reduction in LPS-induced IL-6 production following exercise (Weinstock et al.1997).

LPS-dependent production of IL-8 was increased by exercise in men and TGF-β1 LDR was enhanced in women. Therefore, both sexes showed an enhancement in LPS-dependent production of one of the granulocytotrophic cytokines (TGF-β1 at 30min post-exercise for women and IL-8 at 3h post exercise for men) in the hours after exercise. This agrees well with the improvement of granulocyte function by exercise and suggests that both sexes may achieve same goal through different mechanisms.

The LPS-dependent release of IL-10, MCP-1 and IFN-γ did not change following exercise, suggesting that there was no induction of these cytokines by LPS. It shows that 1h incubation with endotoxin was not long enough to induce these cytokines. But exercise, by itself, significantly increased the secretion of IL-10 and MCP-1 in our cultures, which is in agreement with the findings of previous studies (Sugama et al. 2013; García et al. 2011).

Altogether, our main conclusion to be derived from cytokine protein and parallel gene expression data are: 1- cytokine changes in plasma/whole blood supernatants can only in part be attributed to changes in mRNA expression and 2- IL-10, the most important anti-inflammatory cytokine, is highly likely to be produced in the circulation leukocytes, and the mechanism of its induction is different from that of counter-regulatory reactions to primary inflammatory (LPS) stimulation.

3-2. Exercise affected gene expression profiles in whole blood cells

For our investigations we developed a new technical approach to study mRNA profiles in LPS-stimulated and un-stimulated blood cells. We combined a whole blood culture methods and PaxGene RNA extracting kit in short time culture with and without LPS-stimulation. This

provided us with high quality and sufficient quantity of RNA for our gene expression analysis. Microarray technology was then used to reveal gene expression profiles and pathways in response to exercise. As stated above, the study was designed mainly to reveal the influence of exercise on the early steps of the immune reaction to pathogen contact in comparison to controls.

The half-marathon run significantly changed a row of genes in LPS-stimulated and un-stimulated cultures in both male and female athletes. In the control culture in the absence of LPS, several genes which are involved in innate immune and inflammatory response, metabolic response, apoptosis and stress response, cell cycle, and regulation of transcription factors, were altered in response to exhaustive exercise. We do not expect 1h incubation in absence of stimulus to cause a major gene expression differences. We think that these results can be essentially compared to the published studies which all investigated mRNA expression with no further deliberate stimulation immediately after exercise or after separation procedures (Radom-Aizik et al. 2008, 2009a, b, 2013, 2014; Northoff et al. 2008; Connolly et al. 2004; Neubauer et al. 2013; Carlson et al. 2011; Hilberg et al. 2005; Bütner et al. 2007). According to our results, the majority of genes changed in response to exercise were clustered in innate immunity/inflammatory response. In general, these findings support the old idea (Weinstock et al. 1997) that an acute bout of strenuous exercise could induce both pro-inflammatory and anti-inflammatory responses. The most prominent finding was the up-regulation of genes with predominant anti-inflammatory function and down-regulation of genes with predominant pro-inflammatory function. Among the up-regulated anti-inflammatory genes, ARG1, SOCS3, SAP30, ORMs, DUSP-1, GJB6, and IRAK3 were the most strongly up-regulated ones in cultures of both sexes. Some of these genes including ARG1, ORMs, and GJB6 were also strongly up-regulated in LPS-stimulated cultures. Indeed, these genes were unaffected by LPS, suggesting that neither their habitual expression nor their exercise-induced expression were affected by LPS during short time incubation. The anti-inflammatory impact of these genes has been demonstrated by several studies (Munder, 2009; Hochepied et al., 2003; Libert et al., 1994; Hulsmans et al., 2012; Chanson et al., 2001; Nakase et al., 2004). The strong and identical up-regulation of these genes in both LPS-stimulated and un-stimulated cultures without any LPS effect again indicates that the immunosuppressive effect of exercise during early stages following pathogen contact is

different and independent of possible counter-regulatory effects against primary inflammatory stimulation.

Meanwhile, another type of reaction was shown for genes SOCS3 (Suppressor of cytokine signaling) and DUSP1 (dual specificity phosphatase 1). Both of them were strongly induced by LPS at rest, and exercise did not change this response. In contrast, exercise strongly up-regulated their expression in un-stimulated cultures (11 and 6.5-fold, respectively). SOCS3 and DUSP-1 are both negative regulators in TLRs signaling pathways and can inhibit inflammation via different mechanisms (Bode et al. 2012). The fact that SOCS3 and DUSP-1 genes were significantly up-regulated by 1h exposure to endotoxin, independent of exercise, indicates their role in the early fine tuning of LPS-stimulated TLR signaling. The exercise-induced up-regulation of SOCS3 and DUSP-1 (in absence of LPS) might be explained by the induction of IL-10. It has been demonstrated that SOCS3 and DUSP-1 are IL-10 inducible genes (Bode et al. 2012). The strong induction of IL-10 mRNA and protein by exercise was shown in our studies (Abbasi et al. 2013a). The induction of SOCS3 mRNA in monocytes through brief exercise has been reported very recently (Radom-Aizik et al. 2014).

TNIP3 (TNFAIP3 interacting protein, also called ABIN-3) represented another very interesting pattern of interaction between endotoxin and exercise. While LPS itself could not induce the expression of TNIP3 mRNA under resting situation, exercise significantly induced its expression (up to 16 fold) in LPS-stimulated cultures. TNIP3, which is highly expressed in monocytes/macrophages, is a potent inhibitor of NF-kB activation induced by TNF, IL-1 or LPS. TNIP3 can also be induced by IL-10 (Verstrepen et al., 2008; Weaver et al., 2007). This is the first study reporting this type of interaction, suggesting that some exercise dependent priming factor plus engagement of TLRs was necessary for induction of this gene, at least in our situation. The priming factor could, for example, be IL-6 which is one of the classical exercise-induced factors. Few other genes also followed similar exercise/LPS interaction pattern namely PLAU which codes for a protein that usually converts plasminogen to plasmin and also modulates macrophage phenotype toward M2 macrophages (Meznarich et al. 2013), and HIVEP1 (human immunodeficiency virus type I enhancer binding protein 1) which codes for a protein that participates in the transcriptional regulation of inflammatory target genes by binding specific DNA sequences in their promoter and enhancer regions (Morange et al. 2010).

Some predominantly pro-inflammatory genes, notably IFN-β1, IFN-γ, CXCL10, IL-12B, SPON2, KLRF1, and FCRL6 were also strongly down-regulated through exercise in LPS-stimulated cultures only. Of these, IFN-β1 showed the most interesting pattern. LPS-induced expression of IFN-β1 gene was significantly suppressed (up to 12 fold down-regulation), while exercise itself could not change IFN-β1 gene expression. This is exactly the inverse of the pattern observed with TNIP3. IFN-β1 shows strong pro-inflammatory and antiviral properties and like TNF-α is responsible for part of the LPS toxicity (Karaghiosoff et al., 2003). Due to the nearly complete shutdown by exercise, the down-regulation of IFN-β1 cannot be attributed to cellular shift. In contrast, in the case of IFN-γ and IL-12B, which were significantly but less strongly down-regulated by exercise, cellular shift may play a role. It should however be noted that a very strong suppression of LPS-stimulated IFN-γ following a half-marathon race has been reported previously (Weinstock et al., 1997), suggesting that the transient suppression of these two interferons in circulating leukocytes may be is a good candidate to explain the ``open window`` phenomenon and possibly the beneficial anti-inflammatory effects of exercise.

Taken together, one bout of exhaustive exercise elicited a strong anti-inflammatory response through up-regulation of anti-inflammatory genes and down-regulation of pro-inflammatory ones. We think that this is a coordinated broad protective reaction of the body against possible exercise-induced inflammatory damage. Several factors which are induced by exercise might be involved in this anti-inflammatory gene response. These include catecholamines, cortisol, growth hormones, heat shock proteins and muscle-derived IL-6 (see review Gleeson et al., 2011). In this context, cortisol, which is strongly induced through exercise, is much known for its immunosuppressive/anti-inflammatory functions (Cupps and Fauci, 1982; MacKenzie et al., 2006). In addition, IL-6 which is mainly released from working muscles plays an important role in the anti-inflammatory response of exercise through release of IL-10 and IL-1ra, ACTH/cortisol, and acute phase reactants of hepatocytes (e.g., a1 acid glycoprotein) (Steensberg et al., 2003; Northoff et al., 1995). Apart from the anti-inflammatory role of IL-6, we think that additional mechanisms may also be involved. We propose a hypothesis that exercise-induced modification of TLRs could orchestrate a different pattern of anti-inflammatory response (Abbasi et al. 2013b). Modification of TLRs by exercise induced molecules such as IL-6 or miRNAs, might directly induce SOCS3, DUSP1, IL-10 and others,

and change the reaction cascade of TLRs towards fast induction of TNIP3 upon pathogen contact.

Additional interesting findings of the present study are the regulation of some brain-related genes such as OLIG2, TMEM106B, and NR4A2. The expression of some, like OLIG2, was reported for the first time in blood cells. Until now, OLIG2 was believed to be expressed in neural cells only. OLIG2, which is a marker for certain oligodendrocyte precursors (Takebayashi et al., 2000), was significantly down-regulated following exercise, but this was only observed in LPS-stimulated cultures of male athletes. TMEM106B, which is involved in frontotemporal lobar degeneration (Chen-Plotkin et al., 2012), was significantly down-regulated in LPS-stimulated cultures of females but not males. Whether the changes in the expression of these genes are associated with exercise-induced neuroplasticity needs to be investigated in the future.

Furthermore, exhaustive exercise significantly down-regulated the expression of DNA methylation gene DNMT1 (DNA methyltransferase-1) in LPS-stimulated and un-stimulated blood cultures of both sexes following exercise (~ 4 fold down-regulation). In addition to its role in DNA methylation, recent studies have clearly pointed to a role of DNMT1 in inflammation and inflammation-associated carcinogenesis (Foran et al., 2010). Inside signal transduction DNMT1 has been shown to down-regulate SOCS3 expression, and knockdown of DNMT1 can also increase the expression of SOCS3 (Dhar et al., 2013). Thus, in exercise related regulation too, a possible relationship between DNMT1 and SOCS3 seems possible. The down-regulation of DNMT1 may be an integral factor in the anti-inflammatory effect of exercise. To our knowledge, this is the first study showing a change in DNMT1 mRNA expression in circulating leukocytes in response to exercise.

Using new bioinformatics approaches we performed functional enrichment testing including Gene Ontology (GO) (Ashburner et al., 2000) and Kyoto Encyclopaedia of Genes and Genomes (KEGG; www.genome.jp/kegg/) pathways (Kanehisa et al., 2000) to determine the relative enrichment of genes with common or related functionalities to gain insight into biological processes mediated by LPS or exercise or both. Exhaustive exercise significantly changed several biological pathways in both LPS-stimulated and un-stimulated cultures. The most significantly over-represented KEGG pathways in LPS-stimulated and un-stimulated cultures in response to exercise were hematopoietic cell lineage, natural killer cell mediated

cytotoxicity, T cell receptor signaling pathway, primary immunodeficiency, leishmaniasis and graft-versus-host diseases. Some of these significantly activated KEGG pathways have also been reported by previous studies. These are: activation of hematopoietic cell lineage, natural killer cell signaling, T cell receptor signaling, cancer, cytokine-cytokine receptor interaction, antigen processing and presentation, Type1 diabetes mellitus, JAKSTAT signaling and TLR signaling pathways (Radom-Aizik et al., 2009a, 2009b; Carlson et al., 2011; Radom-Aizik et al., 2013; Königsrainer et al., 2012). Some KEGG pathways were only activated in LPS-stimulated cultures but not in un-stimulated ones. These were prominently associated either with signaling related to chemokines, neurotrophin, MAPK, and mTOR, or with diseases (african trypanosomiasis, lysosome, thyroid cancer, acute myeloid leukemia), suggesting that exercise does indeed interact with early steps in the reaction to pathogen. We could not find any study reporting the activation of these pathways in peripheral blood in response to exercise.

Taken together, it can be concluded, from the results of gene expression profiling, that: 1- In addition to changing gene expression profiles in un-stimulated cells, exhaustive exercise can induce substantial changes in the early gene expression response to endotoxin. 2- Some potentially important effects of exercise can only be detected in relation to pathogen stimulation. 3- There is an anti-inflammatory bias of gene regulation by exercise, including induction of genes involved in the negative regulation of TLR signaling, and knockdown of end products of TLR signaling pathway (IFN-β1).

3-3. Gender differences in the exercise-induced immune response

Recent studies including our work demonstrate significant gender dimorphisms in different aspects of immune responses to exercise (Abbasi et al. 2013a; Northoff et al. 2008; Liu et al. 2010; Timmons et al., 2005, 2006), although studies reporting the impact of sex hormone fluctuations on immunological responses to exercise are quite rare. Here we discuss our new results about protein and gene expression patterns in circulating leukocytes obtained from healthy male and female athletes at before, and after exercise. In a first gender and menstrual phase-controlled microarray study we found a higher extent of gene regulation for females in luteal phase as compared with same females in follicular phase or male athletes. While the pro-inflammatory genes were up-regulated in luteal phase of menstrual cycle, the anti-inflammatory genes were strongly down-regulated in same phase. This reaction was not seen

in the same females at follicular phase or in men. Some genes such as IL-6, IL-1ra, IL-12RB1, PTGDR, and IL-18RAP were clearly regulated in opposite directions in luteal phase as opposed to follicular phase or male. These results were later considerably confirmed by the second independent study, in which male athletes showed significantly higher amounts of LPS-dependent release of IL-6 and IL-1ra as well as higher levels of spontaneously produced IL-10 than females (in luteal phase) following exhaustive exercise (Abbasi et al. 2013a). Our results firstly confirmed the findings of previous studies that sex-specific responses of the immune system to exercise do exist, and secondly, confirmed the role of sex hormones and menstrual cycle in the early steps of immune response to exercise. Before, Timmons et al (2005) had pointed to the gender- and menstrual phase dependent alterations of the reaction of selected cytokines to exercise. The exercise-induced change in IL-6 was approximately 80% greater in non oral contraceptive users (NOC) compared to oral contraceptive users (OC) during follicular phase of menstrual cycle, but it was similar between these groups during luteal phase (Timmons et al., 2005).

It could be concluded, from the results of our 2 independent studies that women in luteal phase show a distinctly different pattern of inflammatory cytokine regulation in response to exercise, compared with women in follicular phase and/or men. The overall direction of cytokine changes of women in luteal phase is clearly pro-inflammatory. It has been previously demonstrated that the luteal phase has an inflammatory bias as compared with the follicular phase (Willis et al., 2003). Lynch et al (1994) reported higher amounts of IL-1ra concentration in follicular phase compared to luteal phase (Lynch et al., 1994). It has also been shown that during luteal phase some leukocytes are changed in phenotype and secretory activity to a more pro-inflammatory and pro-migratory profile (Polan et al. 1994).

3-4. Exercise regulates miRNAs in peripheral cells

The response to exercise of miRNAs in circulating leukocytes was also evaluated to get further insights into the regulation of gene expression (in especial immune genes) by exercise. In a study investigating the effects of 30min moderate exercise before, 30min after and 60min after exercise, microarray technology was used to analyze and compare miRNA and mRNA expression. Previous microarray studies presenting results on miRNA exclusively tested miRNAs at 1 time point after exercise without following the dynamics into the recovery period. The design of our study enabled us to identify 4 dynamically regulated networks

consisting of differentially expressed miRNAs and mRNAs with anti-correlated expression profiles over time. The expression of miR-21-5p, miR-24-2-5p, miR-27a-5p, and miR-181a-5p were changed in response to exercise.

Using network analysis the mRNA targets for each differentially expressed miRNA was identified. Based on anti-correlating analysis, it was suggested that miR-21-5p regulates TGFBR3, PDGFD and PPM1L mRNAs. MiR-24-2-5p was responsible for MYC and KCNJ2 genes and miR-27a-5p for ST3GAL6. The target genes for miR-181a-5p were ROPN1L and SLC37A3. All target genes were prominently associated with exercise response including immune function, apoptosis, membrane traffic of proteins, and transcription regulation (Tonevitsky et al., 2013). These findings confirm the results of previous studies (Radom-Aizik et al., 2010, 2012, 2013) that an acute bout of exercise changes the expression of several miRNAs in circulating leukocytes, suggesting a regulatory role for miRNAs in immune response to exercise.

Notably the up-regulation of circulating miR-21-5p by exercise was first reported by Baggish et al (2011). Although miR-21 has been implicated in some diseases such as in heart failure (Thum et al., 2008) and renal ischemia reperfusion injury (Godwin et al., 2010), its overall action has been described by several authors to be strongly anti-inflammatory (Baggish et al., 2011; O'Neill et al., 2011). Therefore, the up-regulation of miR-21 after 60min recovery in all subjects may reflect the self-protective anti-inflammatory reaction to exercise.

The response of miR-181a-5p to exercise might be even more interesting to discuss. The expression of miR-181a was increased immediately after exercise in all subjects. In line with these findings, Radom-Aizik et al (2010, 2012) reported up-regulation of miR-181 in response to an interval exercise. Since the expression of miR-181 has been shown in different leukocyte subtypes, it could be assumed that this miRNA may play a role in the regulation of adaptive changes in immune system. MiR-181a has been shown to suppress the inflammatory response induced by oxidized low-density lipoprotein in dendritic cells (Wu et al., 2012). The increased levels of miR-181a in whole blood leukocytes during the early inflammatory response have been proposed to be a compensatory event to limit hyperinflammatory reactions (Xie et al., 2013). Therefore, exercise-induced miR-181 can also be interpreted as a compensatory anti-inflammatory response to primary inflammatory stimuli caused by exercise.

In conclusion, our data demonstrate that the expression of miRNAs is changed in response to exercise and that this can be anticorrelated with expression of some of their known target mRNAs. This, in turn, might be involved in the regulation of exercise-responsive genes in the immune system, suggesting another level of regulation of cellular processes mediated by miRNAs.

3-5. Conclusion

In conclusion, as synopsis of all papers published with my contribution several statements can be made to summarize the most important points: 1- exercise has a major influence on the early cytokine response to pathogen. The response has a dramatic anti-inflammatory bias. The exercise-induced IL-10 response is however not influenced by endotoxin. 2- Microarray analysis could reveal a set of exercise reactive genes which can only be detected in presence of pathogen stimulation. To them belongs TNIP3, a regulator of the TLRs cascade. 3- our studies prove that women in luteal phase of their menstrual cycle show significantly less anti-inflammatory regulation than women in follicular phase or men. 4- Exercise does also impact some methylation related and some brain related genes, some of which were not known to be expressed outside of the brain. 5- microRNAs are involved in exercise induced gene expression changes and dynamically regulated miRNA/mRNA networks could be demonstrated. Further investigation of their impact seems warranted.

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Curriculum Vitae

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Education

Apr. 2008- April 2014: PhD student in Molecular Exercise Immunology, University Hospital Tuebingen, Tuebingen, Germany

2005-2007: Master of Science in Exercise Physiology – University of Urmia – IRAN

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Publications

1- Makarova J , Maltseva DV, Galatenko VV, **Abbasi A**, Maximenko DG , Grigoriev AI, Tonevitsky AG, and Northoff H. Exercise Immunology Meets MiRNAs. Accepted for publication in Exerc Immunol Rev. 2014

2- **Abbasi A,** Hauth M, Walter M, Hudemann J, Wank V, Niess AM2, Northoff H. Exhaustive exercise modifies different gene expression profiles and pathways in LPS-stimulated and unstimulated whole blood cultures. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2013.10.023

3- **Asghar Abbasi,** Elvira Fehrenbach, Melanie Hauth, Michael Walter, Jens Hudemann, Veit Wank, Andreas M Niess, Hinnak Northoff. Changes in Spontaneous and LPS-induced ex vivo Cytokine Production and mRNA expression in Male and Female Athletes Following Prolonged Exhaustive Exercise. Exerc Immunol Rev. 2013;19:8-28.

4- Alexander G Tonevitsky, Diana V Maltseva, **Asghar Abbasi**, Timur R Samatov, Dmitry A Sakharov, Maxim U Shkurnikov, Alexey E Lebedev, Vladimir V Galatenko, Anatoly I Grigoriev and Hinnak Northoff. Dynamically regulated miRNA-mRNA networks revealed by exercise. BMC Physiology 2013, 13:9.

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6- Tartibian B, Maleki BH, **Abbasi A**. Omega-3 fatty acids supplementation attenuates inflammatory markers after eccentric exercise in untrained men. *Clin J Sport Med. 2011 Mar; 21(2):131-7.*

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11- Northoff H, Symons S, Zieker D, Schaible EV, Schäfer K, Thoma S, Löffler M, **Abbasi A,** Simon P, Niess AM, Fehrenbach E. Gender- and menstrual phase dependent regulation of inflammatory gene expression in response to aerobic exercise. Exerc Immunol Rev. 2008;14:86-103.

Book chapter

- Hinnak Northof, Markus Löfller, **Asghar Abbasi**. Bewegungstherapie bei internistischen Erkrankungen (Translation: Exercise Therapy for Medical Diseases) (Springer, ISBN: 978-3-642-01331-7).

- Bakhtyar Tartibian, Behzad Hajizadeh Maleki, **Asghar Abbasi**, Mehdi Eghbali, Siamak Asri-Rezaei and Hinnak Northoff. Comparison of Seminal Superoxide Dismutase (SOD) Activity between Elite Athletes, Active and Non Active Men. Source: An International Perspective on Topics in Sports Medicine and Sports Injury. ISBN 978-953-51-0005-8. Edited by: Kenneth R. Zaslav. Publisher: InTech

Honors and Awards

- 2003 Ranked 1st among BSc students at University of Urmia- IRAN
- 2009 Awarded by DAAD (German Academic Exchange Service) for Doctoral Scholarship
- 2013, Travel award for 11th ISEI symposium in Newcastle, Australia.

Supplements

In the following, papers I, II, III, IV, V and VI are reprinted.

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

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Abstract

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

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

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

Introduction

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

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

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

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

Methods and Materials

Subjects

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

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

Preliminary Testing

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

Exercise program

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

Blood sampling

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

In vitro stimulation of whole blood with LPS

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

Table 1. Physical characteristics of the subjects

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

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

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

Measurement of Blood Inflammatory Protein Markers

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

Measurement of plasma TGF-β1

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

Total RNA isolation and cDNA synthesis

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

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

Quantitative real-time PCR

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

Table 2. Primer sequences for qRT-PCR

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

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

Statistical analysis

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

Results

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

Exercise

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

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

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

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

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

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

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

Leukocyte numbers

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

Cytokine concentrations in stimulated and unstimulated whole blood

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

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

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

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

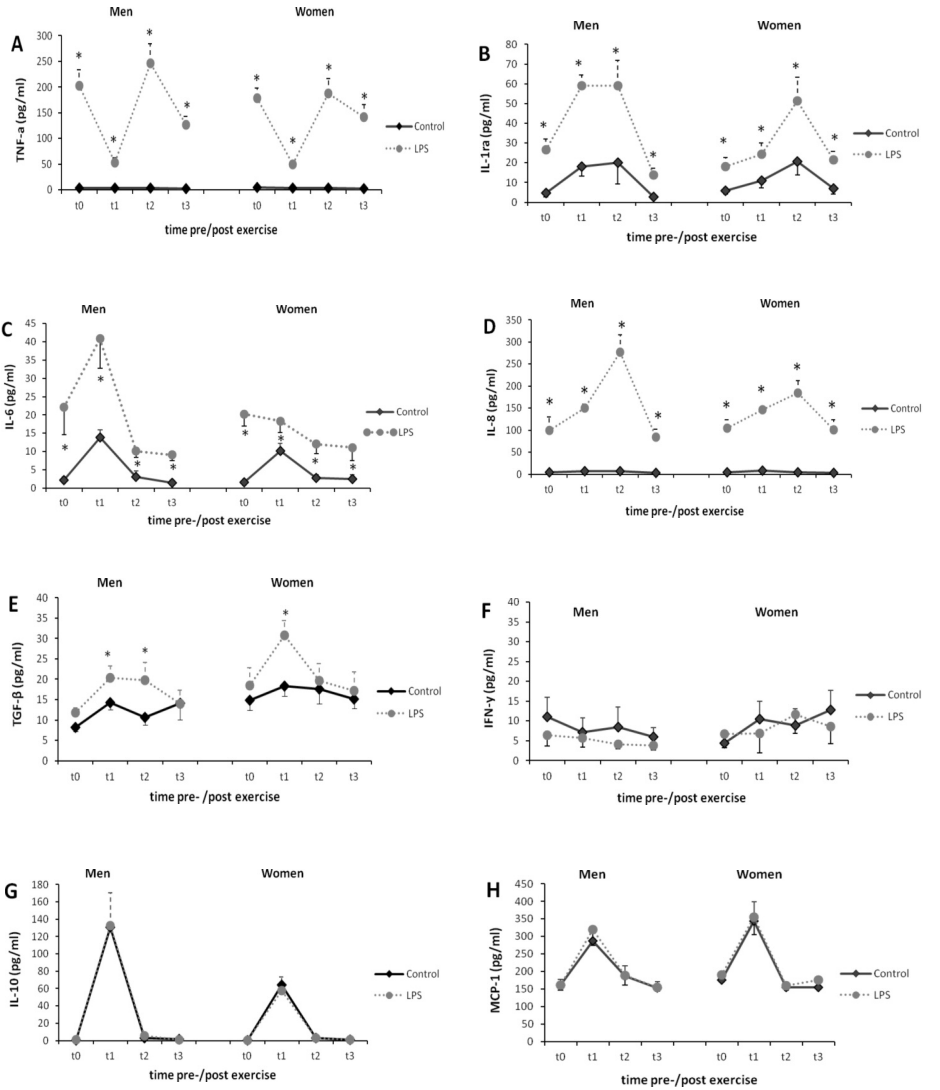
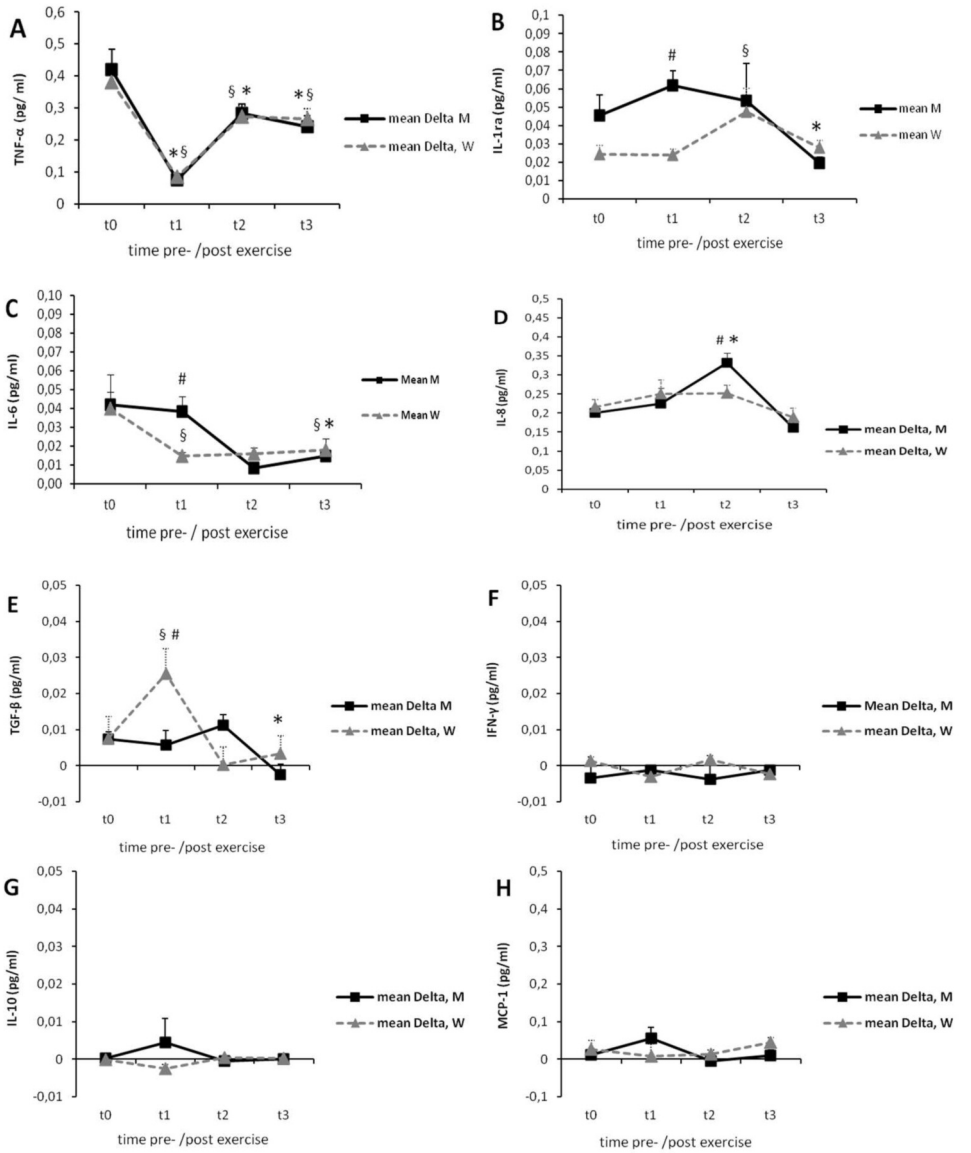


Figure 2. LPS-dependent release per 1000 monocyte (LDR) of cytokines (concentration in stimulated cultures minus concentration in un-stimulated cultures). LPS-dependent release of IFN-γ was normalized to 1000 lymphocytes. Data represent the means ± SE of the values. **M** represents men and **W** represents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



Sex differences in cytokine release

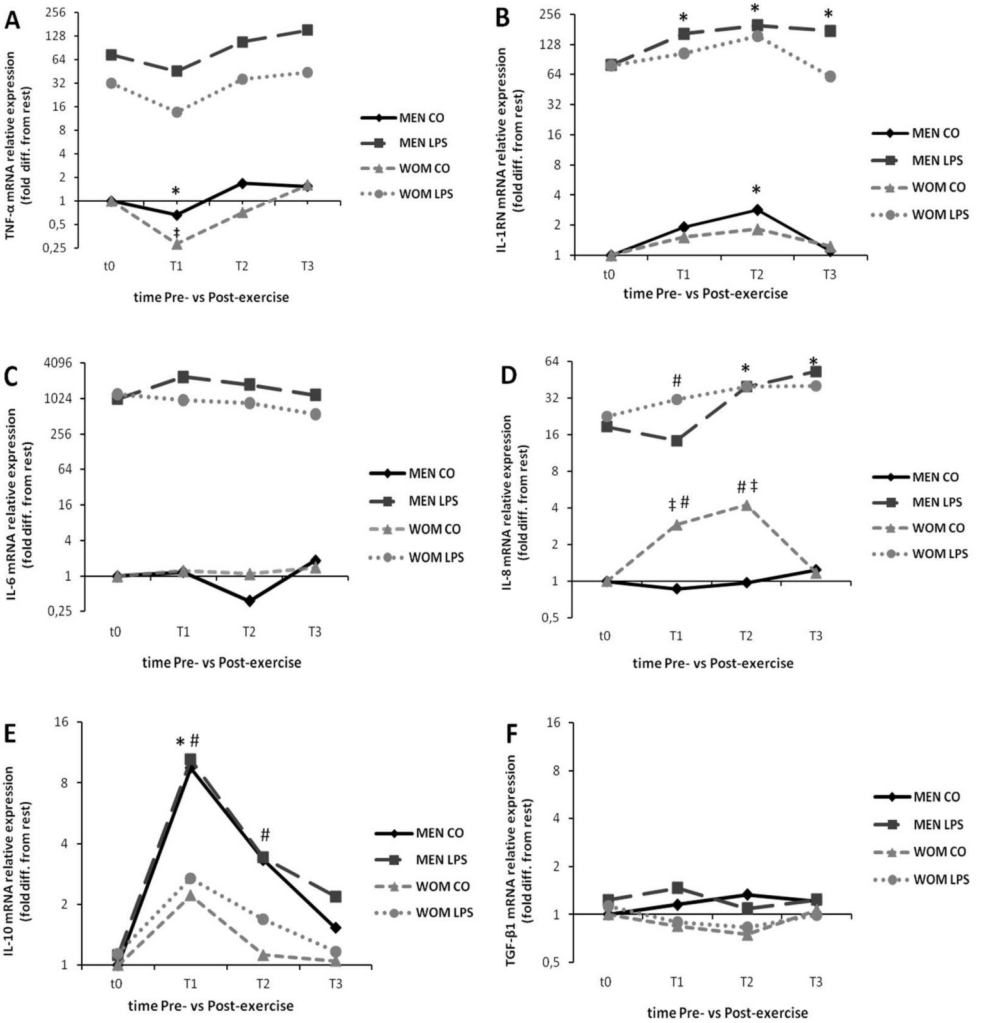
No significant difference was observed between sexes in LDR and unstimulated levels of TNF-α and MCP-1 at either time point (P>0.05) (Figure 2, Table 4). Men showed significantly higher LDR of IL-6 and IL-1ra and also significantly higher unstimulated levels of IL-10 at 30 min post race. As compared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

Cytokine mRNA relative expression in stimulated and unstimulated whole blood

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

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

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



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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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Exhaustive exercise modifies different gene expression profiles and pathways in LPS-stimulated and un-stimulated whole blood cultures

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ABSTRACT

Exhaustive exercise can interfere with immunity, causing transient immunosuppression and infections/inflammation in athletes. We used microarray technology to analyze the gene expression profiles of whole blood in short time (1 h) LPS-stimulated and un-stimulated cultures drawn before, 30 min after, 3 h after and 24 h after a half-marathon run. Four male and 4 female athletes participated. Exercise induced differential expression of genes known to be involved in innate immunity/inflammatory response, metabolic response, DNA methylation, apoptosis and regulation of brain function. Several genes with prominent anti-inflammatory function were up-regulated in un-stimulated cultures, including ARG-1, SOCS3, DUSP-1, ORMs, IRAK3, and GJB6. Some of these genes were also strongly up-regulated in LPS-stimulated cultures (ARG-1, ORM2, and GJB6). Some genes were strongly up-regulated through exercise in LPS-stimulated cultures, but not in un-stimulated cultures (TNIP3, PLAU, and HIVEP1). There was also a row of genes, which were strongly down-regulated by exercise in LPS-stimulated cultures, notably IFN-β1 and CXCL10. Exercise also significantly changed the expression of genes (OLIG2, TMEM106B) which are known to be related to brain function and expression of which has never been documented in peripheral blood. In summary, exhaustive exercise, in addition to modifying gene expression in un-stimulated cells, could also interfere with the early gene expression response to endotoxin. There was an anti-inflammatory bias of gene regulation by exercise, including genes involved in the negative regulation of TLRs signalling. The results of the present study demonstrate that some potentially important effects of exercise can only be detected in relation to pathogen stimulation.

1. Introduction

A growing number of published reports on exercise immunology provide evidence that in contrast to moderate physical activity, an acute bout of prolonged (>1.5 h), exhaustive exercise such as marathon or half-marathon running can cause adverse effects on immunity as reflected by transient immunosuppression and inflammation-like reactions following the event (Nieman, 1995; Nieman et al., 1990; Pedersen and Bruunsgaard, 1995). During this 'open window' of decreased host protection (which may last between 3 and 72 h), viruses and bacteria may gain a foothold, increasing the risk of subclinical and clinical infection (Nieman, 2007). Upper respiratory tract infection (URTI) has regularly been reported as exercise-related infection (viral or bacterial), with increased frequency during periods of heavy training and in the 1- to 2-week period following participation in competitive endurance races (Nieman et al., 1990; Nieman, 2007).

Measurable immune parameters affected by exhaustive exercise comprise changes in peripheral cell numbers; decreases in granulocyte burst activity, NK cytotoxic activity and lymphocyte proliferation; the appearance of some cytokines in plasma (IL-6, IL-8, IL-1, IL-10), and decreased production of some cytokines in stimulated blood cultures (IL-1, TNF-α, IFN-γ) (Stewart et al., 2005; Kakanis et al., 2010; Woods et al., 2000; Shephard and Shek, 1999; Ostrowski et al., 1999; Weinstock et al., 1997; Pyne, 1994). Body temperature changes, increased blood flow and dehydration, and changes in stress hormones including adrenaline and

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glucocorticoids have been discussed as underlying mechanisms (Nieman, 1995). In particular, corticoids are known for their broad immunosuppressive effects and have been shown to be elevated in response to prolonged exhaustive endurance exercise (Keast et al., 1988). Even so, at present, the true sequence of events leading to transient post-exercise immunosuppression is by no means clear. One theory focuses on the strongly suppressed capability of blood leukocytes to respond to LPS in vitro by production of pro-inflammatory cytokines, especially IFN-γ (Northoff et al., 1994). In fact, these experiments have shown that many possibly important effects of exhaustive exercise could neither be detected in native plasma nor in un-stimulated blood cultures, but required in vitro stimulation to become visible.

Stimulation of cells can rapidly be initiated by microbial compounds like endotoxin, also known as lipopolysaccharide (LPS), which engage pattern recognition receptors (in this case TLR-4) leading to activation of NF-κB transcription factor and release of a host of pro-inflammatory cytokines including IFNs and chemokines (Beutler, 2000).

In this light, we decided to use LPS stimulation of whole blood cultures as a model for an in vivo infection at different time points in relation to exercise, and to analyze the effects of exercise, the effects of LPS stimulation, and the effects of their combined action using gene expression microarray technology. The use of whole blood cultures has several advantages in the context of our study, avoiding possible effects from in vitro manipulation (Fannin et al., 2005), allowing us to see collaborative effects, and enabling short and precise timing. To our knowledge, this is the first study to use this complex approach. Until now, only selected cytokines have been investigated in relation to exercise and LPS (Phillips et al., 2012; Martin et al., 2013; Stewart et al., 2005; Weinstock et al., 1997) and several studies have analyzed the effects of exercise on native, un-stimulated blood in relation to cytokine production, but no high throughput analysis of LPS-stimulated cultures in relation to exercise is currently available.

Our approach also differs from previous studies in that we used a very short stimulation time (1 h). Focus on the early events after LPS stimulation may yield results which cannot be detected in longer stimulated cultures (e.g., exercise-related delays of LPS-dependent activation). Such results may still be clinically relevant, since a short-term interference of an exercise bout with the normal process of immune activation may give incumbent or invading infectious agents a foothold to establish an infection.

The central aim of this work was to study the effect of endurance exercise on the early steps of the immune reaction to pathogen contact and compare them to exercise effects in the absence of pathogen. This should lead to the identification of new pathways, candidate genes or interaction patterns of exercise and pathogen effects, ultimately helping to further understand the mechanisms underlying exercise-induced immunosuppression and thus the "open window for infection" phenomenon.

2. Methods and materials

2.1. Materials

K3-EDTA tubes (Vacuette) were obtained from Greiner bio-one (Frickenhausen/Germany), non-pyrogenic 15 ml tubes to perform LPS stimulation from SARSTEDT (Nümbrecht/ Germany). LPS (*Escherichia coli*) was purchased from Sigma–Aldrich (Munich/ Germany). The PaxGene Blood RNA Tubes and the PaxGene Blood RNA kit to stabilize and isolate the RNA were purchased from PreAnalytix (Switzerland). Human Genome U219 Gene Chip® arrays, 3′IVT Express kits and the GeneTitan® Hybridization, Wash, and Stain Kit were all purchased from Affymetrix (United Kingdom).

2.2. Subjects

Eight well-trained male athletes [34.8 ± 9.4 years, body mass index (BMI) 23.41 ± 2.2 kg/m^2] and 8 well-trained female athletes [38.5 ± 5.7 years, body mass index (BMI) 21.9 ± 1 kg/m^2] participated in the study. From these, 4 women who were in the second half of their menstrual phase and 4 randomly selected men were entered into microarray analysis. The individuals had been engaged in specific endurance training for at least 2 years (52.2 ± 25.5 km/week, running). None of the athletes suffered from acute or chronic diseases or reported intake of medicationor antioxidant supplements; all athletes were non-smokers. Informed written consent was obtained from each subject, and the study was approved by the University Ethics Committee. All were experienced athletes with normal dietary habits. The women included in the study had regular menstrual cycles and did not use oral contraception. To confirm in which phase of menstrual cycle they were, the hormonal status of women was determined by measuring plasma estrogen, progesterone, LH, and FSH using the ADVIA Centaur immunoassay system (Siemens Healthcare Diagnostics, Fernwald, Germany).

2.3. Experimental design

Before participating in the main study the athletes performed an incremental exercise test on a treadmill (Saturn, HP Cosmos, Traunstein, Germany) to determine the running velocity at the individual anaerobic threshold (V_{IAT}). Capillary blood for lactate measurement (EBIO, Eppendorf, Hamburg, Germany) was obtained from the earlobe after every stage and heart rate was monitored continuously using a heart rate monitor (Polar Electro, Finland). V_{IAT} was calculated by the method of Dickhuth et al. (1991). All athletes performed an official half-marathon (21.1 km) under competition conditions. The run started at 10:00 AM on a cool and humid December day (1 °C) and took place on a hilly and demanding terrain.

2.4. Blood sampling and ex vivo stimulation of whole blood

Venous blood samples were drawn from the antecubital vein at rest in a sitting position and collected into endotoxin-free K3-EDTA tubes (Vacuette, Greiner bio-one-Frickenhausen, Germany). Samples (a total of 30 ml whole blood) were obtained from each subject at times before ($t0$), 30 min after ($t1$), 3 h after ($t2$) and 24 h ($t3$) after the half-marathon run (Fig. 1). Leukocyte numbers and differential counts before and after the run were determined using an automated Abbott Ruby Coulter counter.

2.5. Whole blood cell cultures

A total of 16 ml whole blood was cultured using a whole-blood culture system as developed in our laboratory. Briefly, 2 × 9-ml tubes containing K3-EDTA were drawn. One tube was stimulated with lipopolysaccharide (final concentration 10 ng/ml). The other tube received the same volume (8 μl) of PBS. After 1 h incubation with slow rotation at 37 °C, both tubes were centrifuged at 1000g for 10 min to obtain plasma. Aliquots were stored at −70 °C until assay. The remainder of the blood including cells was used to extract RNA.

2.6. RNA isolation and quality control

LPS-stimulated and un-stimulated blood samples (2.5 ml/tube, 2 tubes/subject) from male and female athletes were transferred into two PaxGene Blood RNA Tubes (PreAnalytiX/QIAGEN, Switzerland). Total RNA was isolated using the PaxGene Blood RNA kit (PreAnalytix/Switzerland) according to the manufacturer's protocol, with minor modifications. The concentration of the extracted

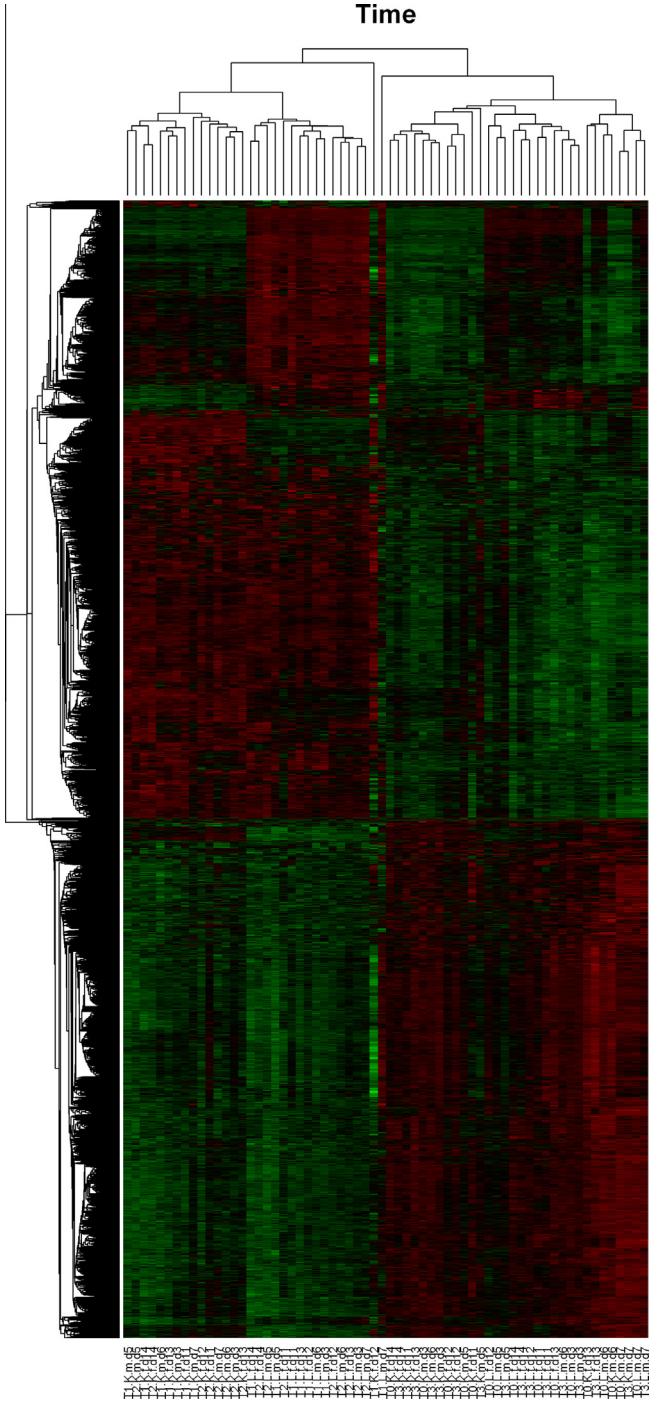

Fig. 1. Hierarchical cluster analysis of all transcripts which were significantly changed by exercise ($n = 5565$). Rows correspond to probeset ids, columns correspond to samples (Label = Timepoint.Treatment.Gender. DonorID). Red: high expression, green: low expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

RNA was measured spectrophotometrically (Nanodrop 1000/Thermo Scientific) and the quality was assessed by a lab-on-a-Chip-System on the Bioanalyzer 2100 (Agilent/Germany) to ensure that samples used for microarray analysis had intact 18S and 28S ribosomal RNA peaks and low degradation factor.

2.7. Transcriptome analysis

For expression profiling 100 ng of total RNA was linearly amplified and biotinylated using the GeneChip HT 3'IVT Express Kit (Affymetrix, UK) according to the manufacturer's instructions. 15 µg of labeled and fragmented cRNA was hybridized onto Human Genome U219 Gene Chip® arrays (Affymetrix). Hybridization, washing, staining and scanning was performed automatically in a GeneTitan® instrument (Affymetrix). Scanned images were subjected to visual inspection to control for hybridization artifacts and proper grid alignment and analyzed with AGCC 3.0 (Affymetrix) to generate CEL files.

2.8. Bioinformatic data analysis

Microarray hybridizations were analysed on the software platform R 2.12.0 with Bioconductor 2.10.0 (Gentleman et al., 2004). Initially, the expression data from all chips were background corrected, quantile normalized and summarized with RMA (Robust Multichip Average) (Irizarry et al., 2003). Due to the design of the experiment, three parameters (treatment, time and gender) have an impact on gene expression, while the influence of inter-individual differences from the 16 different donors has to be taken into account. Therefore, a combined factor from treatment, time and gender was used to design a linear model which captures the influence on gene expression levels while using the donor as random variable. A non specific filter based on overall variance was applied to remove non informative genes before the fitting of the linear model was performed. The coefficients describing the expression profiles of the remaining probe sets were calculated and the standard errors were moderated using an empirical bayesian approach (Smyth, 2004). From the F statistic the resulting *p*-values were established and corrected for multiple testing with "Benjamini-Hochberg" (Benjamini and Hochberg 1995). To attribute significant regulations to individual contrasts, a decision matrix was generated based on the function 'decide tests' within the limma package, where significant up- or down-regulations are represented by values of 1 or −1, respectively.

Cluster analysis for selected probesets was performed in R 2.15.1. Signal intensities were scaled and centered and the distance between two expression profiles was calculated using euclidian distance measure. Hierachical cluster analysis was performed with average linkage. Heatmaps were generated with Bioconductor package *geneplotter*.

2.9. Gene ontology and KEGG pathway analysis

In the lists of genes that were significantly differentially expressed with LPS and exercise in our study, we conducted functional enrichment testing including Gene Ontology (GO) (Ashburner et al., 2000) and Kyoto Encyclopaedia of Genes and Genomes (KEGG; www.genome.jp/kegg/) pathways (Kanehisa and Goto, 2000) to determine the relative enrichment of genes with common or related functionalities to gain insight into biological processes mediated by LPS or exercise or both. This might give an overview which biological and molecular processes are responsible for the observed changes in transcription. A one-sided conditional hypergeometric test was used to analyze the lists of differentially regulated transcripts for over-representation of GO categories in the two GO main branches "biological process" and "molecular function". GO categories with a *p*-value of less than 0.01 were called significantly enriched. In the same way, the lists were analyzed for over-representation of known signal transduction and metabolic pathways from the KEGG data base.

3. Results

3.1. Exercise

All runners successfully completed the half-marathon race (21.1 km) in an average running time of 95.5 ± 8 min (86–116 min)

for men and 114 ± 12 min (96–129 min) for women. The individual characteristics of the subjects are shown in Table 1.

3.2. Blood cell count in response to exercise

The effect of half-marathon running on total leukocyte, granulocyte, lymphocyte and monocyte counts are illustrated in Table 2. Total leukocyte counts were increased significantly 30 min after exercise. Throughout the 3 h recovery period, the total white cell count remained significantly elevated ($P < 0.0001$). The prolonged exercise bout induced a pronounced granulocytosis, which was largely responsible for the changes in total white cell count. Changes were significant at 30 min ($P < 0.0001$) and remained elevated at 3 h post exercise ($P < 0.0001$). The circulating monocyte count also rose mildly but significantly after exercise at the same time points as granulocytes. Circulating total lymphocyte count decreased 30 min after exercise and remained attenuated for at least 3 h post competition ($P < 0.0001$). The total leukocyte, granulocyte, monocyte and lymphocyte counts reached pre-exercise levels at 24 h post-exercise (Table 2).

3.3. Gene transcription and pathway regulation induced by exercise in LPS-stimulated and un-stimulated cultures (exercise effect)

Fig. 1 shows the global transcriptional profiles in LPS-stimulated and un-stimulated whole blood cultures in response to exhaustive exercise in both sexes (Cluster analysis – Fig. 1).

Exercise induced significant changes in LPS-stimulated and un-stimulated whole blood gene expression in both sexes. The total numbers of altered genes in each group are shown in Table 3. In total, more genes were activated in men than women, but women had more activated genes at 24 h post-exercise. The detailed list of genes that were altered by exercise in each group is shown in Supplementary Table S3, which is available with the online version of this article. Table S1 shows the 10 most significantly up- and 10 most significantly down-regulated genes which were differentially expressed in response to exercise in LPS-stimulated and un-stimulated whole blood cultures.

We used the KEGG pathway analysis to summarize specific biological pathways that were significantly over-represented by genes following exercise in LPS-stimulated and un-stimulated cultures in both sexes. A complete detailed list of pathways which were significantly overrepresented in each group is shown in Table 4. Activated pathways only partially overlap between stimulated and un-stimulated cultures and between sexes. More pathways were activated in LPS-stimulated compared to un-stimulated cultures. Contrasting with total number of activated genes, women showed more activated pathways than men. Prolonged activation (24 h) was only found in women.

The Toll-like receptor signaling pathway was the most significantly regulated pathway 24 h after exhaustive exercise.

Table 1
Physical characteristics of the subjects.

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

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

3.4. Gene transcription and pathway regulation induced by exercise in LPS-stimulated cultures only or in un-stimulated cultures only (exercise effect)

One of the central aims of this study was to characterize the expression of genes and the pathways that are significantly altered in the LPS-stimulated group only but not in the un-stimulated group, and vice versa. Supplementary Table S2 shows the top 10 most significantly up- and down-regulated genes in each group. A complete list of gene sets is shown in Supplementary Table S4. Table 5 shows the complete list of KEGG pathways which were significantly over-represented in only LPS-stimulated or in only un-stimulated cultures following exercise in both sexes. Here, too, more pathways were activated in LPS-stimulated compared to un-stimulated cultures, and women showed more activated pathways than men. Again, prolonged activation (24 h) was only found in women. Interestingly, in LPS-stimulated cultures, under this algorithm of calculation, no pathway was found to be activated at $t2$ while prominent activation was seen at $t1$ and $t3$ (Table 5).

Altogether, different patterns of gene regulation through exercise were observed in stimulated and un-stimulated cultures. For a better illustration, six of the prominently regulated genes (ARG1, SAP30, SOCS3, DUSP1, TNIP3 and IFN-β1), which show somewhat prototypical patterns are depicted in Fig. 2.

3.5. Gene transcriptional and pathway regulation induced by exercise and differentially affected by LPS (Time × Treatment interaction)

In this section, the changes in gene expression (up- or down-regulation) from pre-exercise ($t0$) to post-exercise ($t1$,$t2$,$t3$) in the LPS-stimulated whole blood in relation to the same status in un-stimulated whole blood are described [(LPS post Ex. − LPS pre Ex.) − (Con post Ex. − Con pre Ex.)].

It should be noted that following this Time × Treatment interaction (T × T interaction) no regulation results when up- or down-regulation through exercise occurs in LPS and control cultures in parallel. Regulation signifies that LPS and control cultures were affected by exercise in different ways. Thus, up-regulation of this T × T interaction can mean that LPS cultures were up-regulated through exercise with no change in controls, or that controls were down-regulated through exercise with no changes in LPS, or a mixture of both movements. Vice versa, down-regulation of T × T interaction means down-regulation of LPS cultures or up-regulation of controls in response to exercise.

Several genes were significantly altered in terms of this T × T interaction. Table S5 shows a detailed list of regulated genes in both male and female athletes. Significantly over-represented KEGG pathways on the basis of the T × T interaction calculation are shown in Table S6. Like with the other algorithms used, again, a higher degree of regulation was found in women than in men and prolonged activation was presented in women only.

4. Discussion

In recent years, microarray technology has increasingly been used to measure genome-wide changes in gene expression in response to exercise. Gene expression profiling of blood may better elucidate the mechanisms of exercise-related transient immunosuppresion (ERTI).

Our study is unique from several viewpoints. To our knowledge, it is the first study to use gene expression profiling of whole blood cultures in response to prolonged exhaustive exercise. Second, we used short duration (1 h) stimulation with LPS and third, we developed a new method combining the whole blood culture model and the PaxGene RNA extraction kit.

Table 2
Changes in peripheral blood leukocyte numbers [$\times 10^9$ cells/l] at before, 30 min after, 3 h after and 24 h after exhaustive exercise.

	Pre-exercise (*t*0)	30 min after Exe. (*t*1)	3 h after Exe. (*t*2)	24 h after Exe. (*t*3)
Men				
Total cells	5.68 ± 0.72	13.29 ± 1.27*	14.01 ± 0.98*	6.32 ± 0.55
Neutrophils	2.84 ± 0.28	11.09 ± 0.02*	11.83 ± 0.09*	3.41 ± 0.01
Neutrophils, %	50.02 ± 3.98	83.52 ± 1.51*	84.48 ± 1.01*	54.02 ± 3.61
Lymphocytes	2.08 ± 0.24	1.33 ± 0.12*	1.26 ± 0.07*	1.20 ± 0.02
Lymphocytes, %	35.36 ± 3.40	10.01 ± 0.97*	9 ± 0.77*	31.56 ± 2.72
Monocytes	0.46 ± 0.05	0.70 ± 0.02*	0.83 ± 0.42*	0.54 ± 0.04
Monocytes, %	8.19 ± 0.82	5.26 ± 0.45*	5.95 ± 0.42*	8.70 ± 0.88
Women				
Total cells	6.47 ± 0.67	13.10 ± 1.03*	14.83 ± 0.90*	6.94 ± 0.73
Neutrophils	3.45 ± 0.03	11.07 ± 0.01*	12.51 ± 0.09*	3.90 ± 0.02
Neutrophils, %	53.33 ± 4.56	84.56 ± 1.01*	84.3 ± 1.04*	56.31 ± 2.91
Lymphocytes	2.31 ± 0.02	1.34 ± 0.05*	1.43 ± 0.06*	2.20 ± 0.02
Lymphocytes, %	35.78 ± 4.09	10.27 ± 0.81*	10.38 ± 0.68*	33.07 ± 2.75
Monocytes	0.48 ± 0.04	0.54 ± 0.03*	0.70 ± 0.48*	0.50 ± 0.04
Monocytes, %	7.54 ± 0.65	4.18 ± 0.37*	4.76 ± 0.48*	7.85 ± 0.66

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

Table 3
Summary of the number of regulated transcripts in LPS-stimulated and un-stimulated cultures for each time point after exercise compared to pre-exercise (*t*0) in both sexes.

	*t*1	*t*2	*t*3
Men			
−LPS	3062	2544	5
+LPS	2695	3096	10
Women			
−LPS	2388	1736	11
+LPS	2279	1920	58

*t*1: 30 min post-exercise, *t*2: 3 h post-exercise, *t*3: 24 h post-exercise.
−LPS: unstimulated cultures, +LPS: LPS-stimulated cultures.

This combination minimizes potential gene expression changes, RNA degradation and losses that accompany leukocyte isolation protocols (Debey et al., 2004; Fannin et al., 2005), but allows natural interaction and fast, stringent kinetics. Consequently, it provided us with RNA of high quality and sufficient quantity to perform microarray analysis, yielding high level information about the effect of exercise on the early events in the response to a wide spread pathogen-derived endotoxin. We think our model comes as close as possible to the in vivo situation.

Interpretation of the data has to account for exercise-associated cellular shifts. From certain aspects this could be viewed as a disadvantage. In any case it would have to be considered as the price to be paid in order to achieve the aforementioned advantages. It can, however, also be seen as kind of reality marker, since it provides a snap-shot of the physiological situation at the time of analysis. Still, many of the observed gene expression changes clearly exceed the magnitude of the parallel shifts in cell composition. Furthermore, most of the relevant changes concerned monocyte associated parameters and the significance of any up-regulation is strengthened rather than corrupted by this situation.

Exhaustive exercise significantly altered a row of genes in LPS-stimulated and un-stimulated blood cultures of male and female athletes. Exercise induced, in both male and female cultures, the differential expression of genes encoding products known to be involved in innate immune/inflammatory responses, metabolic responses, the cell cycle, apoptosis and regulation of transcription. The results presented herein revealed that the majority of genes that were enriched in response to exercise (up- or down-regulated in expression) were clustered in biological categories that are recognized to have, or potentially have, a role in innate immunity/inflammation (see Supplementary Table S3). Depending on which aspect the calculation was based, different pathways came up as being activated. Overlap between un-stimulated and stimulated cultures was only partial. Consistent general findings were, however, that women showed a higher degree of pathway activation than men and still showed some regulation after 24 h, while men did not. Cellular shifts cannot explain the differences between sexes in our study since men and women exhibited virtually identical shifts in circulating cell numbers. This probably reflects the fact that the relative intensity of exercise was identical for men and women in this competitive situation.

The higher numbers of activated pathways in women somehow contrast with the lower number of activated genes (Table 3). We have no explanations for this finding so far. In a previous study we have shown that women in their late luteal phase (like the ones investigated in this study) showed much higher numbers of regulated genes immediately following 60 min of moderate exercise compared with women in the follicular phase or men (Northoff et al., 2008). Thus, the general finding that women in the luteal phase of their menstrual cycle show a different regulation following exercise is consistent. The exact kinetic, however, needs to be analyzed in more detail, and we will not focus on possible differences between sexes in the following part of the discussion.

The most activated KEGG pathways in LPS-stimulated and un-stimulated cultures in response to exercise were associated with hematopoietic cell lineage, natural killer cell mediated cytotoxicity, T cell receptor signaling pathway, primary immunodeficiency, leishmaniasis and graft-versus-host diseases (Table 4). Some of the significantly over-represented pathways are consistent with the finding of previous studies, including activation of hematopoietic cell lineage, natural killer cell signaling, T cell receptor signaling, cancer, cytokine–cytokine receptor interaction, antigen processing and presentation, Type1 diabetes mellitus, JAK-STAT signaling and TLR signaling pathways (Radom-Aizik et al., 2009a,b; Carlson et al., 2011; Radom-Aizik et al., 2013; Königsrainer et al., 2012). Some KEGG pathways were only over-represented in LPS-stimulated cultures but not in un-stimulated ones. These were prominently associated either with diseases (african trypanosomiasis, lysosome, thyroid cancer, acute myeloid leukemia) or with signaling related to chemokines, neurotrophin, MAPK, and mTOR, suggesting that exercise does indeed interact with early steps in the reaction to pathogen. We could not find any previous reports regarding the activation of these pathways in peripheral blood in response to exercise.

Table 4
Exercise activated KEGG Pathways in un-stimulated and LPS-stimulated cultures in male and female athletes.

KEGG Pathways	−LPS (con)						+LPS (con)					
	Men			*Women*			*Men*			*Women*		
	*t*1–*t*0	*t*2–*t*0	*t*3–*t*0	*t*1–*t*0	*t*2–*t*0	*t*3–*t*0	*t*1–*t*0	*t*2–*t*0	*t*3–*t*0	*t*1–*t*0	*t*2–*t*0	*t*3–*t*0
Hematopoietic cell lineage	$P = 0.001^{2}$	$P = 0.000^{1}$		$P = 0.001^{2}$	$P = 0.000^{1}$		$P = 0.000^{1}$	$P = 0.000^{1}$		$P = 0.000^{1}$	$P = 0.000^{1}$	
Natural killer cell mediated cytotoxicity	$P = 0.002^{4}$	$P = 0.000^{2}$		$P = 0.003^{6}$	$P = 0.000^{3}$		$P = 0.000^{3}$	$P = 0.000^{2}$		$P = 0.000^{2}$	$P = 0.000^{2}$	
T cell receptor signaling pathway	$P = 0.000^{1}$	$P = 0.000^{3}$		$P = 0.002^{4}$	$P = 0.000^{2}$		$P = 0.001^{4}$	$P = 0.000^{3}$		$P = 0.000^{4}$	$P = 0.001^{3}$	
Leishmaniasis	$P = 0.002^{3}$			$P = 0.000^{1}$	$P = 0.006^{14}$		$P = 0.006^{9}$	$P = 0.002^{7}$		$P = 0.000^{3}$	$P = 0.003^{5}$	
Primary immunodeficiency	$P = 0.007^{5}$	$P = 0.008^{7}$			$P = 0.002^{7}$		$P = 0.009^{13}$					
Graft-versus-host disease	$P = 0.009^{6}$			$P = 0.003^{5}$			$P = 0.000^{2}$					
Antigen processing and presentation				$P = 0.002^{3}$						$P = 0.002^{6}$	$P = 0.004^{7}$	
Dorso-ventral axis formation		$P = 0.000^{4}$			$P = 0.000^{4}$			$P = 0.000^{4}$			$P = 0.003^{6}$	
Acute myeloid leukemia		$P = 0.004^{5}$									$P = 0.008^{8}$	
Jak-STAT signaling pathway		$P = 0.006^{6}$						$P = 0.003^{8}$				
Chagas disease					$P = 0.000^{5}$		$P = 0.006^{8}$	$P = 0.005^{9}$		$P = 0.003^{7}$		$P = 0.003^{4}$
Toll-like receptor signaling pathway					$P = 0.002^{6}$							$P = 0.002^{1}$
Pathways in cancer					$P = 0.002^{8}$							
MAPK signaling pathway					$P = 0.003^{9}$					$P = 0.004^{10}$	$P = 0.009^{9}$	
Chronic myeloid leukemia					$P = 0.003^{10}$							
Amoebiasis					$P = 0.004^{11}$		$P = 0.008^{11}$	$P = 0.007^{10}$				
Prion diseases					$P = 0.005^{12}$							
Prostate cancer					$P = 0.005^{13}$							
Bladder cancer						$P = 0.009^{1}$	$P = 0.008^{10}$					
Allograft rejection							$P = 0.001^{5}$			$P = 0.004^{11}$		
Type I diabetes mellitus							$P = 0.003^{6}$					
Cytokine-cytokine receptor interaction							$P = 0.003^{7}$	$P = 0.001^{5}$		$P = 0.001^{5}$	$P = 0.002^{4}$	$P = 0.006^{5}$
Malaria							$P = 0.009^{12}$			$P = 0.003^{9}$		$P = 0.002^{3}$
Drug metabolism - other enzymes										$P = 0.003^{8}$		
Chemokine signaling pathway								$P = 0.001^{6}$				
Cytosolic DNA-sensing pathway												$P = 0.002^{2}$
Wnt signaling pathway												$P = 0.007^{6}$

The table shows the significantly activated KEGG pathways in each group, indicating *P* values with 3 digits after the point, and the index number giving the rating of the significance level. For example, $P = 0.000^{1}$ in *t*1–*t*0 column means it was the most significantly regulated pathway in this group and $P = 0.007^{5}$ means it was the fifth significantly regulated pathway in this column. *t*1–*t*0 represents comparing 30 min after exercise with before exercise, *t*2–*t*0 represents comparing 3 h after exercise with before exercise, and *t*3–*t*0 represents comparing 24 h after exercise with before exercise.

4.1. Exercise and the inflammatory response

Our study supported, in general, the original idea (Weinstock et al., 1997) that an acute bout of exercise could stimulate both, pro- and anti-inflammatory responses. In our study the most prominent finding was the up-regulation of a row of genes with predominant anti-inflammatory function following exercise. These included ARG1, SOCS3, SAP30, ORM1///ORM2, DUSP-1, GJB6, and IRAK3 which were all highly up-regulated in un-stimulated cultures of both sexes. Of these, ARG1, ORM1///ORM2, GJB6, and IRAK3 showed identical behavior in that they were unaffected by LPS. Thus, LPS could neither induce these genes nor modify their exercise-induced expression during the short-term stimulation employed in our study. ARG1 is expressed by activated macrophages and exerts its potent anti-inflammatory activation by hydrolyzing L-arginine and inhibiting iNOS, NF-κB and TNF-α production (see review Munder, 2009). Orosomucoids (ORMs), also called α-1 acid glycoproteins, are produced by hepatocytes during the acute phase reaction, inhibit superoxide generation and MAPK signaling (Hochepied et al., 2003) and protect mice from TNF-α-induced lethality (Libert et al., 1994). Interleukin-1 receptor associated kinase-3 (IRAK3; also referred to as IRAKM) is a kinase-deficient member of the TLR/IRAK family, negatively regulates IRAK1 and IRAK4 signaling and is a key inhibitor of inflammation in obesity and metabolic syndrome (Hulsmans et al., 2012). Finally, GJB6 (also called Connexin 30), a gap junction protein, is involved in regulation of ion homeostasis, apoptosis, and negative regulation of neuroinflammation, and inhibits inflammation through limiting intracellular diffusion of signaling molecules (Chanson et al., 2001; Nakase et al., 2004). Our study provides the first evidence showing that the induction of the GJB6 gene is up-regulated (up to 16-fold) in response to exercise.

The fact that ARG1, ORM1///ORM2, GJB6, and IRAK3 which are all very strongly induced by exercise, represent different modes of anti-inflammatory signaling and are not reversed by LPS is indicative of the broad immunosuppressive potential of exercise at least during the early stages following pathogen contact. In contrast, LPS could partly suppress exercise-induced expression of other genes like SAP30 (Fig. 2). As part of the histone deacetylase complex, SAP30 (Sin3A-associated protein, 30 kDa) mediates transcriptional repression and inhibits IFN-β1 (see below) expression in infected cells (Le May et al., 2008). The underlying mechanisms and the biological significance of this type of interaction of endotoxin with the exercise effect remain to be elucidated. It should, however, be noted that LPS-induced IFN-β1 gene expression was massively suppressed by exercise (see below).

A third type of relation between exercise effects and endotoxin effects became apparent with SOCS3 (Suppressor of cytokine signaling) and DUSP1 (dual specificity phosphatase 1) (Fig. 2). Both were strongly stimulated by endotoxin at rest, and exercise could not modify this reaction. Exercise could, however, induce their expression (~11 and 6.5-fold, respectively) up to the ceiling marked by LPS. SOCS3 and DUSP1 are both expressed by macrophages and are negative regulators of LPS/TLR signaling albeit at different points of the signaling cascade. Both require STAT3 for induction, and while induction of SOCS3 requires p38MAPK pathway in addition, DUSP1 can in turn inhibit the p38MAPK pathway (Bode et al., 1999). The fact that SOCS3 and DUSP1 genes were both strongly activated by 1 h exposure to LPS, independent of exercise, points to their role in the early fine tuning of LPS-induced TLR signaling. SOCS3 and DUSP1 can also be induced by IL-10 (Bode et al., 1999) although it is unlikely that LPS-induced SOCS3 and DUSP1 expression was IL-10 dependent. LPS could neither induce IL-10 protein, nor mRNA within 1 h (Abbasi et al., 2013). Exercise,

Table 5
Exercise activated KEGG Pathways in only un-stimulated or in only LPS-stimulated cultures in male and female athletes.

KEGG pathways	Only CON (−LPS)						Only LPS					
	Men			*Women*			*Men*			*Women*		
	*t*1–*t*0	*t*2–*t*0	*t*3–*t*0	*t*1–*t*0	*t*2–*t*0	*t*3–*t*0	*t*1–*t*0	*t*2–*t*0	*t*3–*t*0	*t*1–*t*0	*t*2–*t*0	*t*3–*t*0
Ribosome	$P = 0.002^{1}$	$P = 0.000^{1}$										
Pyrimidine metabolism		$P = 0.002^{2}$										
Purine metabolism		$P = 0.003^{3}$										
Rheumatoid arthritis		$P = 0.005^{4}$										$P = 0.001^{1}$
Fatty acid metabolism		$P = 0.007^{5}$										
Leishmaniasis				$P = 0.008^{1}$	$P = 0.003^{6}$							
Chagas disease					$P = 0.000^{1}$		$P = 0.006^{5}$	$P = 0.003^{4}$		$P = 0.0061^{7}$		$P = 0.002^{5}$
Toll-like receptor signaling pathway					$P = 0.000^{2}$			$P = 0.002^{2}$				$P = 0.002^{2}$
Chronic myeloid leukemia					$P = 0.001^{3}$					$P = 0.0092^{0}$		
Pathways in cancer					$P = 0.002^{4}$		$P = 0.001^{2}$			$P = 0.0081^{9}$		
Bladder cancer					$P = 0.002^{5}$	$P = 0.008^{1}$				$P = 0.001^{2}$		
VEGF signaling pathway					$P = 0.004^{7}$							
Melanoma					$P = 0.004^{8}$					$P = 0.002^{7}$		
Malaria					$P = 0.005^{9}$	$P = 0.010^{2}$						$P = 0.002^{4}$
Long-term potentiation					$P = 0.005^{10}$					$P = 0.0092^{1}$		
Prostate cancer					$P = 0.006^{11}$					$P = 0.001^{5}$		
Dorso-ventral axis formation					$P = 0.006^{12}$							
Glioma					$P = 0.009^{13}$					$P = 0.001^{4}$		
Amoebiasis					$P = 0.010^{14}$		$P = 0.001^{1}$	$P = 0.002^{3}$				
African trypanosomiasis							$P = 0.002^{3}$					
Lysosome							$P = 0.006^{4}$					
Thyroid cancer							$P = 0.008^{6}$			$P = 0.0041^{4}$		
Acute myeloid leukemia							$P = 0.010^{7}$			$P = 0.0041^{3}$		
Chemokine signaling pathway								$P = 0.002^{1}$				
Neurotrophin signaling pathway								$P = 0.007^{5}$				
Endocytosis										$P = 0.001^{1}$		
Cytokine–cytokine receptor interaction										$P = 0.001^{3}$		$P = 0.004^{6}$
Renal cell carcinoma										$P = 0.002^{6}$		
Non-small cell lung cancer										$P = 0.002^{8}$		
Pantothenate and CoA biosynthesis										$P = 0.002^{9}$		
MAPK signaling pathway										$P = 0.0031^{0}$		
Toxoplasmosis										$P = 0.0031^{1}$		
mTOR signaling pathway										$P = 0.0031^{2}$		
Endometrial cancer										$P = 0.0051^{5}$		
Focal adhesion										$P = 0.0051^{6}$		
Jak-STAT signaling pathway										$P = 0.0081^{8}$		
Cytosolic DNA-sensing pathway												$P = 0.002^{3}$
Wnt signaling pathway												$P = 0.006^{7}$

The table shows the significantly activated KEGG pathways in each group, indicating *p* values with 3 digits after the point, and the index number giving the rating of the significance level. For example, $P = 0.000^{1}$ in *t*2–*t*0 column means it was the most significantly regulated pathway in this group and $P = 0.007^{5}$ means it was the fifth significantly regulated pathway in this column. *t*1–*t*0 represents comparing 30 min after exercise with before exercise, *t*2–*t*0 represents comparing 3 h after exercise with before exercise, and *t*3–*t*0 represents comparing 24 h after exercise with before exercise.

however, can do it. Therefore, it is possible that the exercise-induced SOCS3/DUSP1 activation was due to IL-10. The fact that exercise could not increase or otherwise modify endotoxin-induced SOCS3 or DUSP-1 mRNAs does not necessarily mean that it cannot have functional consequences. Other stimulants, with kinetics of less than 1 h or concentrations below 10 ng LPS may result in different outcomes. Here it should be mentioned that induction of DUSP1 was significantly prolonged in women as compared to men, contrasting with TNIP3, which is discussed in the following paragraph.

TNIP3 (TNFAIP3 interacting protein 3) represents a fourth, highly interesting pattern of exercise/endotoxin interaction. Exercise up-regulated (up to 16-fold) TNIP3 in LPS-stimulated cultures only, while LPS itself (in resting pre-exercise blood samples) did not significantly increase the expression of this gene under the short-term incubation conditions used in the present study. Giving credit to the sequence of events we can also say that LPS only stimulated this gene in "exercised" cells. Up-regulation of TNIP3 was observed in both sexes but was only significant in male athletes. TNIP3 (also known as ABIN3) negatively regulates NF-κB activation in monocytes in response to TNF-α and LPS, but also to IL-10 (Verstrepen et al., 2008; Weaver et al., 2007). To our knowledge this is the first report to show this type of interaction, suggesting that in our situation some exercise dependent priming factor plus engagement of TLRs was necessary for induction of this gene. The priming factor could be one of the classical exercise-induced factors like IL-6, but modification of TLRs by exercise-induced miRNAs (Tonevitsky et al., 2013) may also be considered as a possible mechanism.

A few genes with mixed (PTGES), unclear (SLED1) or presumed pro-inflammatory function were also strongly up-regulated by exercise, either in stimulated and un-stimulated cultures (SLED1), in un-stimulated only (BMX, CD177) or in LPS-stimulated cultures only (END1, PTGES). SLED1 probably plays a role in Systemic Lupus Erythematosus (Ishii et al., 2005). BMX is a cytosolic tyrosine kinase which is required for activation of p38MAPK and NF-κB signaling, and is essential for IL-8 induction (Gottar-Guillier et al., 2011). CD177 is a neutrophil-specific gene involved in cellular transmigration (Sachs et al., 2007). Thus, both, BMX and CD177 are involved in boosting granulocyte function. There are no other reports yet regarding their up-regulation through exercise.

Among the genes most significantly down-regulated by exercise, IFN-β1 seems to be the most interesting one. While exercise alone did not modify IFN-β1 gene expression, LPS-induced-expression of the IFN-β1 gene was strongly suppressed (12.1-fold down-regulation) by previous exercise. This pattern of behavior

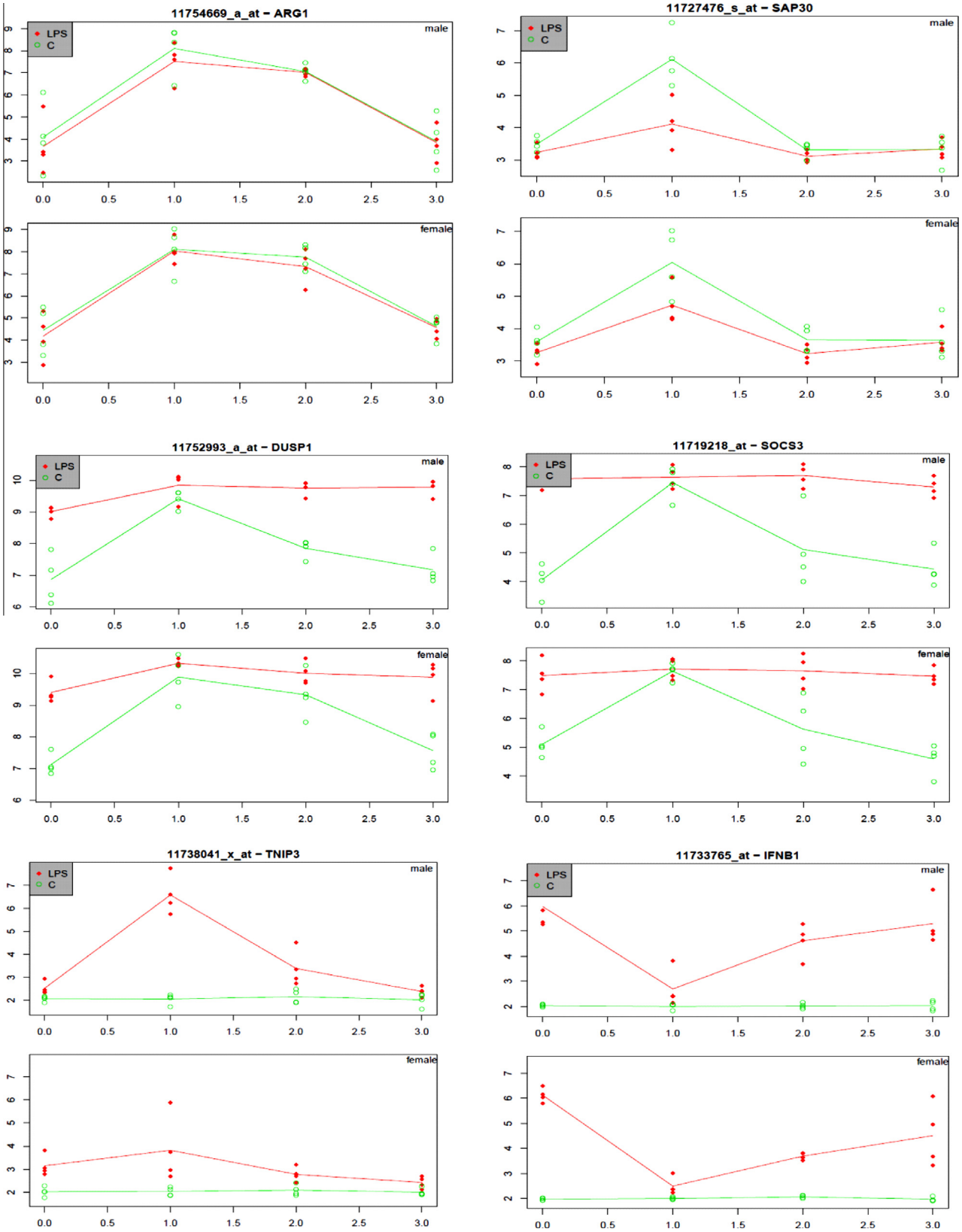

Fig. 2. Significant regulation of some genes in LPS-stimulated and un-stimulated cultures in response to exhaustive exercise. *X* axis shows the time points with 0,0 corresponing to *t*1 (before Ex); 1,0 corresponding to *t*1 (30 min post-Ex); 2,0 corresponding to *t*2 (3 h post-Ex); 3,0 corresponding to *t*3 (24 h post-Ex). *Y* axis shows normalized signal intensities (log2). ARG1: Arginase-1; SAP30: Sin3A-associated protein, 30 kDa, SOCS3: suppressor of cytokine signaling 3; DUSP1: dual specificity phosphatase 1; TNIP3: TNFAIP3 interacting protein 3; interferon, beta 1.

is exactly the inverse of the pattern seen with TNIP3, and since TNIP3 regulates different steps of TLR signaling and since IFN-β1 is one of the TLR activation end products it can be hypothesized that TNIP3, at least in men, may be involved in regulating IFN-β1. In addition to its antiviral properties, IFN-β1 is extremely pro-inflammatory and responsible for part of the LPS toxicity, similar to TNF-α (Karaghiosoff et al., 2003).

This is the first study providing evidence that exhaustive exercise significantly down-regulates LPS-induced IFN-β1 gene expression. Since down-regulation came close to a complete shutdown, it cannot be attributed to changes in monocyte percentage. Shifts of monocyte subgroups may play a role (Booth et al., 2010) but are also unlikely to account for the full extent of the observed regulation.

A similar pattern to IFN-β1 was also seen for IL-12B (also known as IL-12 p40 subunit) and IFN-γ (up to −5-fold). In these cases cellular shifts may play a role. It has, however, been shown earlier that following a marathon run, IFN-γ production in response to 24 h stimulation with LPS was almost completely abrogated (Weinstock et al., 1997). We think that the transient but close to complete shutdown of these two interferons in peripheral blood is a good candidate for explaining the "open window for infection" phenomenon and possibly also the beneficial anti-inflammatory effects of exercise.

Other strongly down-regulated genes were SPON2 (mindin), a pattern recognition molecule essential in initiating the innate immune response to certain microbes (He et al., 2004), and a row of granzymes (A, M, B, K, H) which belong to the killing machinery of lymphocytes and NK cells but also induce pro-inflammatory cytokines in different target cells. All were down-regulated in un-stimulated and LPS-stimulated cultures up to an extent (9- to 12-fold) which cannot be explained by cellular shifts alone. Other studies have reported mild up-regulation or down-regulation following moderate exercise (Radom-Aizik et al., 2008; Connolly et al., 2004).

In addition, two chemokines, CXCL10 and CX3CR1, were also significantly down-regulated, a finding that has not been reported by others yet. Both chemokines have potent pro-inflammatory functions in addition to their chemotactic properties, and have been implicated in causing and perpetuating inflammation in autoimmune diseases (Lee et al., 2013; Mionnet et al. 2010). In contrast, IL-8 mRNA was mildly increased by exercise which is in line with previous findings (Nieman et al., 2006).

In summary, among the top 10 significantly regulated genes, exercise evoked a strong anti-inflammatory impact either through up-regulation of anti-inflammatory ones (TNIP3, ARG1, SOCS3, DUSP1, IRAK3) or through down-regulation of pro-inflammatory ones (IFN-β1, SPON2, IL-12B, KLRF1, TRA@///TRD@, FCRL6). In our interpretation this constitutes a coordinated broad protective reaction of the body against possible exercise-induced inflammatory damage. Much of the reaction happens in the absence of exogenous pathogen. However, other changes, notably altered expression of TNIP3, IFN-β1 and IFN-γ clearly interfere with early steps of pathogen signaling, further increasing the likelihood that these effects will interfere with the defense against invading microorganisms.

Many factors and hormones which are induced by exercise may be involved in organizing this broad anti-inflammatory gene reaction. To these belong catecholamines, cortisol, growth hormones, heat shock proteins and muscle-derived IL-6 (see review Gleeson et al., 2011). Especially cortisol is known for its vast array of immunosuppressive/anti-inflammatory functions and is highly likely to play an important role in this context (Cupps and Fauci, 1982;

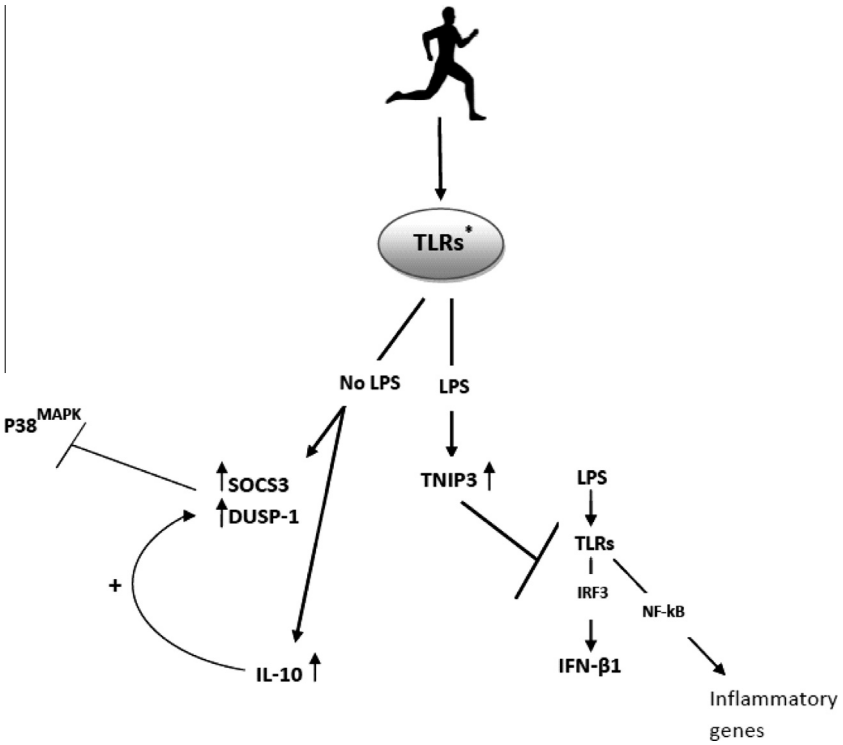

Fig. 3. The schematic presentation of the effect of exercise on TLR signaling: a hypothesis. Exercise modifies the TLRs, possibly through attachment of some regulatory molecules. If no further signal ensues, this will lead to induction of the anti-inflammatory molecules SOCS3, DUSP1, IL-10 and others. If a pathogen signal – here LPS – ensues, TNIP3 is rapidly induced which mitigates/delays the normal signaling cascade which would ensue when the unmodified TLR is hit by LPS and which would lead to rapid and massive activation of pro-inflammatory molecules including IFNβ1, only later followed by regulatory coactivation of TNIP3 and IL-10. *Candidate molecules to modify TLRs through exercise are miRNAs. miRNAs are known to be used by cancer cells to create an anti-inflammatory environment by docking to the TLRs of surrounding leukocytes. miRNAs also rapidly appear in leukocytes and plasma following exercise (Tonevitsky et al., 2013; Baggish et al., 2011).

MacKenzie et al., 2006). It seems, however, safe to say that IL-6 probably is the key player in orchestrating this broad anti-inflammatory reaction. IL-6 is released from working muscle and causes release of IL-10 and IL-1ra, ACTH/cortisol, and acute phase reactants of hepatocytes (e.g., α1 acid glycoprotein) (Steensberg et al., 2003; Northoff et al., 1995). It is noteworthy to mention that IL-10 mRNA, other than SOCS3 or DUSP1 is not increased during the early fine tuning of LPS/TLR signaling (Abbasi et al., 2013). Its induction through exercise/IL-6 may therefore represent more of a direct, preemptive anti-inflammatory event rather than a balancing counter regulation against some primary inflammatory stimulus.

Although IL-6 and its followers can eventually explain most of the anti-inflammatory reaction reported, we think it is possible that additional mechanisms may also be at work. Knowing that exercise results in fast induction of miRNAs (Tonevitsky et al., 2013) and that miRNAs can interfere with TLRs (see review Fabbri, 2012), it is tempting to speculate that such a fast mechanism might also be instrumental in organizing the anti-inflammatory response to exercise. This hypothesis, assuming that exercise-induced miRNAs may modify TLRs, could unify the diverging patterns we see with induction of different regulators of TLR signaling through exercise (TNIP3, IFN-β1 vs. SOCS3 and DUSP1) (Fig. 3).

4.2. Exercise and brain-related genes

We also found significant changes in some of the brain-related genes, such as OLIG2, TMEM106b, and NR4A2. OLIG2 was among the most significant down-regulated genes (−3.02-fold) following exercise, but this was significant in LPS-stimulated cultures of male athletes only. OLIG2 is a marker for certain oligodendrocyte precursors and is also up-regulated in proliferating glial cells (Takebayashi et al., 2000). Until now it was believed that the expression of the OLIG2 gene is restricted to neural tissues. The significance of the striking differences between males and females remains to be elucidated. The same holds true for TMEM106B which was significantly down-regulated in LPS-stimulated cultures of females but not males. TMEM106B has been associated with frontotemporal lobar degeneration (Chen-Plotkin et al., 2012), and again we are not aware of any report of its expression in blood. Our findings are underlined by the fact that the neurotrophin signaling pathway was significantly activated by exercise in LPS-stimulated cultures (Table 5). Whether there is any relation of these changes to the known effects of exercise on neuroplasticity needs to be investigated in the future.

4.3. Exercise and DNA methylation

Among the most significantly regulated genes in the present study, we observed a significant down-regulation in DNA methyltransferase-1 (DNMT1) gene expression in LPS-stimulated and un-stimulated blood cultures of male and female athletes following exercise (~4-fold down-regulation). While classically DNMT1 is considered a maintenance methyltransferase, its role in inflammation and inflammation-associated carcinogenesis has also been established recently (Foran et al., 2010). DNMT1 down-regulates SOCS3 expression, and vice versa knockdown of DNMT1 increases SOCS3 expression (Dhar et al., 2013). Therefore, a relation between down-regulation of DNMT1 and up-regulation of SOCS3 as observed in the present study seems possible. The mechanism underlying the down-regulation of DNMT1 gene in response to exercise is unclear. However, it has been demonstrated that DNMT1 is a target mRNA of the mi-RNA-223 (Milagro et al., 2013) and it has also been reported that exercise induces miRNA-223 in blood leukocytes (Radom-Aizik et al., 2010). Thus, DNMT1 down-regulation may be an integral factor in the anti-inflammatory effect of exercise. To our knowledge, this is the first study reporting a change in DNMT1 gene expression in peripheral blood cells in response to exercise.

A limitation of our study is that we did not include a control group of non-exercising athletes. It would have exceeded our financial and practical capabilities. We do however think that the pre-run control is an excellent basis for comparing the effect of exercise.

In summary, this is the first study to show exercise-induced changes in gene expression profiling in short time (1 h) LPS-stimulated and un-stimulated cultures of athletes. Our data indicate that exhaustive exercise induced substantial changes in the early gene expression response to endotoxin in addition to modifying gene expression in un-stimulated cells. Several of the genes regulated in LPS-stimulated cultures (e.g., TNIP3 and IFN-β1) have not been reported before. These results demonstrate that some potentially important effects of exercise can only be detected in relation to pathogen stimulation. In general, there was an anti-inflammatory bias of gene regulation by exercise, including induction of genes involved in the negative regulation of TLR signaling, and knockdown of IFN-β1, one of the end products of the TLR signaling pathway. A hypothesis which could unify the diverse findings concerning regulation of TLR signaling by exercise is developed (Fig. 3). Moreover, exercise also modified several biological pathways associated with innate immune/inflammatory responses, metabolic responses, the cell cycle, DNA methylation and brain function.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2013.10.023.

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RESEARCH ARTICLE Open Access

Dynamically regulated miRNA-mRNA networks revealed by exercise

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Abstract

Background: MiRNAs are essential mediators of many biological processes. The aim of this study was to investigate the dynamics of miRNA-mRNA regulatory networks during exercise and the subsequent recovery period.

Results: Here we monitored the transcriptome changes using microarray analysis of the whole blood of eight highly trained athletes before and after 30 min of moderate exercise followed by 30 min and 60 min of recovery period. We combined expression profiling and bioinformatics and analysed metabolic pathways enriched with differentially expressed mRNAs and mRNAs which are known to be validated targets of differentially expressed miRNAs. Finally we revealed four dynamically regulated networks comprising differentially expressed miRNAs and their known target mRNAs with anti-correlated expression profiles over time. The data suggest that hsa-miR-21-5p regulated TGFBR3, PDGFD and PPM1L mRNAs. Hsa-miR-24-2-5p was likely to be responsible for *MYC* and *KCNJ2* genes and hsa-miR-27a-5p for *ST3GAL6*. The targets of hsa-miR-181a-5p included ROPN1L and SLC37A3. All these mRNAs are involved in processes highly relevant to exercise response, including immune function, apoptosis, membrane traffic of proteins and transcription regulation.

Conclusions: We have identified metabolic pathways involved in response to exercise and revealed four miRNA-mRNA networks dynamically regulated following exercise. This work is the first study to monitor miRNAs and mRNAs in parallel into the recovery period. The results provide a novel insight into the regulatory role of miRNAs in stress adaptation.

Keywords: Exercise, Regulation, miRNA-mRNA networks

Background

MiRNAs are one family of small (20–22 nucleotides) non-coding RNAs. They regulate gene expression post-transcriptionally through binding to the complementary sites of target mRNAs in the 3′-UTR, and play an important role in regulating diverse biological processes [1].

Recently, miRNA have been demonstrated as regulators of processes involved in physiological stress adaptation, including inflammation [2], angiogenesis [3], mitochondrial metabolism [4], muscle force generation [5]. However, just a few studies were published to date describing the changes in miRNA expression during exercise of different intensity [6-12]. They did not include the analysis of post-exercise recovery period and thus provided no information concerning dynamics of the predicted miRNA-mRNA regulatory pairs. Detailed investigation of miRNA-mRNA networks specifically regulated by exercise could reveal important biomarkers of exercise physiology and would provide for deep insight into the molecular control of the stress response.

MiRNAs regulate target gene expression in different ways including mRNA degradation and translation inhibition [1]. The target genes which were regulated by miRNAs through mRNA degradation are anti-correlated with the miRNA regulators. In this study, for the first time whole transcriptome changes were monitored during exercise followed by 30 min and 60 min of recovery period and differentially expressed mRNAs and miRNAs were analysed resulting in identification of four dynamically regulated miRNA-mRNA networks.

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Results and discussion

Anthropometric and physiological data

To exclude possible effects of gender, only male subjects were recruited for this study. Anthropometric and physiological parameters of athletes are presented in Table 1. Before exercise the serum lactate level was 1.7 ± 0.4 mM. After exercise, it was mildly elevated, but still below 4.0 mM, confirming that the exercise performed was moderate without transgression of the anaerobic threshold.

Branched-chain amino acids (BCAA) include three structurally related amino acids Leucine (Leu), Isoleucine (Ile), and Valine (Val). The initial steps of their degradation are catalyzed by the same set of mitochondrial enzymes, and therefore, the BCAA behave as a very homogenous group. Their regulation is performed by short-term metabolic control reflecting consuming of energy sources. It has been shown previously that an acute bout of prolonged exercise increases the rate of BCAA oxidation by skeletal muscle [13]. We observed a slight increase in the BCAA level immediately after exercise followed by a decline below base level during recovery (Table 2). The ratio of citrulline (Cit) to ornithine (Orn) is indicative of the ornithine carbamoylphosphate transferase activity and characterizes the regulation of the urea cycle pathway [14]. This ratio had a tendency to increase (Table 2). The ratios of methionine sulfoxide (Met-SO) to methionine (Met) and tyrosine (Tyr) to phenylalanine (Phe) indicate oxidative stress [15,16]. We found a decrease in Met-SO/Met and a slight increase in Tyr/Phe (Table 2).

The data summarized in Table 2 confirmed that the exercise was moderate and athletes reacted as normal healthy subjects [17].

Flow cytometry analysis

Changes in white blood cell subpopulations in response to exercise are presented in Table 3. Total white blood cell counts revealed the expected exercise-induced leukocytosis. NK lymphocytes (defined as CD3–, CD16/56+) substantially contributed to the observed changes which was consistent with the published data [18]. However NK-specific mRNAs (e.g., coding for KIR receptors) in the whole blood did not demonstrate similar nearly 3-fold increase (see Additional file 1) thus confirming that our subsequent microarray analysis showed true changes in RNA expression.

Table 1 Anthropometric and physiological data

Parameter	Value
Age (year)	21.7 ± 2.6
Body mass (kg)	74.9 ± 2.3
Height (cm)	185.3 ± 3.5
Body mass index (kg/m^2)	21.8 ± 1.2
Heart rate before exercise(bpm)	57.3 ± 1.9
Heart rate after exercise (bpm)	179.4 ± 3.2
Blood pressure before exercise (mmHg)	120/66 ± 3/2
VO_{2max} (ml min^{-1} kg^{-1})	74.8 ± 3.3

Values are mean ± SD.

Table 2 Aminoacids before and in response to exercise

	BCAA, μM	Cit/Orn	Met-SO/Met	Tyr/Phe
Pre	567.63 ± 172.16	0.35 ± 0.09	2.28 ± 1.73	0.83 ± 0.15
Post (E)	612.83 ± 99.68	0.34 ± 0.11	0.92 ± 0.21	0.91 ± 0.18
Rest 30 min (R1)	496.89 ± 128.31	0.42 ± 0.09	1.71 ± 0.92	0.91 ± 0.19
Rest 60 min (R2)	417.75 ± 58.2	0.5 ± 0.15	1.21 ± 0.47	0.9 ± 0.16

Abbreviations: *BCAA* Branched-Chain Amino Acids, *Cit* Citrulline, *Orn* ornithine, *Met-SO* Methionine Sulfoxide, *Met* Methionine, *Tyr* Tyrosine, *Phe* Phenylalanine.

miRNA and mRNA differential expression profiles

The miRNA and mRNA expression profiles in the whole blood for each time point and each athlete were determined using microarray analysis. PAXgene RNA tubes enable the isolation of intracellular RNA of circulating leukocytes, including B cells, T cells, neutrophils, monocytes, and other less abundant cell types. Furthermore, a large proportion of reticulocyte-derived globin mRNA is prepared from PAXgene blood RNA tubes, as it has been demonstrated previously [19]. Affymetrix GeneChip Human Gene 1.0 ST arrays contain both miRNA and mRNA probes and are capable of measuring about 20,000 mRNAs and 200 miRNAs.

The data are presented in Additional file 1. 298 mRNAs and 5 miRNAs were changed significantly (at least 40% change with the adjusted P-value threshold of 0.05), including hsa-miR-21-5p, hsa-miR-24-2-5p, hsa-miR-27a-5p, hsa-miR-181a-5p and hsa-miR-181b-5p. Remarkably, hsa-miR-24-2-5p is clustered with hsa-miR-27a-5p, and hsa-miR-181a-5p is clustered with hsa-miR-181b-5p. Consistently, the clustered miRNAs exhibited similar expression profiles over time (see Additional file 1 and Figures 1, 2, 3 and 4).

Pathway analysis of differentially expressed mRNAs

All 298 differentially expressed mRNAs were analysed for enriched metabolic pathways. Table 4 indicates the revealed pathways including immune response and glycoproteins. As expected, they were previously reported to be associated with exercise [6,8] thus confirming the relevance of our experimental model.

Pathway analysis of mRNA targets of differentially expressed miRNAs

All 5 differentially expressed miRNAs have 1136 validated target mRNAs in total. We performed pathway enrichment analysis for these mRNAs (Table 5). Again, the revealed pathways are highly relevant to exercise, e.g. transcription regulation, apoptosis, response to stress etc.

It has been demonstrated that the same mRNAs can be targeted by more than one miRNA which provides

Table 3 Changes in white blood cell subpopulations in response to an exercise

	Pre		Post (E)		Fold change relative
	Absolute, cells/ul	Relative, %	Absolute, cells/ul	Relative, %	
WBC	5710 ± 1170		7705 ± 2000		
LY	2387 ± 674	41.8 ± 11.8	3989 ± 932	69.9 ± 12.1	1.67
CD3+	1616 ± 549	28.3 ± 9.6	2349 ± 901	41.1 ± 11.7	1.45
CD3 + CD4+	1020 ± 413	17.9 ± 7.2	1189 ± 419	20.8 ± 5.4	1.17
CD3 + CD8+	596 ± 265	10.4 ± 4.6	1124 ± 532	19.7 ± 6.9	1.89
CD3-CD16+	494 ± 203	8.7 ± 3.6	1380 ± 523	24.2 ± 6.8	2.79
CD3-CD56+	393 ± 217	6.9 ± 3.8	1111 ± 468	19.5 ± 6.1	2.83
CD19+	227 ± 124	4.0 ± 2.2	308 ± 221	5.4 ± 2.9	1.36

Values are mean ± SD.

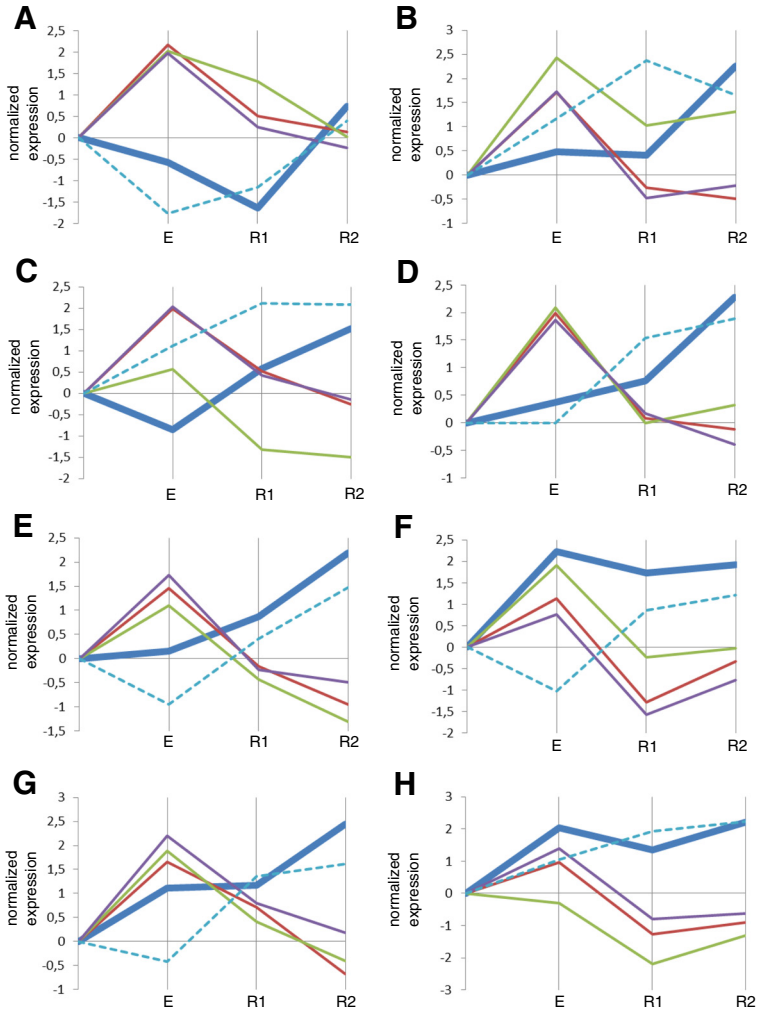

Figure 1 Expression profiles of hsa-miR-21-5p and its target mRNAs. (**A**-**H**) the tested athletes. **E**, after 30 min of exercise; R1, after 30 min relaxation; R2, after 60 min relaxation. Bold blue, hsa-miR-21-5p; brown, TGFBR3; green, PDGFD; violet, PPM1L; light blue, RHOBTB3. Solid lines indicate validated mRNA targets and dashed line indicates predicted potential mRNA target.

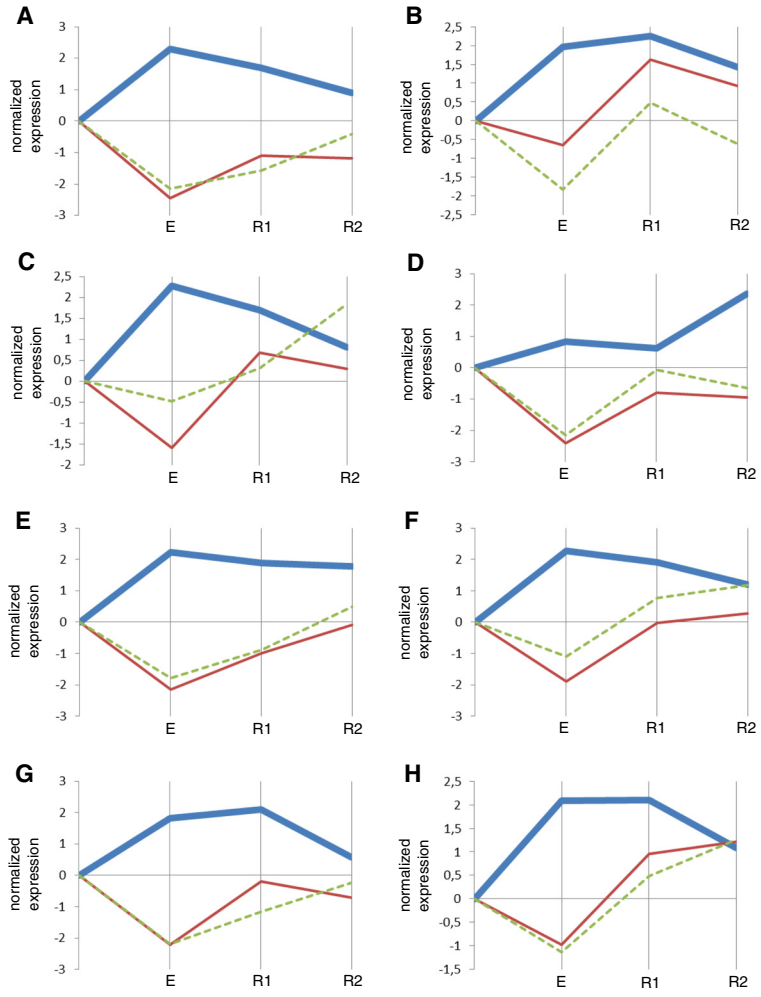

Figure 2 Expression profiles of hsa-miR-24-2-5p and its target mRNAs. (**A**-**H**) the tested athletes. **E**, after 30 min of exercise; R1, after 30 min relaxation; R2, after 60 min relaxation. Bold blue, hsa-miR-24-2-5p; brown, MYC; green, KCNJ2. Solid line indicates validated mRNA target and dashed line indicates predicted potential mRNA target.

for more efficient and specific regulation [20,21]. We found 49 mRNAs which are known to be validated targets for 2 or even 3 differentially expressed miRNAs. They have higher potential to be involved in exercise-induced regulation. Table 6 shows the pathways enriched with some of these mRNAs. Notably, these exercise-relevant pathways (cell death, stress response, proliferation) comprise significant number of intersecting mRNAs. Figure 5 presents the identified regulatory miRNA-mRNA network for all 3 revealed pathways. Interestingly, some of these genes are known to interact with each other. Namely transcription factor MYC was reported to be functionally associated with RNA helicase DDX3X [22], apoptosis regulator BCL2 [23] and tumor suppressor BRCA1 [24]. BRCA1 in turn interacts itself with BCL2 [25] and transcription factor E2F1 [26]. The presented data support the regulatory role of identified miRNAs in response to exercise.

Dynamically regulated miRNA-mRNA networks

We monitored the transcriptome expression level before and following exercise and this allowed us to reveal dynamically regulated miRNA-mRNA networks. We used a two-step approach to identify the mRNA targets for the differentially expressed miRNAs. First, we looked for anti-correlating groups of miRNAs and mRNAs expression of which over time tended to have opposite profiles. Target mRNA degradation is one of the mechanisms of miRNA action when their perfect complementarity occurs [1,27]. Thus, the second step of our strategy was either analysis of published data and selection of experimentally validated target mRNAs for a given miRNA or theoretical prediction of miRNA targets based on their complementarity, using one of the most popular web resources TargetScan [28]. The expression profiles and biological function of selected miRNAs and mRNAs were analysed in more detail. Based on this analysis, our final

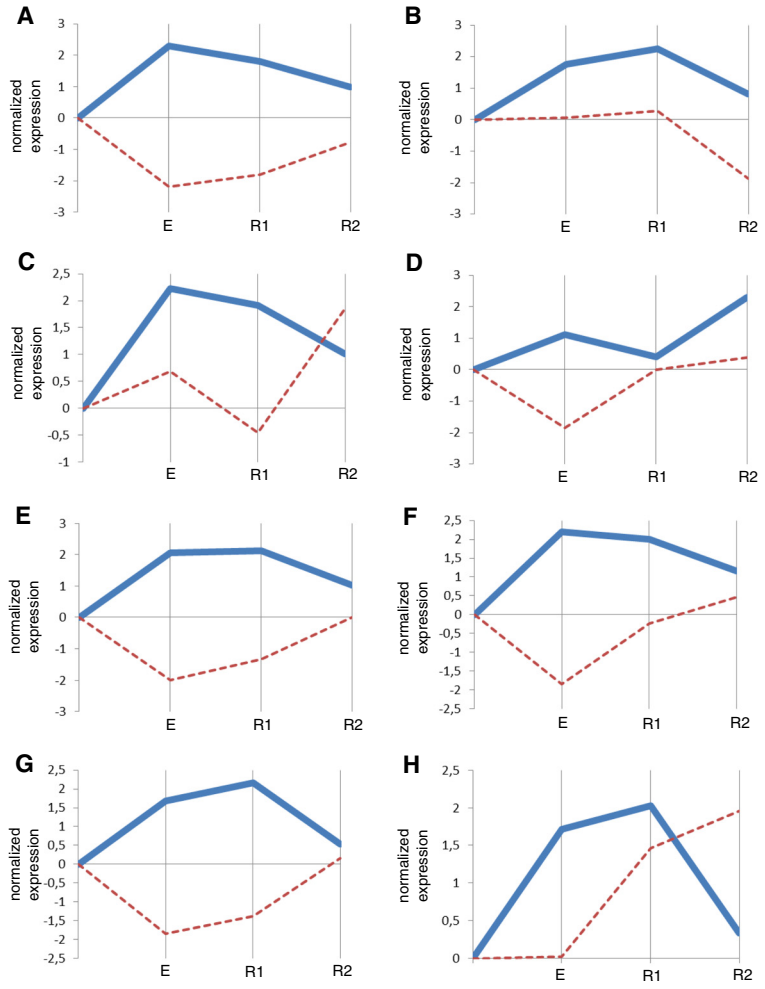

Figure 3 Expression profiles of hsa-miR-27a-5p and its target mRNA. (**A**-**H**) the tested athletes. **E**, after 30 min of exercise; R1, after 30 min relaxation; R2, after 60 min relaxation. Bold blue, hsa-miR-27a-5p; dashed brown, predicted potential target mRNA ST3GAL6.

identified miRNA-mRNA pairs have a high probability of being involved in the regulation of exercise-related physiological processes.

hsa-miR-21-5p

MiRNA hsa-miR-21-5p demonstrated different expression profiles over time (adjusted P-value 0.0039) however remarkably anti-correlating with experimentally validated target mRNAs TGFBR3 (adjusted P-value 1.079E-06), PDGFD (adjusted P-value 1.28E-06) and PPM1L (adjusted P-value 6.78E-07) (Figure 1). RHOBTB3 mRNA (adjusted P-value 0.002) predicted by TargetScan to be a potential target for hsa-miR-21-2-5p behaved similarly. We observed the up-regulation of hsa-miR-21-5p one hour after exercise. The differences in kinetics can be potentially explained by individualities of each athlete.

The expression level of hsa-miR-21-5p itself is known to be stress-responsive and play an important role in heart failure [29] and renal ischemia reperfusion injury [30]. Notably the up-regulation of circulating hsa-miR-21-5p was recently reported to occur in plasma upon exercise [6]. The overall action of hsa-miR-21-5p has been described by several authors to be strongly anti-inflammatory [6,31]. Note, that 60 min into relaxation, there was an up-regulation of hsa-miR-21-5p in all 8 subjects. This may reflect the self-protective anti-inflammatory reaction to exercise.

We identified four pairing targets for this miRNA namely TGFBR3, PDGFD, PPM1L, and RHOBTB3. TGFBR3 is a transforming growth factor (TGF)-beta type III receptor, mRNA of which is known to be up-regulated in the peripheral blood leukocytes in allograft rejection-prone recipients after intestinal transplantation thus mediating innate and adaptive inflammatory functions of leukocytes [32]. PDGFD encodes for platelet derived growth factor D [33], a member of the platelet-derived growth factor family which can regulate many cellular processes, including cell proliferation, apoptosis, transformation, migration,

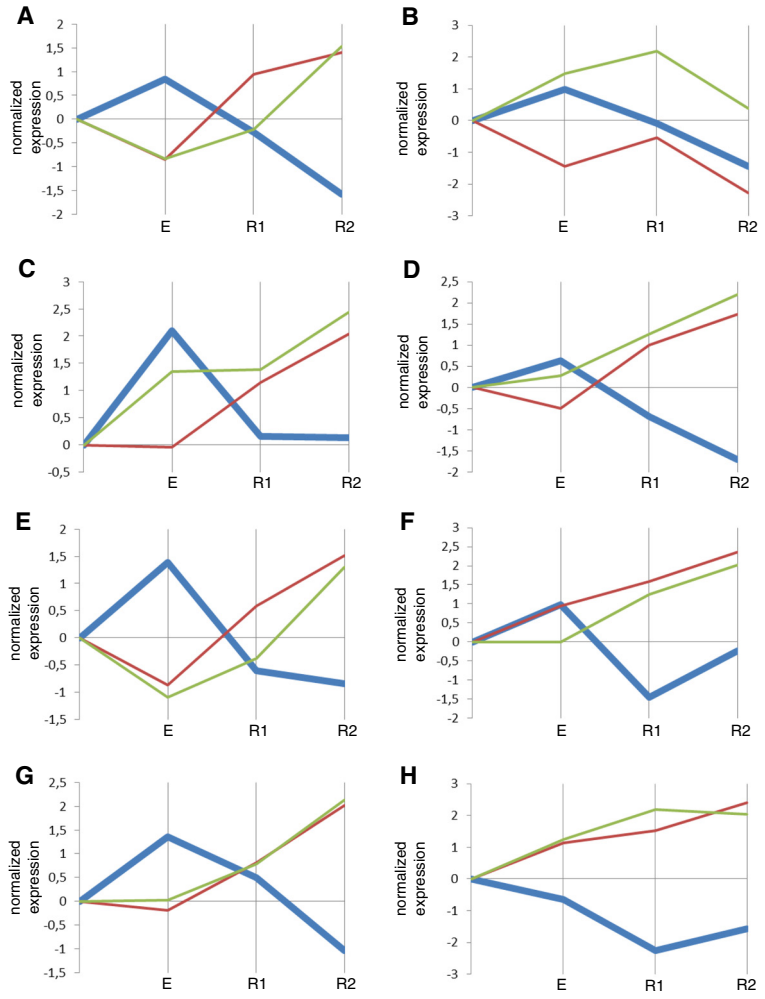

Figure 4 Expression profiles of hsa-miR-181a-5p and its validated target mRNAs. (**A**-**H**) the tested athletes. **E**, after 30 min of exercise; R1, after 30 min relaxation; R2, after 60 min relaxation. Bold blue, hsa-miR-181a-5p; brown, ROPN1L; green, SCL37A3.

invasion, angiogenesis and metastasis [34]. PPM1L encodes a protein phosphatase gene [35] responsible for the regulation of stress-activated protein kinase signaling cascade and apoptosis [36]. Finally, the fourth identified target for hsa-miR-21-5p is RHOBTB3 mRNA [33] which encodes for the Rho GTPase regulating the membrane traffic of proteins [37]. Interestingly, this mRNA proved to be a blood biomarker of psychosis and shows a decreased expression level in high hallucinations states [38].

hsa-miR-24-2-5p

MiRNA hsa-miR-24-2-5p (adjusted P-value 0.00017) was up-regulated immediately after exercise, then tended to decrease during the recovery period except athlete D (Figure 2). In our study for the first time we report the reaction to exercise of this miRNA which is known to have protective effects on myocytes against myocardial ischaemia/reperfusion-induced apoptosis [39].

MYC mRNA (adjusted P-value 0.00013) which is known to be the target for this miRNA from the literature, and KCNJ2 mRNA (adjusted P-value 0.00023) predicted by TargetScan to be a potential target essentially followed anti-profile of hsa-miR-24-2-5p. The protein encoded by the *MYC* gene is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. It functions as a transcription factor that regulates transcription of specific target genes. Interestingly, hsa-miR-24-2-5p is known to be up-regulated during hematopoietic cell terminal differentiation suppressing MYC expression [40]. Thus the increase of this miRNA we observed (Figure 2) may reflect the regulation of hematopoiesis upon exercise. Remarkably, MYC mRNA is among those which are regulated by 2 differentially expressed miRNAs, namely hsa-miR-21-5p and hsa-miR-24-2-5p (Figure 5), however its expression profile is anti-correlated with only the profile of hsa-miR-24-2-5p (Figures 1 and 2).

Table 4 Pathway analysis of differentially expressed mRNAs

Pathway	Number of genes	Adjusted P-value
Glycoprotein	76	3.00E-09
Natural killer cell mediated cytotoxicity	22	1.80E-15
Immune response	13	3.10E-03
Response to wounding	7	8.70E-01
Inflammatory response	7	1.90E-01
Regulation of lymphocyte mediated immunity	5	1.40E-01
Leukocyte activation	5	8.90E-01
Response to mechanical stimulus	3	3.80E-02
Stress response	3	5.90E-02
Cytolysis	3	3.20E-02

KCNJ2 protein is an integral membrane protein and inward-rectifier type potassium channel participating in establishing the action potential waveform and excitability of neuronal and muscle tissues [41]. This mRNA expressed in peripheral blood lymphocytes is a biomarker for Parkinson's disease [42].

hsa-miR-27a-5p

MiRNA hsa-miR-27a-5p is clustered with hsa-miR-24-2-5p and behaved similarly to it increasing after exercise and decreasing during the recovery period except athlete D (Figure 3) with the adjusted P-value 0.00012. This miRNA was reported to promote myoblast proliferation by reducing the expression of myostatin [43].

The only mRNA target identified is predicted by TargetScan ST3GAL6. The encoded protein belongs to the sialyltransferase family and is responsible for the synthesis of selectin ligands [44].

hsa-miR-181a-5p

hsa-miR-181a-5p tended to increase after exercise and then to down-regulate during the first as well as the second period of the relaxation time (Figure 4) with an adjusted P-value of 5.83E-05. The observed differential expression of hsa-miR-181a-5p in our athletes is consistent with previously published results [7,8]. This miRNA is characterized as a regulator of hematopoietic lineage differentiation [45] and a modulator of T cell sensitivity and selection [46]. Radom-Aizik showed up-regulation of this miRNA after 30 min interval exercise. They related it to increased T cell responsiveness and reduced susceptibility to infection due to physical activity. In our study 7 subjects showed up-regulation immediately after exercise (Figure 4).

Table 5 Pathway analysis of all validated mRNA targets of differentially expressed miRNAs

Pathway	Number of genes	Adjusted P-value
Transcription regulation	283	2.03E-13
Apoptotic process	157	2.59E-11
Cell cycle	148	5.95E-12
Regulation of kinase activity	76	1.08E-08
Regulation of cellular response to stress	34	1.63E-04
p53 signaling pathway	19	4.26E-08
E2F transcription factor network	19	1.51E-07
Validated targets of C-MYC transcriptional activation	14	1.08E-03

Table 6 Pathway analysis of mRNAs validated to be targets for 2 or 3 differentially expressed miRNAs

Pathway	Number of genes	Adjusted P-value
Cell death	17	7.64E-07
Regulation of cell proliferation	15	2.10E-07
Cellular response to stress	12	5.35E-05

The mRNAs ROPN1L (adjusted P-value 0.00024) and SLC37A3 (adjusted P-value 0.0019) were previously validated to be targets for hsa-miR-181a-5p and demonstrated pronounced anti-correlation with the miRNA expression profile. The *ROPN1L* gene encodes a member of the ropporin family. The encoded protein is involved in the targeting towards specific physiological substrates of Protein Kinase A, regulating glycogen, sugar, and lipid metabolism [47]. The SLC37A3 protein belongs to transmembrane sugar transporters and is responsible for sugar metabolism [48].

Conclusion

We have identified metabolic pathways enriched with differentially expressed mRNAs and with mRNA targets of differentially expressed miRNAs, including mRNAs known to be regulated by 2 or 3 miRNAs described here. The result supports previously published data. Moreover, we revealed four miRNA-mRNA networks dynamically regulated following exercise. These observations provide a novel insight into the potential regulatory role of miRNAs in the numerous physiological processes involved in stress adaptation.

Methods

Ethical approval and study participants

Eight national level ski athletes took part in this study. None of them suffered from acute or chronic diseases or reported intake of medication. Participants were informed about the nature, purpose, and potential risks of the experiments and signed an informed consent statement approved by the ethics committee of Scientific Research Center Bioclinicum (Moscow, Russia).

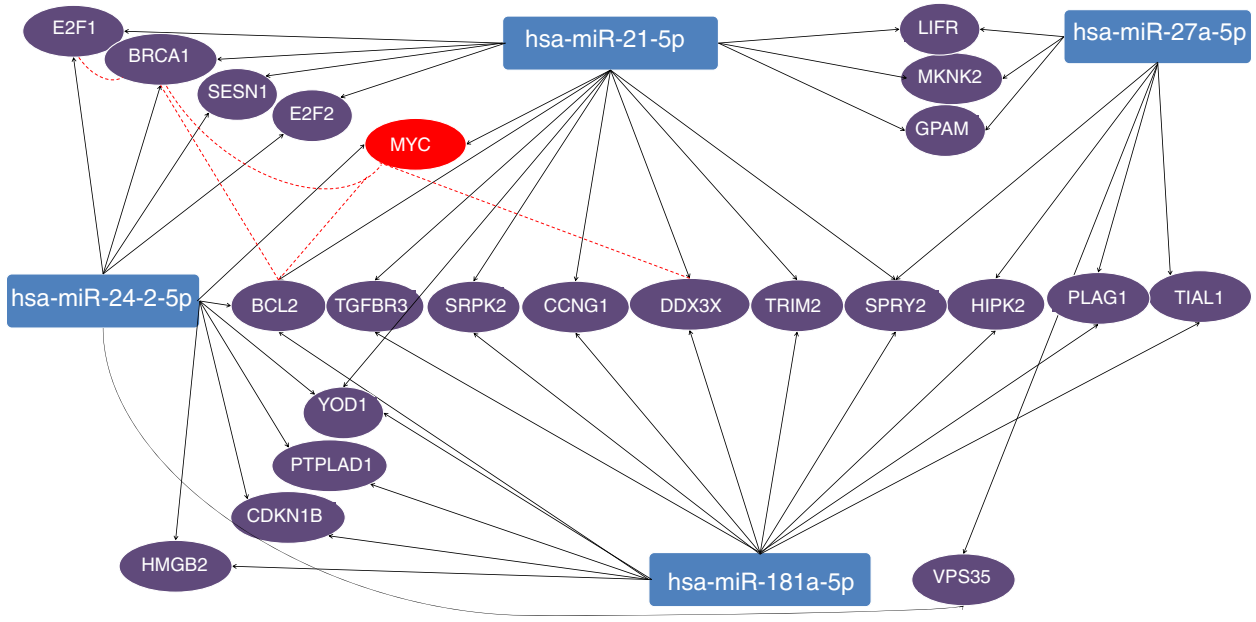

Figure 5 Regulatory network of mRNAs validated to be the targets for 2 or 3 differentially expressed miRNAs. All mRNAs belong to three enriched pathways listed in Table 6. Red dashed lines connect mRNAs of proteins reported to functionally interact with each other. MYC is highlighted with red oval indicating that this mRNA was further identified to be a component of dynamically regulated miRNA-mRNA network.

Anthropometric measurements

Height, weight, medical historical data and resting vital signs were recorded at the time of enrolment.

Exercise test protocol

In order to determine the VO_{2max} values, each subject performed a treadmill test with an incremental step protocol until exhaustion as described previously [47]. VO_{2max} was calculated as described [49]. The anaerobic threshold was calculated using the standard V-slope method [50].

Two weeks later, athletes participated in the main exercise, consisting of running at 80% VO_2 peak for 30 min on a treadmill. The exercise was performed during the morning hours (between 8 and 11 a.m.) keeping the exact test time for each participant constant.

Lactate concentration

Lactate concentration in capillary blood was measured electrochemically using the automated analyzer Biosen C_Line (EFK Diagnostic, Germany).

Analysis of aminoacids

Sera derived from the venous blood samples were analyzed for aminoacids in Genome Analysis Centre (Helmholtz Zentrum, Munich, Germany) using the BIOCRATES AbsoluteIDQ p150 Kit (Biocrates Life Sciences AG, Innsbruck, Austria) in a combined FIA-MS/MS and LC-MS/MS assay as recommended by the manufacturer and described previously [51].

Blood sampling

Venous blood was collected at four time points during the exercise. A 20-gauge intravenous catheter was placed antiseptically into a dorsal hand vein or a vein in the distal forearm as dictated by favourable anatomy using the Seldinger technique and then secured with tape. During ME 2.5 ml of blood was collected in PAXgene blood RNA tube, 7 ml in a Serum separator tube (BD, USA) and 4.5 ml in a tube containing buffered tri-sodium citrate (BD, USA) for flow cytometry analysis at baseline (prior to exercise testing) and immediately post-exercise (within 1 min of completion of exercise testing). After 30 min of rest and after 60 min of rest following exercise testing 2.5 ml of blood was collected in PAXgene blood RNA tubes.

RNA extraction

According to the Affymetrix Manual P/N 701880 Rev. 4 total RNA was extracted using the PAXgene Blood RNA kit as recommended by the manufacturer. RNA concentrations were determined by the Nanodrop photometer (NanoDrop, USA). RNA quality was checked using the Agilent Bioanalyser 2100 System (Agilent Technologies, USA). For all samples RNA integrity number (RIN) was greater than 7.

Flow cytometry analysis

Flow cytometry analysis of blood samples was performed using fluorescently labeled antibodies against B and T cell

receptors and natural killer (NK) cell markers. Cells were labeled with antibodies against CD3 (FITC), CD4 (PE), CD8 (PE), CD16 (FITC), CD56 (PE), CD19 (FITC) (Sorbent, Russia), where NK cells were distinguished from the rest of the lymphocytes via positive expression of CD56 and negative expression of CD3.

The samples were analyzed on a FACScan Calibur flow cytometer (BD Biosciences, USA) and leukocytes were gated based on forward and side scatter properties. Events in the range of 40,000–200,000 were collected depending on the occurrence of the investigated leukocyte population, and analyzed with CELLQuest Pro analysis software (BD Biosciences, USA). To ensure flow cytometric standardization, the voltage settings were updated daily using 'Calibrate Beads' (BD Biosciences, USA).

Microarray analysis

RNA samples were prepared according to manufacturer's instructions (Affymetrix Manual P/N 701880 Rev. 4) as described elsewhere [52]. The samples were hybridized on GeneChip Human Gene 1.0 ST Arrays containing both miRNA and mRNA probes (Affymetrix, USA) for 16 h at 45°C. Arrays were washed to remove non-specifically bound nucleic acids and stained on Fluidics Station 450 (Affymetrix) using FS450_0007 protocol followed by scanning on a GeneChip Scanner 3000 7G system (Affymetrix). The microarray CEL files have been deposited in the GEO database (accession GSE46075).

Microarray data processing

Microarray data was processed using bioconductor [53] xps package implementation of RMA [54]. At the first step background correction was performed based on a global model for the distribution of probe intensities [54]. Then a quantile normalization algorithm [55] (so-called probe-level normalization) was applied to the preprocessed data. Finally, fitting a robust linear model using Tukey's median polish procedure [56] was done to convert probe intensities to the expression levels of probesets.

The statistical analysis of microarray data was performed using bioconductor [53] package limma [57]. The analysis was based on a generalized linear model [57,58] approach. In this approach one constructs a linear data model with a structure determined by the experiment layout, and then fits this model to the actual data. The linear model is defined in terms of a so-called design matrix. The number of rows in this matrix coincides with the number of experiment samples, and the number of columns coincides with the number of factors that have an essential influence on the measurable values. The value at the i-th row and j-th column of a design matrix specifies an effect of a factor j on a sample i. Each measurable value (i.e., each probeset) in this approach is analysed independently. For each probeset a vector of its expression values E is represented in the form $E = D\beta + \varepsilon$, where D is a design matrix, β is a vector of coefficients indicating values of each factor's actual influence on the analyzed probeset, ε is a vector of error, and model fitting consists in the minimization of "error term" ε by finding optimal coefficients β. After the coefficients β are computed for each probeset, one can test various hypotheses on the structure of considered factors. For example, in order to find probesets that are affected by a factor i, one should search for the probesets with βi statistically different from zero.

The general linear model was applied in the analysis of the studied transcriptome changes as follows. The model that takes into account both experiment time points and athletes individual features was used. Thus, the total number of factors was eleven: c1, …, c8 correspond to an expression level of each athlete in a normal state (for all samples the effect of these factors is set to 1 if a sample and a factor correspond to the same athlete, and set to 0 otherwise), and d1, d2, d3 correspond to the changes induces by the exercises, exercises and 30-minutes relaxation, and exercises and 60-minutes relaxation respectively (for all samples the effect of these factors is set to 1 if a sample and a factor correspond to the same experiment time point, and set to 0 otherwise). The total number of samples was 32: 4 samples for each athlete.

For each pair of experiment time points the detection of probesets with reliable difference between time points was performed. The probeset was considered to have a reliable difference between time points k, m if an adjusted p-value of an equality $\beta k = \beta m$ (where βl was an actual influence of exercises at experiment time point for $l = 1,2,3,$ and $\beta 0 = 0$) was less than 0.05, and a log-fold change was greater than 0.484 (this threshold corresponds to an intensity change by more than 40%). The Benjamini-Hochberg [59] algorithm was used for multiple testing adjustment. The minimum adjusted p-value for all pairs of time points is indicated for each differentially expressed probeset in the Table of differentially expressed mRNAs and miRNAs (Additional file 1) as a statistical significance value.

Pathway analysis

Bioinformatic analysis was performed using DAVID online tool (http://david.abcc.ncifcrf.gov) as described elsewhere [60]. So all the analyzed genes were classified into several functional groups, and the groups that may be potentially associated with physiological stress were considered and listed in the tabs of excel document. P-values on the tabs are modified Fisher Exact P-Values. When members of two independent groups can fall into one of two mutually exclusive categories, Fisher Exact test is used to determine whether the proportions of those falling into each category

differs by group. In DAVID annotation system, Fisher Exact test is adopted to measure the gene-enrichment in annotation terms.

Additional file

Additional file 1: Differentially expressed mRNAs and miRNAs.

Abbreviations

miRNA: microRNA; mRNA: messenger RNA.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conception and design of the experiments: AGT, AIG. Collection, analysis and interpretation of data: DVM, DAS, MUS, AEL, VVG. Drafting the article and revising it critically TRS, AGT, AA, HN. All authors read and approved the final manuscript.

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Position Statement
Part one: Immune function and exercise

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CONSENSUS STATEMENT

An ever-growing volume of peer-reviewed publications speaks to the recent and rapid growth in both scope and understanding of exercise immunology. Indeed, more than 95% of all peer-reviewed publications in exercise immunology (currently >2, 200 publications using search terms "exercise" and "immune") have been published since the formation of the International Society of Exercise and Immunology (ISEI) in 1989 (ISI Web of Knowledge^{SM}). We recognise the epidemiological distinction between the generic term "physical activity" and the specific category of "exercise", which implies activity for a specific purpose such as improvement of physical condition or competition. Extreme physical activity of any type may have implications for the immune system. However, because of its emotive component, exercise is likely to have a larger effect, and to date the great majority of our knowledge on this subject comes from exercise studies.

In this position statement, a panel of world-leading experts provides a consensus of current knowledge, briefly covering the background, explaining what we think we

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know with some degree of certainty, exploring continued controversies, and pointing to likely directions for future research. Part one of this position statement focuses on 'immune function and exercise' and part two on 'maintaining immune health'. Part one provides a brief introduction and history (Roy Shephard) followed by sections on: respiratory infections and exercise (Maree Gleeson); cellular innate immune function and exercise (Jeffrey Woods); acquired immunity and exercise (Nicolette Bishop); mucosal immunity and exercise (Michael Gleeson and Nicolette Bishop); immunological methods in exercise immunology (Monika Fleshner); anti-inflammatory effects of physical activity (Charlotte Green and Bente Pedersen); exercise and cancer (Laurie Hoffman-Goetz and Connie Rogers) and finally, "omics" in exercise (Hinnak Northoff, Asghar Abbasi and Perikles Simon).

The focus on respiratory infections in exercise has been stimulated by the commonly held beliefs that the frequency of upper respiratory tract infections (URTI) is increased in elite endurance athletes after single bouts of ultra-endurance exercise and during periods of intensive training. The evidence to support these concepts is inconclusive, but supports the idea that exercised-induced immune suppression increases susceptibility to symptoms of infection, particularly around the time of competition, and that upper respiratory symptoms are associated with performance decrements. Conclusions from the debate on whether sore throats are actually caused by infections or are a reflection of other inflammatory stimuli associated with exercise remains unclear.

It is widely accepted that acute and chronic exercise alter the number and function of circulating cells of the innate immune system (e.g. neutrophils, monocytes and natural killer (NK) cells). A limited number of animal studies has helped us determine the extent to which these changes alter susceptibility to herpes simplex and influenza virus infection. Unfortunately, we have only 'scratched the surface' regarding whether exercise-induced changes in innate immune function alter infectious disease susceptibility or outcome and whether the purported anti-inflammatory effect of regular exercise is mediated through exercise-induced effects on innate immune cells. We need to know whether exercise alters migration of innate cells and whether this alters disease susceptibility. Although studies in humans have shed light on monocytes, these cells are relatively immature and may not reflect the effects of exercise on fully differentiated tissue macrophages. Currently, there is very little information on the effects of exercise on dendritic cells, which is unfortunate given the powerful influence of these cells in the initiation of immune responses.

It is agreed that a lymphocytosis is observed during and immediately after exercise, proportional to exercise intensity and duration, with numbers of cells (T cells and to a lesser extent B cells) falling below pre-exercise levels during the early stages of recovery, before returning to resting values normally within 24 h. Mobilization of T and B cell subsets in this way is largely influenced by the actions of catecholamines. Evidence indicates that acute exercise stimulates T cell subset activation *in vivo* and in response to mitogen- and antigen-stimulation. Although numerous studies report decreased mitogen- and antigen-stimulated T cell proliferation following acute exercise, the interpretation of these findings may be confounded by alterations in the relative proportion of cells (e.g. T, B and

NK cells) in the circulation that can respond to stimulation. Longitudinal training studies in previously sedentary people have failed to show marked changes in T and B cell functions provided that blood samples were taken at least 24 h after the last exercise bout. In contrast, T and B cell functions appear to be sensitive to increases in training load in well-trained athletes, with decreases in circulating numbers of Type 1 T cells, reduced T cell proliferative responses and falls in stimulated B cell Ig synthesis. The cause of this apparent depression in acquired immunity appears to be related to elevated circulating stress hormones, and alterations in the pro/anti-inflammatory cytokine balance in response to exercise. The clinical significance of these changes in acquired immunity with acute exercise and training remains unknown.

The production of secretory immunoglobulin A (SIgA) is the major effector function of the mucosal immune system providing the 'first line of defence' against pathogens. To date, the majority of exercise studies have assessed saliva SIgA as a marker of mucosal immunity, but more recently the importance of other antimicrobial proteins in saliva (e.g. α-amylase, lactoferrin and lysozyme) has gained greater recognition. Acute bouts of moderate exercise have little impact on mucosal immunity but prolonged exercise and intensified training can evoke decreases in saliva secretion of SIgA. Mechanisms underlying the alterations in mucosal immunity with acute exercise are probably largely related to the activation of the sympathetic nervous system and its associated effects on salivary protein exocytosis and IgA transcytosis. Depressed secretion of SIgA into saliva during periods of intensified training and chronic stress are likely linked to altered activity of the hypothalamic-pituitary-adrenal axis, with inhibitory effects on IgA synthesis and/or transcytosis. Consensus exists that reduced levels of saliva SIgA are associated with increased risk of URTI during heavy training.

An important question for exercise immunologists remains: how does one measure immune function in a meaningful way? One approach to assessing immune function that extends beyond blood or salivary measures involves challenging study participants with antigenic stimuli and assessing relevant antigen-driven responses including antigen specific cell-mediated delayed type hypersensitivity responses, or circulating antibody responses. Investigators can inject novel antigens such as keyhole limpet haemocyanin (KLH) to assess development of a primary antibody response (albeit only once) or previously seen antigens such as influenza, where the subsequent antibody response reflects a somewhat more variable mixture of primary, secondary and tertiary responses. Using a novel antigen has the advantage that the investigator can identify the effects of exercise stress on the unique cellular events required for a primary response that using a previously seen antigen (e.g. influenza) does not permit. The results of exercise studies using these approaches indicate that an acute bout of intense exercise suppresses antibody production (e.g. anti-KLH Ig) whereas moderate exercise training can restore optimal antibody responses in the face of stressors and ageing. Because immune function is critical to host survival, the system has evolved a large safety net and redundancy such that it is difficult to determine how much immune function must be lost or gained to reveal changes in host disease susceptibility. There are numerous examples where exercise alters measures of immunity by 15-25%. Whether changes of this magnitude are sufficient to alter host defence, disease susceptibility or severity remains debatable.

Chronic inflammation is involved in the pathogenesis of insulin resistance, atherosclerosis, neurodegeneration, and tumour growth. Evidence suggests that the prophylactic effect of exercise may, to some extent, be ascribed to the anti-inflammatory effect of regular exercise mediated via a reduction in visceral fat mass and/or by induction of an anti-inflammatory environment with each bout of exercise (e.g. via increases in circulating anti-inflammatory cytokines including interleukin (IL)-1 receptor antagonist and IL-10). To understand the mechanism(s) of the protective, anti-inflammatory effect of exercise fully, we need to focus on the nature of exercise that is most efficient at allieviating the effects of chronic inflammation in disease. The beneficial effects of endurance exercise are well known; however, the anti-inflammatory role of strength training exercises are poorly defined. In addition, the independent contribution of an exercise-induced reduction in visceral fat versus other exercise-induced anti-inflammatory mechanisms needs to be understood better. There is consensus that exercise training protects against some types of cancers. Training also enhances aspects of anti-tumour immunity and reduces inflammatory mediators. However, the evidence linking immunological and inflammatory mechanisms, physical activity, and cancer risk reduction remains tentative.

In the very near future, genomics, proteomics, and metabolomics may help exercise immunologists to better understand mechanisms related to exercise-induced modulation of the immune system and prevention (or reduced risk) of diseases by exercise training. In addition, these technologies might be used as a tool for optimizing individual training programmes. However, more rigorous standardization of procedures and further technological advances are required before practical application of these technologies becomes possible.

Key Words: exercise; sport; training; immune; pathogen; infection; innate; acquired; mucosal; saliva; leukocyte; monocyte; neutrophil; granulocyte; lymphocyte; immunoglobulin; method; cytokine; interleukin; inflammation; cancer; genomics; proteomics; metabolomics

INTRODUCTION AND HISTORY

Two recent papers have summarized the scientific history of exercise immunology (263) and its development as a specific discipline (264) with its own international society and a dedicated journal. Exercise immunology has quite a short history relative to many branches of the exercise sciences, the modern era of careful epidemiological investigations and precise laboratory studies beginning in the mid 1980s. However, an ever-growing volume of peer-reviewed publications speaks to a rapid growth in both scope and understanding of the topic since that date. In addition to enquiries into many areas of intrinsic scientific interest, exercise immunologists have found diverse applications for their talents in augmenting population health and maintaining high performance athletes in peak physical condition.

From early during the 20th century, clinicians had pointed to what seemed adverse effects of prolonged heavy exercise upon both resistance to and the course of various viral and bacterial diseases (25, 261). These concerns were seemingly sub-

stantiated by a 2-6 fold increase in the reported symptoms of upper respiratory infection (URTI) for several weeks following participation in marathon or ultra-marathon events (200, 224). The influence of exercise on the risks of URTI is discussed in the following section. A transient fall in the circulating natural killer (NK) cell count following a sustained bout of vigorous exercise (270) seemed to offer a mechanism explaining the increase in risk; the temporary lack of NK cells and killer cell activity offered an "open window," a period when a reduced resistance to viral infections allowed easier access to infecting micro-organisms. Innate immunity is discussed in detail later in this part of the position statement. In one report, the reduction in NK cell count persisted for seven days following exercise (259), but in most studies, circulating NK cell numbers and activity have been described as being depressed for only a few hours, raising doubts as to whether the "window" was open long enough to account for the increased vulnerability to infection. Moreover, technical advances (particularly in automated cell counting and identification) (85) have underlined that exercise does not destroy NK cells; rather, they are temporarily relocated to reservoir sites such as the walls of peripheral veins in response to the exercise-induced secretion of catecholamines and activation of adhesion molecules (266). A more plausible explanation for the reported increase in URTI during heavy training and following participation in long-distance events appeared as attention shifted to immunoglobulins in general, and in particular to a depression of front-line defences through a decrease in the mucosal secretory functions of the nose and salivary glands (152, 298). The influence of exercise on mucosal immunity is discussed in more detail later in this part of the position statement.

The hypothesis of a U-shaped relationship between physical activity and resistance to disease, although based on a relatively limited amount of laboratory and epidemiological data (202, 267), has made intuitive sense, jibing with the more general belief that although regular moderate doses of physical activity have beneficial effects on health, excessive amounts or intensities of physical activity have negative consequences. In the case of the immune system, one suggestion has been that an excess of physical activity provokes something analogous to clinical sepsis, with tissue destruction from an excessive inflammatory reaction (260). Although initially conceived simply in the context of URTI (201), the concept of a U-shaped response has now been extended to cover the effects of physical activity upon a variety of clinical disturbances of immune function. In terms of cancer prevention and therapy (268), regular moderate physical activity has been shown to reduce the risk of developing certain forms of the disease (265); it also limits the risk of metastasis, at least in experimental animals (156). Exercise and cancer is discussed in more detail in this part of the position statement. On the other hand, excessive exercise has been shown to cause DNA damage and apoptosis (176, 186). Ageing is increasingly considered in part as an expression of disturbed immune function; high concentrations of pro-inflammatory cytokines are seen in the elderly, and seemingly contribute to such problems of ageing as sarcopenia, neural degeneration and Alzheimer's Disease. Moreover, appropriate amounts of physical activity can control levels of pro-inflammatory cytokines, and appear to have a beneficial effect on these manifestations of ageing (188). Certain autoimmune conditions also respond to carefully regulated physical activity programmes, although it has yet to be established clearly whether benefit occurs

through some direct modulation of cell counts and cytokines, or through changes in the activity of transcription factors for pro-inflammatory cytokines (9).

Developments in fluorescent antibodies have allowed exercise immunologists to identify an ever-growing number of cell sub-types and receptors. At the same time, new cytokine identification kits and methods in molecular technology (173) have allowed the examination of humoral factors that are present in the body for very short periods and in extremely low concentrations; an increasingly complex range of pro- and anti-inflammatory cytokines has been revealed. The exercise immunologist seems drawn into the main streams of sports medicine, physiology and even psychology. A fascinating cascade of cytokines is now thought to have an important role not only in controlling exercise-induced inflammation, but also in regulating the release and necessary flow of metabolites (221). Development of the sub-discipline of psycho-neuroimmunology (141) has emphasized that vigorous exercise should be considered as but one example of the body's reaction to a variety of stressors (221), with an important two-way communication between peripheral immunocytes and hypophyseal centres, involving a wide variety of hormones and autonomic pathways (157). A section in the second part of the position statement deals with stress and immune function.

On the sports field, exercise immunologists are increasingly asked to develop procedures to detect such abuses as blood doping (185) and gene transfer (11) (see "Omics" section in this part of the position statement). However, attempts to pinpoint immunological markers of over-training have as yet proved inferior to traditional indices such as mood state and physical performance (as discussed in the second part of this position statement). A variety of nutritional supplements to date seem to have had only limited success in blunting the immune impairment associated with heavy exercise (as discussed in the second part of this position statement).

These are a few of the important topics on which a panel of world experts provide a succinct consensus of current knowledge, briefly covering the relevant background, exploring continued controversies, and pointing to likely directions of future research.

RESPIRATORY INFECTIONS AND EXERCISE

Background

There are more uncertainties than evidence based facts on the nature of upper respiratory tract infections (URTI) associated with exercise, particularly in high performance athletes. Although URTI or 'sore throats' are the most common reason for presentation of elite athletes to a sports medicine clinic (62, 77, 80), the debate on whether sore throats are actually caused by infections, or are a reflection of other inflammatory stimuli associated with exercise remains unclear (48, 106, 242).

The costs associated with identification of the underlying causes of upper respiratory symptoms (URS) and the delay in obtaining results of investigative tests

means that infections are not usually verified by pathology examinations. Physician confirmation of an infective cause of the symptoms, based on clinical signs and symptoms, has until recently been considered the 'gold standard' for exercise studies, but the involvement of physicians in assessments and diagnosis is not common in research settings. Recently, the 'gold standard' of physician verified diagnosis of URTI has also come under scrutiny, and been found less than ideal (48). Very few studies have examined the underlying causes of URS and extensive clinical investigations of athletes are rare (48, 242).

The focus on respiratory infections in exercise has been stimulated by the commonly held beliefs that the frequency of URTI is increased in elite endurance athletes and that their incidence is associated with more intensive training (201). The evidence to support these concepts is inconclusive, but does, support the idea that exercised-induced immune suppression increases susceptibility to symptoms of infection and that URS are associated with performance decrements.

Evidence based consensus and uncertainties

Over the past thirty years, there have been numerous investigations examining the association between changes in immune parameters and the risk of URTI in athletic and non-exercising populations. The only immune measures to date to show consistent relationships with URS in exercising populations have been changes in salivary IgA concentrations and secretion rates (19, 89, 263). A section focusing on exercise and mucosal immunity appears later in this part of the position statement.

Altered mucosal immunity and risk of symptoms of URTI

The inverse relationship between salivary IgA concentrations and risk of URTI in exercising and non-exercising populations has demonstrated differences between these two populations (76, 89, 98, 232). The different population risk profiles are predominantly due to differences in the levels of intensity and quantum of exercise undertaken by very fit elite athletes and non-elite exercising or sedentary populations. The impact of exercise intensity on salivary IgA concentrations and secretion rates has demonstrated greater decreases in salivary IgA associated with prolonged high intensity exercise, whereas moderate increases in salivary IgA occur in response to short duration moderate intensity exercise (6, 19, 23, 98, 129, 148, 163, 232).

Although study populations vary, the association of an increased risk of URS and/or URTI with lower concentrations of salivary IgA and secretion rates has been consistent for high-performance endurance athletes undertaking intensive training (64, 91, 92, 95, 97, 148, 187, 195-198, 201, 320). Similarly, the increases in salivary IgA observed after moderate exercise training may contribute to the reduced susceptibility to URTI associated with regular moderate exercise (3, 129).

Symptoms and frequency

Although there are many anecdotal reports that URTIs are more common in elite athletes, there is very little reported evidence to support this commonly held belief. This uncertainty is compounded by the current uncertainty around whether the URS are due to infections or other inflammatory stimuli mimicking URTI (48, 242).

Retrospective and prospective longitudinal studies have identified that the majority of elite athletes experience symptoms of URTI at a rate similar to the general population (48, 78, 234). However, the episodes of URS in elite athletes do not follow the usual seasonal patterns of URTI observed in the general population, but rather occur during or around competitions (97, 160, 198, 224). Symptoms occur more frequently during the high intensity training and taper period prior to competitions in some sports, such as swimming (79, 89, 91), but in other endurance sports, such as long distance running, URS appear more frequently after a competition (49, 198, 224). Illness-prone athletes may also be susceptible to URS during regular training periods or following increases in training load (80). The commonly reported short-term duration of URS (1-3 days) in most studies suggests that in most instances a primary infection is unlikely and the symptoms may be due to viral reactivation (97, 242) or other causes of exercise-induced inflamma-

Table 1. Pathogens identified and the number of cases in comprehensive prospective studies of athletes presenting with symptoms of upper respiratory infections in 1) a cohort of high performance triathletes during training and competitions (282); 2) a study of elite athletes from a variety of sports undertaking routine training presenting to a sports clinic with URS (48); and 3) a cohort of elite athletes experiencing recurrent episodes of URS associated with fatigue and performance decrements (242). Where investigations were not performed this is recorded as (-).

Pathogen identified by microbial and viral investigation	Triathletes (n=63) undertaking routine training and competitions Spence et al. (282)	Elite athletes (n=70) presenting to a sports clinic Cox et al. (48)	Elite athletes (n=41) with persistent fatigue and poor performance Reid et al. (242)
Rhinovirus	7	6	-
Influenzae (A & B)	7	1	-
Parainfluenzae (1, 2 & 3)	4	3	-
Adenovirus	0	2	-
Coronavirus	2	0	-
Metapneumovirus	1	0	-
Epstein Barr virus (primary infection)	1	1	3
EBV reactivation	-	1	8
Cytomegalovirus	0	0	5
Herpes simplex virus (types 1 & 2)	0	-	-
Ross River virus	-	-	1
Toxoplasmosis	-	-	1
Mycoplasma pneumoniae	0	1	1
Streptococcus pneumonia	2	1	-
Staphylococcus pyogenes	0	1	-
Haemophilus influenzae	0	0	-
Moraxella catarrhalis	0	0	-
Enterococcus spp	0	0	-

tion. The evidence that URS are associated with poor performance is also limited. In the month prior to an international competition URS have been associated with decrements in performance in elite swimmers (235), suggesting that regardless of whether the URS are due to infections or other inflammatory stimuli, they can impact on performance at an elite level. However, a small proportion of high-performance endurance athletes experience recurrent episodes of URS at significantly higher rates than the incidence in the general population (92, 234), and in these athletes the URS are associated with significant persisting fatigue and poor performance (79, 91, 93, 242).

Infections versus inflammation

The few studies that have undertaken pathology testing to identify infectious from non-infectious causes of the episodes of URS in high-performance athletes have revealed that bacterial infections account for about 5% of the episodes (48, 94, 242, 282). Most episodes of URS with an identified infectious cause are of viral origin, but these account for only about 30-40% of the episodes in each study (48, 282). The bacterial and viral pathogens identified in these comprehensive studies indicate that the infections are caused by the usual respiratory pathogens associated with URTI (246) in the general population (Table 1).

However, the profile of infections in a study of elite athletes experiencing recurrent URS associated with long-term fatigue and poor performance identified a high percentage as having herpes group viruses (e.g. cytomegalovirus) or evidence of Epstein Barr Virus (EBV) reactivation (242) (Table 1). Epstein Barr viral reactivation has also been demonstrated in association with URS in some endurance sports (97, 242), which may account for the short duration of the symptoms reported in most studies, resulting from viral reactivation rather than primary infection. However, in a study examining the prophylactic use of an antiviral treatment in elite runners, it was shown that not all episodes of URS were associated with EBV expression (50) and that the frequency of EBV expression differed between sports (50, 97). Although an anti-herpes virus treatment was effective in reducing EBV expression in elite long-distance runners, it was not effective in reducing the frequency of episodes of URS, once again suggesting other non-infective causes for the URS in elite athletes (50).

Physician diagnosis of infections as the cause of the URS has recently come under scrutiny (48) and in conjunction with a previous study by Reid et al. (242) has identified that elite athletes suffering recurrent episodes of URS need more exhaustive clinical assessments to exclude non-infectious yet treatable causes of the symptoms, such as asthma, allergy, autoimmune disorders, vocal cord dysfunction and unresolved non-respiratory infections. In these studies, other diseases with an inflammatory basis accounted for 30-40% of episodes of URS in elite athletes. These studies identified that URS were divided into approximately one-third proportions as having an infectious cause, non- infectious medical cause and an unknown aetiology. The speculative causes of the ‘unknown-aetiology’ group could include physical or mechanical damage such as drying of the airways (16); asthma and allergic airway inflammation (106); psychological impacts of exercise on membrane integrity and immunity (22); and the migration

to the airways of inflammatory cytokines generated during damage to muscles susained in eccentric exercise (214, 222). Multiple stressors experienced by athletes, biological, physical and psychological, are likely to induce neurological and endocrine responses in addition to alterations in immune parameters; these share common exercise-induced pathways (207) that may result in URS. However, there is currently little direct evidence to support any of these mechanisms being associated with URS, respiratory infections or susceptibility to infections in athletes.

Cytokine regulation

Cytokine responses to exercise (particularly those associated with micro-trauma and or glycogen depletion of muscle tissue (27, 214, 222, 294)) are reasonably well characterised (as discussed in the section on anti-inflammatory effects of physical activity later in this part of the position statement). They are likely to play an important role in modulating post-exercise changes in immune function that increase the risk of infection or the appearance of inflammatory symptoms (294). The pro-inflammatory responses to exercise have the potential to be involved in expression of URS that mimic URTI. A study comparing cytokine responses to exercise in illness-prone distance runners demonstrated impaired anti-inflammatory cytokine regulation compared to runners who did not suffer frequent episodes of URS (51). A recent cytokine gene polymorphism study by Cox et al. (47) identified an underlying genetic predisposition to high expression of the pro-inflammatory interleukin-6 in athletes prone to frequent URS. These studies add further weight to the evidence that suggests infections are not the only cause of the symptoms of 'sore throat'. They are supported by studies examining the prophylactic use of topical anti-inflammatory sprays to prevent URS in long-distance runners which demonstrate a reduction in the severity of the symptoms but not the frequency of episodes following marathon races (49, 257).

Conclusions and future directions

Interpreting the findings of studies on the role of respiratory infections in exercise is often limited by the lack of pathogen identification. Regardless of the underlying stimulus for the inflammatory symptoms the implications of the upper airway symptoms for athletes may be the same. However, unless the symptoms are confirmed as infections, reference to symptoms as URS rather than as infections or URTI should become the accepted reporting standard, particularly when there is no physician assessment.

The current consensus is that the cause of URS in athletic populations is uncertain. Physician identification can no longer be considered the gold standard and symptoms should only be referred to as infection if a pathogen has been identified. Although diagnostic pathology is rarely performed, in the few studies that have examined pathology, the infections identified in most athletes have been the common respiratory pathogens observed in the general population.

Inflammation from non-infective causes is common among athletes and many will have underlying treatable conditions. As differentiation between the inflammatory causes of URS is currently not feasible in most research settings, appro-

priate treatments are difficult to prescribe universally. Athletes with recurrent URS associated with long-term fatigue and poor performance do, however, warrant more exhaustive clinical investigations, including assessment for possible involvement of the herpes group viruses. Identifying athletes with an underlying genetic predisposition to pro-inflammatory responses to exercise may be useful in managing the training regimens of elite athletes, particularly those who suffer recurrent episodes of URS associated with fatigue and poor performance.

The two main questions to be resolved about the relationship between respiratory infections and exercise are: 1) whether the upper respiratory tract symptoms are actually infections and if so whether they can be prevented or treated; and 2) if the symptoms are not due to infections can the different causes of the inflammation be segregated in the complex paradigm of elite training to optimise the illness-prone athlete's training and performance.

CELLULAR INNATE IMMUNE FUNCTION AND EXERCISE

Background

Innate immunity is our first line of defence against infectious pathogens and is intimately involved in tissue damage, repair and remodeling. The major difference between innate immune responses and adaptive responses is that innate responses do not strengthen upon repeated exposure (there is no memory function). In addition, innate responses are less specific in terms of pathogen recognition. So, whereas innate responses recognize classes of pathogens (e.g. gram-negative bacteria) through toll-like receptors (TLRs), lymphocytes exhibit exquisite specificity for epitopes of individual pathogens (e.g. influenza virus). The innate branch of the immune system includes both soluble factors and cells. Soluble factors include complement proteins which mediate phagocytosis, control inflammation and interact with antibodies, interferon α/β which limits viral infection, and anti-microbial peptides like defensins which limit bacterial growth. Major cells of the innate immune system include neutrophils which are first line defenders against bacterial infection, dendritic cells (DCs) which serve to orchestrate immune responses, macrophages (Mϕ's) that perform important phagocytic, regulatory and antigen presentation functions, and natural killer (NK) cells which recognize altered host cells (e.g. virally infected or transformed). However, many host cells, not just those classified as innate immune cells, can initiate responses to pathogenic infection. Although partitioning the immune system into innate and adaptive systems makes the system easier to understand, in fact, these branches are inextricably linked with each other. For example, the innate immune system helps to develop specific immune responses through antigen presentation, whereas cells of the adaptive system secrete cytokines that regulate innate immune cell function. This section will focus on the influence of acute and chronic exercise on cellular components of innate immunity (Figure 1). A later section in this part of the position statement will focus on exercise and inflammatory cytokines which constitute the products of innate immune and other cells.

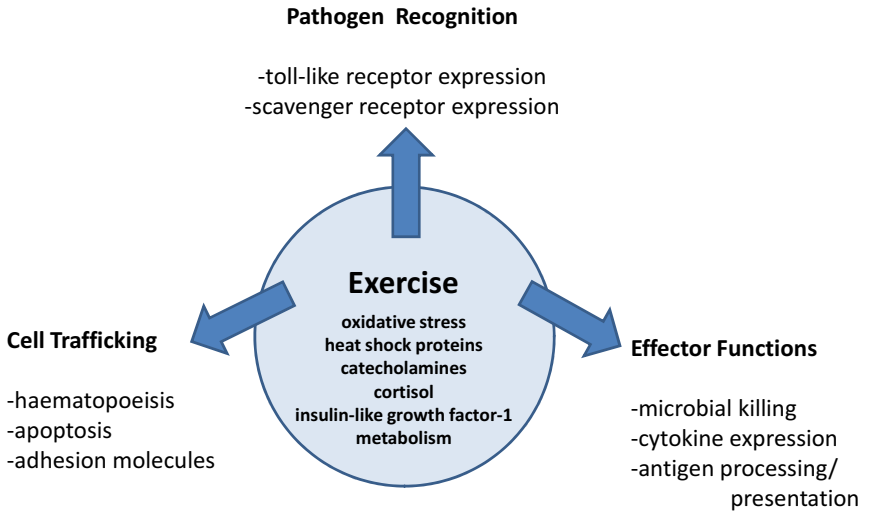

Figure 1. Potential mechanisms whereby acute/chronic exercise affects innate immunity. Exercise-induced factors such as oxidative stress, increased metabolic rate, heat shock proteins, catecholamines, cortisol and insulin-like growth factor can influence: pathogen recognition by altering expression of recognition molecules such as toll-like or scavenger receptors; cell trafficking by altering haematopoieisis, cell death and adhesion molecule expression; and effector functions like oxidative burst, cytokine expression and antigen processing and presentation. This list of potential mechanisms is not all-inclusive and very few have been definitively tested.

Consensus

Acute exercise and cellular innate immune function

Neutrophils

Acute exercise results in a first, rapid and profound neutrophilia (increase in blood neutrophil number) followed by a second, delayed increase in blood neutrophil count a few hours later, the magnitude of which is related to both the intensity and duration of exercise (216, 247). The initial increase is likely due to demargination caused by shear stress and catecholamines, whereas the later increase may be due to cortisol-induced release of neutrophils from the bone marrow (162). Unstimulated neutrophil degranulation, phagocytosis and oxidative burst activity are increased by an acute bout of exercise but there is a reduced degranulation and oxidative burst in response to bacterial stimulation that can last for many hours (215, 216, 247). This indicates that although exercise may mobilize highly functional neutrophils into the circulation, in recovery, their ability to respond to exogenous stimuli may be diminished. Marginated neutrophils are more mature than recently released neutrophils and this likely has implications for the study of exercise on neutrophil function, although this does not appear to influence respiratory burst activity (276).

Monocytes/Macrophages

Many studies have examined the influence of acute exercise on human $CD14^+$ blood monocytes (Mo's) which are relatively immature cells destined to become

tissue Mϕ's. Acute exercise results in a transient (~2 h) monocytosis and most likely represents a shifting of Mo's from the marginated to the circulating pool (206). This could occur as a result of haemodynamic and/or cortisol or catecholamine-induced release from the vascular endothelium (136). Indeed, administration of the beta-blocker propranolol can reduce exercise-induced monocytosis (2) and adrenaline (epinephrine) administration causes monocytosis (307). There are also reports that exercise can affect Mo phenotype, cell surface protein, and cytokine expression. For example, in response to acute exercise, there is a preferential mobilization of $CD14^+/CD16^+$ expressing Mo's (115, 289) that exhibit a pro-inflammatory phenotype relative to $CD14^+/CD16^-$ classical Mo's. It may be that these marginated cells have a more mature inflammatory function for entry into tissues and are knocked off the endothelium in response to exercise. Interestingly, the percentage of these $CD14^+/CD16^+$ cells is reduced in recovery, perhaps indicating remarginalization or tissue recruitment (272). Acute exercise also reduces expression of TLRs 1, 2 and 4 on $CD14^+$ Mo's (140). However, the extent to which these changes reflect a true decrease versus Mo population shifts is unclear. In an attempt to reconcile this, Simpson et al. (272) examined cell surface proteins on Mo subpopulations in response to acute exercise. They found that TLR4 and HLA.DR (major histocompatibility molecule II important in antigen presentation) expression were altered on total $CD14^+$ Mo's but also on individual Mo populations, indicating that changes in cell surface expression are not influenced solely by exercise-induced changes in Mo subpopulations in blood. Several studies have examined Mo cytokine production after acute exercise, finding that, although spontaneous cytokine levels in $CD14^+$ cells change little (245, 285), acute exercise reduces TLR ligand-stimulated interleukin (IL)-6, IL1-α, and tumour necrosis factor-alpha (TNF-α production (140, 286), perhaps as a consequence of reduced TLR expression. Further studies regarding the effects of acute exercise on Mo TLR signaling may clarify these observations.

Because Mo's are relatively immature, exercise-induced changes in their function may not reflect actual tissue Mϕ function which is central to inflammation and immune responses. For this reason, animal studies have examined the influence of exercise on tissue Mϕ number and function. Both moderate and intense acute exercise have potent stimulatory effects on phagocytosis (210), anti-tumour activity (52, 327, 328), reactive oxygen and nitrogen metabolism (327, 328), and chemotaxis (206, 209). However, not all functions are enhanced by exercise. We have documented prolonged exercise-induced reductions in Mϕ MHC II expression (325) and antigen presentation capacity (35, 36). Some effects may be dose-dependent as exhaustive exercise was shown to decrease alveolar Mϕ anti-viral function; this effect was correlated with increased susceptibility to Herpes simplex virus (HSV)-1 infection (133, 134) and related to increased release of adrenal catecholamines, but not corticosterone (133). Thus, it appears that exercise, perhaps dependent on dose with respect to some functions, can affect tissue Mϕ's and, in some studies, disease outcomes in animals. Whether these same effects can be generalized to humans is unknown.

Dendritic cells

The effect of acute exercise on DCs has received little attention despite the important emergent role of these cells in the initiation of immune responses. There are

only two studies reporting that exercise can increase circulating numbers of DCs (59, 109) and, to our knowledge, nothing is known about acute effects of exercise on DC function.

Natural killer (NK) cells

There is a vast literature on the acute effects of exercise on circulating NK ($CD3^{-}CD16^{+}CD56^{+}$) cells, perhaps because of their ease of study and large magnitude change in response to exercise. Like other blood leukocytes, NK cells are rapidly mobilized into the circulation in response to acute exercise, most likely by increased shear stress and catecholamine-induced down-regulation of adhesion molecule expression (15, 122, 301). There appears to be a differential mobilization such that $CD56^{bright}$ NK cells are less responsive than $CD56^{dim}$. Perhaps this indicates a reduced ability to defend against pathogens during acute exercise, as $CD56^{bright}$ cells are more cytotoxic. However, the health significance of exercise-induced changes in circulating NK cells, like other leukocytes, remains unknown. After prolonged exercise, the numbers of circulating NK cells are reduced in blood (87), perhaps as a consequence of remarginalization or tissue migration, but there is a relative increase in the $CD56^{bright}$ subset (302).

NK cell cytotoxicity (NKCC) is a major functional measure of NK activity. Early studies demonstrated that unstimulated NKCC was dependent upon the intensity and duration of the exercise bout (87). Immediately after a single bout of moderate or exhaustive exercise there is a 50-100% increase in human peripheral blood NKCC (87, 329). The exercise-induced increase in NKCC is largely due to an increase in the absolute number and percentage of blood NK cells (87). NKCC expressed on a per cell basis does not appear to change much after acute exercise unless the bout is intense and prolonged, in which case NKCC can be depressed for several hours, possibly indicating an enhanced period of susceptibility to infection (90). Only a few studies have examined whether NK cells mobilized into the circulation in response to exercise have altered sensitivity to stimulating agents like interferon-α or IL-2 (68, 329); however, like unstimulated NKCC, these effects are likely mediated by distributional shifts in NK cell subsets and should not necessarily be interpreted as altered NK cell function on a per cell basis.

Exercise training and cellular innate immune function

Neutrophils

Regular exercise training does not appear to alter blood leukocyte counts, including neutrophils appreciably (90). However, there are a few reports that exercise training reduces blood neutrophil counts in those with chronic inflammatory conditions or neutrophils in sites of chronic inflammation (171) raising the possibility that such exercise acts in an anti-inflammatory fashion in those with inflammation. This effect could be beneficial or deleterious, dependent upon the context. Although there is little known about the influence of exercise training on neutrophil function, regular exercise, especially heavy, intense training, may attenuate neutrophil respiratory burst (103, 233). This could reflect a sustained effect of previous acute exercise, as attenuation of respiratory burst has been documented to last several days post-exercise (295).

Monocytes/Macrophages
Both longitudinal exercise training and cross-sectional studies have shown that physically active people exhibit reduced blood Mo inflammatory responses to lipopolysaccharide, lower TLR4 expression, and a lower percentage of $CD14^+/CD16^+$ 'inflammatory' Mo's (73, 165, 166, 273, 290, 300). The extent to which these effects on the relatively small blood Mo pool contribute to the anti-inflammatory effect of exercise training is unknown. In contrast, animal studies have demonstrated that exercise training can increase induced inflammatory responses of peritoneal Mϕ's (128, 151, 292), indicating a possible difference between the effects of training on blood Mo's when compared with differentiated tissue Mϕ's. Animal studies have the potential to shed additional light on the source of the anti-inflammatory effect of regular exercise, especially in populations that exhibit inflammation. Indeed, in two recent studies, we have shown that exercise training, with or without a low fat diet, reduces visceral adipose tissue (e.g. Mϕ infiltration and pro-inflammatory cytokine gene expression) and systemic inflammation in high fat diet-fed mice (309, 310). Regular exercise may also reduce Mϕ infiltration into other sites of chronic inflammation, including growing tumours (336), and this could be interpreted as a benefit given the tumour supporting role of these cells. In contrast, reduced infiltration of Mϕ's into sites of chronic infection could lead to morbidity, although this has not been demonstrated. In fact, Mϕ's appear to play a definitive role in mediating the beneficial effects of regular moderate exercise as it relates to intranasal infection with HSV-1 in mice (181).

Dendritic cells
There are two reports from the same group demonstrating an effect of exercise training on rat dendritic cells. Liao et al. (147) reported that dendritic cell number increased after training, with no difference in costimulatory molecule (CD80 or CD86) expression, while Chiang et al. (40) found that MHC II expression, mixed leukocyte reaction and IL-12 production were increased in DCs from exercise trained rats. Clearly, given the importance of DCs in early immune regulation, this is an area ripe for investigation.

Natural killer (NK) cells
Despite much research regarding the effects of exercise training on NK cell number and function, there appears to be much controversy regarding its effect. Early cross-sectional or intervention studies with limited subject numbers reported modest increases in NKCC after moderate exercise training in previously sedentary subjects (167, 194, 202, 223, 269, 326). In larger trials, one study (65) found that 15 weeks of moderate exercise training increased NKCC compared with sedentary controls, while another 12-month trial found no change in NKCC in 115 post-menopausal women (31). However, intense training has been shown to alter NK cell subsets and reduce NKCC (93, 293). Studies in animals have demonstrated that regular exercise can increase *in vivo* cytotoxicity (119, 120, 155); however, the specific contribution of NK cells in mediating this exercise effect is unclear (119).

Controversies

Based upon the body of literature, it appears that both acute and chronic exercise have the potential to alter both the number and function of cells of the innate immune system (Figure 1). A limited number of animal studies have helped us determine the extent to which these changes alter susceptibility to herpes simplex (181) and influenza virus (149, 150, 271) infection. Unfortunately, we have only 'scratched the surface' regarding whether exercise-induced changes in immune function alter infectious disease susceptibility or outcome. In addition, although some progress has been made, we know relatively little about how acute and chronic exercise affect innate immune cell trafficking. We need to determine whether exercise alters migration of these cells and whether this alters disease susceptibility. Given the important role of innate immune cells in inflammatory states and the relationship between inflammation and chronic disease, we need to clarify whether the purported anti-inflammatory effect of regular exercise is mediated through exercise-induced effects on innate immune cells. In this regard, it is of interest to know whether exercise affects Mϕ phenotype (e.g. classical versus alternative). Although studies in humans shed light on Mo's, these cells are relatively immature and may not reflect the effects of exercise on fully differentiated tissue Mϕ's. Lastly, there is very little information on the effects of exercise on DCs, which is unfortunate given the powerful influence of these cells early in immune responses.

ACQUIRED IMMUNITY AND EXERCISE

Background

Acquired immunity (also known as adaptive or specific immunity) is designed to combat infections by preventing colonisation of pathogens and destroying invading micro-organisms. With only a few exceptions, it is initiated by the presentation of antigen to T helper ($CD4^+$) lymphocytes within the peptide binding groove of major histocompatibility complex class II molecules on antigen presenting cells $CD4^+$ T cells form a key part of the cell-mediated immune response, since they orchestrate and direct the subsequent response. Helper T cell clones can be divided into two main phenotypes, type 1 (Th1) and type 2 (Th2) cells, according to the cytokines that they produce and release. Th1 cells play an important role in defence against intracellular pathogens, e.g. viruses, the release of the cytokines interferon-γ (IFN- γ) and interleukin-2 (IL-2) stimulating T cell activation and proliferation of clones of effector cells. Memory T cells are also generated, allowing a rapid secondary response upon subsequent exposure to the same antigen. Th2 cells release IL-4, IL-5, IL-6 and IL-13 and appear to be involved in protection against extracellular parasites and stimulation of humoral immunity (production of antibody and other soluble factors that circulate in the blood and other body fluids). Therefore, cytokines released from Th2 cells can activate B lymphocytes, leading to proliferation and differentiation into memory cells and plasma cells (although some antigens can activate B cells independently of $CD4^+$ cells). Plasma cells are capable of secreting vast amounts of immunoglobulin (Ig) or antibody specific to the antigen that initiated the response. The binding of Ig to its target antigen forms an antibody-antigen complex and both free Igs and anti-

body-complexes circulate in the body fluids. $CD8^+$ cells can also be classified into type 1 (Tc1) and type 2 (Tc2) cells according to their cytokine profiles, as described above, but the functional significance of these cells is at yet unclear. A further set of T-cells, the naturally-occurring regulatory T-cells (Tregs) express the phenotype CD4+CD25+ and can suppress the functional activity of lymphocytes by mechanisms that most likely involve secretion of cytokines including IL-10 and TGF-β1.

Consensus: acute exercise and acquired immune function

T and B cell number

Acute exercise elicits characteristic transient biphasic changes in the numbers of circulating lymphocytes. Typically, a lymphocytosis is observed during and immediately after exercise, with numbers of cells falling below pre-exercise levels during the early stages of recovery, before steadily returning to resting values. This pattern of mobilisation is observed for T cells (and T cell subpopulations) and to a lesser extent, B cells. Changes are proportional to exercise intensity and duration, although the effect of intensity is more marked (161, 258). Insufficient recovery between prolonged exercise bouts appears to exaggerate the biphasic response (251). Mobilization of T and B cell subsets in this way is largely influenced by the actions of adrenaline (epinephrine) both directly on the expression of cell adhesion molecules particularly those of the integrin and selectin families, and indirectly via sympathetically mediated influences on cardiac output and the

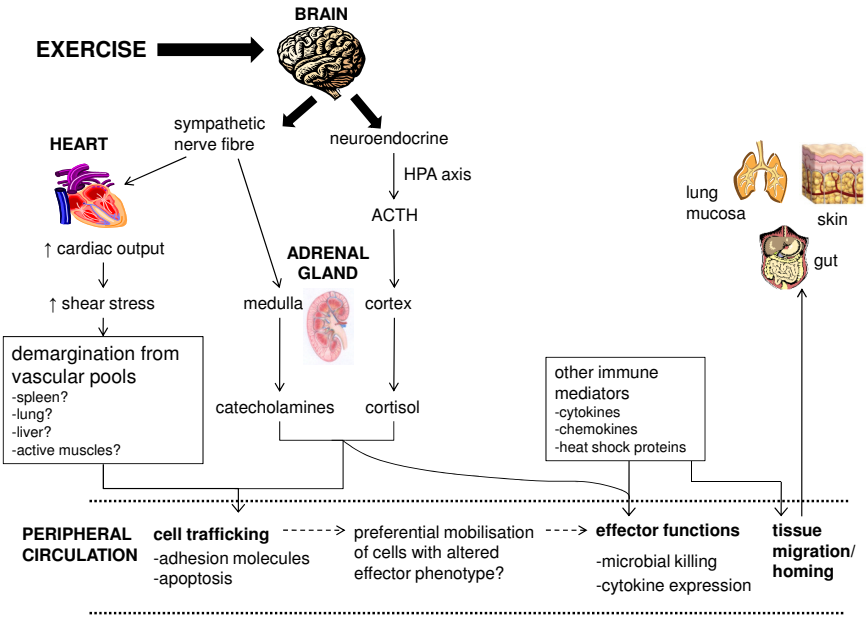

Figure 2. Potential mechanisms by which acute and chronic exercise affects acquired/adaptive immunity. HPA = hypothalamic pituitary adrenal; ACTH = adrenocorticotropic hormone.

subsequent increase in shear stress associated with enhanced blood flow (262) (Figure 2). Lymphocytes express a high density of β_2-adrenergic receptors and the density of these receptors increases with both exercise and exposure to catecholamines (262). The greatest expression of these receptors is found on the surface of NK cells, with fewer on $CD8^+$ and B cells and least of all on $CD4^+$ cells; the differing effects of intense exercise on the relative magnitude of mobilization of the lymphocyte subsets reflects this differential density of adrenergic receptor expression. The decrease in T cell number following exercise is largely due to a decrease in type 1 T cells, since intensive physical activity decreases the percentage of circulating Type 1 T cells but has little effect on the percentage of circulating Type 2 T cells (118, 287). It is unclear whether these changes are due to apoptosis or, as seems more likely, a redistribution of cells to other compartments. A decrease in the percentage of type 1 $CD4^+$ and $CD8^+$ T cells alone does not necessarily indicate that defence against intracellular pathogens such as viruses is suppressed; cytokine production is just one step of the multi-stage process that ultimately leads to lymphocyte proliferation or cytotoxicity. It is possible that any increase or decrease in cell number is countered by a diminished or enhanced response of other aspects of immune cell function. Moreover, the addition of a subpopulation of cells from the marginated pool into the circulation in response to exercise may influence lymphocyte function simply because the mobilized cells may have different functional abilities to those already in the circulation (Figure 2).

T and B cell function

T cells play a fundamental role in the orchestration and regulation of the cell-mediated immune response to pathogens. One important consequence of a defect in T cell function is an increased incidence of viral infections (63). With this in mind, it has been speculated that the apparent increased susceptibility of sportsmen and women to upper respiratory tract infections may be due to exercise-induced decreases in T cell function.

There is evidence that acute exercise stimulates T cell subset activation *in vivo* and in response to mitogen- and antigen-stimulation, as assessed by expression of cell surface markers of T cell activation, including CD69, CD25, the HLA-DR antigen, CD45RO and CD45RA (84, 86, 100). It is not clear whether such increases in activation are due to the recruitment of activated cells into the circulation, or are an effect on the state of activation of individual cells themselves. Most likely it is a combination of both. Numerous studies report decreased mitogen- and antigen-stimulated T cell proliferation following acute exercise, but interpretation of these findings may be confounded by the presence of NK cells and B cells within the cell cultures; alterations in relative numbers of T, B and NK cells in blood samples obtained before and after exercise may affect the proportion of cells that can respond to stimulation in a given volume of blood or number of peripheral blood mononuclear cells (102). Furthermore, *in vitro* stimulation with mitogen does not necessarily reflect the more subtle responses of cells following a specific antigen encounter within the body (20). Moreover, exercise may alter T cell function *in vitro* through an increase in the rate of apoptosis in cell culture rather than a decrease in T cell proliferation rate (101).

Upon stimulation, B cells proliferate and differentiate into memory cells and plasma cells, with plasma cells localised primarily in lymphoid or mucosal tissue and able to produce and secrete vast amounts of Ig (or antibody) specific to the antigen that initiated the response. The binding of Ig to its target antigen forms antibody-antigen complexes; Ig and antibody-antigen complexes circulate in the body fluids. The effect of exercise on humoral immune function has been assessed through measurements of serum and mucosal Ig concentration *in vivo* and serum Ig synthesis following *in vitro* mitogen-stimulation. Serum Ig concentration appears to remain either unchanged, or slightly increased, in response to either brief or prolonged exercise (184, 203, 229). Mitogen-stimulated IgM concentration appears to increase in response to exercise independently of changes in T or B cell number, although there are contrasting findings concerning IgA and IgG (258, 306).

Consensus: exercise training and acquired immune function

In the true resting state (i.e. more than 24 h after their last training session) circulating lymphocyte numbers and functions of athletes appear to be broadly similar to those of non-athletes (192). Longitudinal studies in which previously sedentary people undertake weeks or months of exercise training fail to show any marked changes in T and B cell functions, provided that blood samples are taken at least 24 h after their last exercise bout. In contrast, T and B cell functions appear to be sensitive to increases in training load in well-trained athletes undertaking a period of intensified training, with decreases in circulating numbers of Type 1 T cells, reduced T cell proliferative responses and falls in stimulated B cell Ig synthesis reported (7, 139, 308). This suggests that athletes engaging in longer periods of intensified training can exhibit decreases in T cell functionality. The cause of this depression in acquired immunity appears to be related to elevated circulating stress hormones, particularly cortisol, and alterations in the pro/anti-inflammatory cytokine balance in response to exercise (Figure 2). This appears to result in a temporary inhibition of Type 1 T cell cytokine production, with a relative dampening of the Type 1 (cell-mediated) response.

Conclusions

Acute intensive exercise elicits a depression of several aspects of acquired immune function. This depression is transient and cell numbers and functions usually return to pre-exercise values within 24 h. If recovery between exercise sessions is insufficient, as during prolonged periods of intensified training in elite athletes, this temporary decrease in cell function can become a chronic depression of acquired immunity. Although not clinically immune deficient, it is possible that the combined effects of small changes in several aspects of host defence may compromise resistance to minor illnesses, such as respiratory infections. The clinical significance of these alterations requires more detailed investigation.

MUCOSAL IMMUNITY AND EXERCISE

Background
Mucosal surfaces such as those in the gut, urogenital tract, oral cavity and respiratory system are protected by a network of organised structures known as the Common Mucosal Immune System (96). These structures include Peyer's patches and isolated lymphoid follicles in gut-associated, nasal-associated, and bronchial/tracheal-associated lymphoid tissues and salivary glands. The production of immunoglobulin A (IgA), specifically secretory IgA (SIgA), is the major effector function of the mucosal immune system, SIgA together with innate mucosal defences such as α-amylase, lactoferrin and lysozyme, provides the 'first line of defence' against pathogens present at mucosal surfaces. In addition, secretory IgM and locally produced IgG play a less significant role in protection of mucosal surfaces (96). The transepithelial transport of the polymeric Ig receptor (pIgR)-IgA complex into secretions such as saliva affords three potential ways in which IgA provides an effective defence against microbial pathogens: through prevention of pathogen adherence and penetration of the mucosal epithelium, by neutralising viruses within the epithelial cells during transcytosis and by excretion of locally formed immune complexes across mucosal epithelial cells to the luminal surface (138).

Consensus
A high incidence of infections is reported in individuals with selective deficiency of SIgA (105) or very low saliva flow rates (75). Moreover, high levels of saliva SIgA are associated with low incidence of URTI (252) and low levels of saliva SIgA in athletes (64, 95) or substantial transient falls in saliva SIgA (187) are associated with increased risk of URTI.

Levels of saliva SIgA vary widely between individuals. Although some early studies indicated that saliva SIgA concentrations are lower in endurance athletes compared with sedentary individuals (304), the majority of studies indicate that there are no differences between athletes compared with non-athletes except when athletes are engaged in heavy training (19, 96).

Falls in saliva SIgA concentration can occur during intensive periods of training (4, 32, 64, 93, 95, 97, 187, 303, 304) and some studies (32, 64, 93, 95, 187), though not all (4, 303, 320) have observed a negative relationship between saliva SIgA concentration and occurrence of URTI. Several of the above cited studies examined changes in saliva SIgA during intensive periods of military training (32, 303, 320). However, this often involves not only strenuous physical activity, but also dietary energy deficiency (see section on nutritional countermeasures in part two of the position statement), sleep deprivation (see section on sleep disruption in part two of the position statement) and psychological challenges (see section on the effects of stress on immune function in part two of the position statement). These multiple stressors are likely to induce a pattern of immunoendocrine responses that amplifies the exercise-induced alterations (207).

Increases in saliva SIgA have been observed after a period of regular moderate exercise training in previously sedentary individuals and may, at least in part, con-

tribute to the apparent reduced susceptibility to URTI associated with regular moderate exercise (3, 129).

The saliva SIgA response to acute exercise is variable and may be influenced by exercise mode, intensity and duration as well as the fitness of the subjects, unstimulated versus stimulated saliva collection methods, how saliva SIgA is expressed (e.g. absolute concentration, as a secretion rate or as a ratio to total protein or osmolality) and other factors that may be present such as reduced food intake, dehydration, sleep deprivation, altitude, and psychological stress (19). Levels of saliva SIgA are generally unchanged with resistance exercise sessions (130) and moderate aerobic exercise lasting less than 1 h (19).

The saliva SIgA response to exercise is generally not affected by environmental temperature (116, 137, 312), short periods (<24 h) of fasting (5) or food restriction (207), carbohydrate intake during exercise (18, 146, 199), up to 30 h of sleep deprivation (243), or by time of day (4, 57, 145).

Salivary α-amylase is another antimicrobial protein (317) and its secretion is stimulated by increased activity of the sympathetic nervous system (37), with the majority of this protein produced by the parotid gland (281). In accordance with this, several studies have found that exercise increases the α-amylase activity of saliva in a manner that is dependent on exercise intensity (6, 18, 145, 317).

Controversies

Secretion of saliva and its constituent proteins is regulated by the autonomic nervous system. The secretion of SIgA in rats can be increased by both parasympathetic and sympathetic nerve stimulation and adrenaline has recently been shown to increase the transport of human IgA into saliva by rat salivary cells via increased mobilisation of the pIgR (33, 34). Since intensive exercise is associated with enhanced sympathetic nervous system activation, it seems surprising that some studies report a decrease in saliva SIgA concentration following a bout of high intensity exercise (>80%$\dot{V}O_2$max) that recovers to resting levels within 1 h of exercise completion (154, 164). Other studies have reported either no change (163, 243, 299) or increases (6, 23, 313) in saliva SIgA concentration after single or repeated bouts of high intensity exercise.

Saliva SIgA concentration (or secretion rate) in response to prolonged (>1.5 h) moderate intensity exercise (50-75%$\dot{V}O_2$max) is more consistently reported to decrease (153, 199, 213, 288, 304) or remain unchanged (23, 116, 163, 195, 255). Different methods of saliva collection and differences in hydration status of subjects may contribute to the discrepancies in the literature (19, 144, 207, 291).

A few small-scale studies have reported that female athletes have lower saliva SIgA concentration (95) and secretion rate (4, 5) compared with their male counterparts, but confirmation of this possible gender difference is required in a larger subject population.

There is little data available regarding changes in salivary lysozyme and lactoferrin concentrations with acute or chronic exercise, although intense and exhaustive

exercise of both short and long duration is associated with increases in salivary lysozyme (6, 316, 317) and lactoferrin secretion (316). These effects also appear to be dependent on exercise intensity, since no change was seen following ~20 min of cycling at 50%$\dot{V}O_2$max (6). Prolonged cycle ergometer exercise at 60%$\dot{V}O_2$max caused a significant increase in salivary α-defensin concentrations and secretion rates (53).

The mechanisms by which exercise influences salivary responses remain to be fully elucidated (Figure 3). The rate of secretion of saliva SIgA is dependent on the production of IgA by the plasma cells in the submucosa and/or the rate of IgA transcytosis across the epithelial cell which is determined by the availability of the pIgR (24). The time-course (minutes) of the alterations in saliva SIgA secretion that are observed in response to acute exercise suggest that this is the princi-

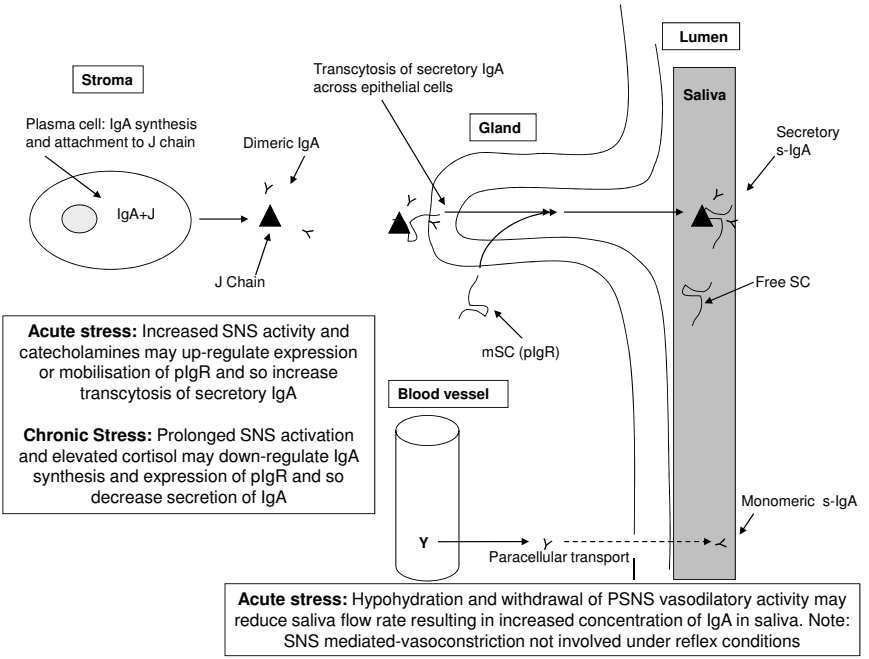

Figure 3. Effects of acute and chronic stress on receptor-mediated transport of locally produced dimeric IgA and paracellular transport of serum derived monomeric IgA into saliva. mSC = membrane secretory component; pIgR = polymeric Ig receptor; SNS = sympathetic nervous system; PSNS = parasympathetic nervous system.

pal mechanism by which acute intensive exercise influences saliva SIgA secretion. In anaesthetised rats, acute stimulation of β-adrenoreceptors above a certain threshold increases saliva SIgA secretion in a dose-independent manner via elevated transcytosis from the glandular pool (230) and this is associated with increased availability of the pIgR (34). Although such a mechanism has not yet

been demonstrated in humans, the finding that increases in saliva SIgA secretion rate are associated with elevations in plasma adrenaline following caffeine ingestion lends some support to this suggestion (21).

Although enhanced IgA transcytosis probably accounts for elevations in saliva SIgA secretion observed after exercise, it cannot account for the findings of either no change or decreases in saliva SIgA secretion rate with intense physical activity. The observation that increased mobilisation of the pIgR only occurred above a certain threshold frequency of stimulation (230) could account for the finding of little change in saliva SIgA levels at more moderate intensities of exercise. However, the finding of decreased concentrations of saliva SIgA in response to acute exercise is harder to explain. Nevertheless, a study in rats demonstrated that following a prolonged treadmill run to exhaustion, decreases in saliva SIgA concentration were associated with a decline in pIgR mRNA expression (127). Although highly speculative, this might imply that there is a second critical threshold (or duration) of stimulation, above which pIgR expression becomes downregulated.

It is unlikely that cortisol plays a major role in the regulation of saliva SIgA secretion in response to acute exercise, because changes in both saliva SIgA concentration and secretion rate have been observed in the absence of any alterations in plasma or salivary cortisol (6, 145, 146, 256, 299) and there appears to be no correlation between saliva SIgA and cortisol responses to exercise (164).

Modification of IgA synthesis could play a major role in the changes in saliva SIgA secretion observed in response to long term intensive training and chronic psychological stress (19, 24, 226). In addition, it may be that repeated mobilisation of the pIgR could deplete the available formed IgA pool, leading to decreases in saliva SIgA output. However, to date there is scant research in either animals or humans to support these speculations.

Conclusions

To date the majority of exercise studies have assessed saliva SIgA as a marker of mucosal immunity but more recently the importance of other antimicrobial proteins in saliva including α-amylase, lactoferrin and lysozyme has gained greater recognition. Acute bouts of moderate exercise have little impact on mucosal immunity, but very prolonged exercise and periods of intensified training can result in decreased saliva secretion of SIgA. Mechanisms underlying the alterations in markers of mucosal immunity with acute exercise are probably largely related to the activation of the sympathetic nervous system and its associated effects on salivary protein exocytosis and IgA transcytosis. Depressed secretion of SIgA into saliva during periods of intensified training and chronic stress are likely linked to altered activity of the hypothalamic-pituitary-adrenal axis, with inhibitory effects on IgA synthesis and/or transcytosis. There is reasonable evidence to indicate that reduced levels of saliva SIgA are associated with increased risk of URTI.

IMMUNOLOGICAL METHODS IN EXERCISE IMMUNOLOGY

Background

There are many examples in the literature and reviewed in this consensus paper that acute exercise and exercise training can alter host defence, leading to changes in disease susceptibility and severity. One important mechanism for such changes is alterations in *immune function*. Herein lies a primary challenge for exercise immunologists; how does one measure immune function in a meaningful way? The immune system is comprised of a large variety of cells, occurs in diverse tissues (i.e., lymph node, Peyer's patches, spleen and liver), and involves the orchestration of hundreds of soluble and cell membrane associated proteins. Successful host defence is the end product of these responses.

Consensus

Exercise immunology experiments test the impact of acute exercise and/or regular exercise training on a number of measures of the immune system. The types of immunological assessments most commonly reported, especially in the human exercise studies involve analyses of blood borne circulating immune proteins (e.g., interleukin (IL)-6, IL-1β, C-reactive protein, IL-8, tumour necrosis factor alpha (TNFα) chemokines), circulating blood leukocytes (e.g., CD4+ T cells, CD8+ T cells, Th1, Th2, Th17, Treg, B cells, neutrophils, monocytes), and salivary/plasma antibody or immunoglobulin (Ig) concentrations. Some studies document dynamic changes in the composition of blood leukocyte populations (e.g., decreases in peripheral blood CD4+ T cells and increases in neutrophils), and some studies isolate the peripheral blood leukocytes and put them in culture with various exogenous stimuli, such as mitogens, that stimulate large populations of immune cells to produce immune products. Using these types of measures, there are many reported examples of robust dynamic changes produced both with acute exercise and after exercise training. As discussed in other sections of this position statement, the nature of the reported changes measured depends on a number of variables that include the training status of the individual, the intensity of the exercise bout, the nutritional status of the individual, the timing of the blood/saliva sample collection and the nature of the specific immunological measure. Due to the reported dynamic changes in such blood borne and salivary measures, it is essential that multiple samples are taken, including pre-, during-, and post- exercise timepoints. Non-exercised, time-matched controls must also be sampled to control for circadian, seasonal, and environmental changes in these dynamic measures. The majority of studies in exercise immunology are sensitive to these aspects of experimental design, making these methodological features strengths of the field.

Another approach to assessing immune function extends beyond blood or salivary soluble proteins, circulating cells, total Ig or *in vitro* stimulated responses. It involves challenging experimental subjects with antigenic (immune stimulating, not disease capable) or pathogenic (immune stimulating, possible disease producing) stimuli and assessing relevant antigen-driven responses including antigen specific cell-mediated delayed type hypersensitivity (DTH) responses or antibody responses and in some instances, changes in disease susceptibility, duration, and

severity. This approach allows assessment of *in vivo* immune function and has several advantages over the previously described measures. Firstly, the generation an antigen specific Ig response reflects a functionally important end product of a multicellular *in vivo* immunological response. For example, the generation of a primary antibody response to a novel antigen like keyhole limpet haemocyanin (KLH) requires antigen presentation (likely by a B cell given KLH is a low dose soluble protein) to CD4+ T cells. KLH specific T cells then provide T cell help in the form of both co-stimulation and cytokines to KLH specific B cells to stimulate the production of anti-KLH IgM and promote isotype switching to anti-KLH IgG1 (driven by Th2 cytokines) and IgG2a (driven by Th1 cytokines). If an acute exercise bout or exercise training impacts *in vivo* immune function, then changes in the generation of KLH specific Ig will be detected. In addition, if there are selective changes in isotype switching, for example an impact on anti-KLH IgG1 and not on anti-KLH IgG2a, or *vice versa*, this suggests selective effects on Th1 and Th2 responses (70, 88, 159, 177). This approach has been successfully used in both humans (274, 275, 278) and animals (55, 69, 71, 82, 179, 311).

The results of the exercise immunology studies that measure *in vivo* anti-KLH Ig responses support the general conclusion that an acute bout of intense exercise suppresses anti-KLH Ig production (178), however, moderate exercise training can restore optimal antibody in the face of stressors (69, 72) and ageing (99, 277). Interestingly, the majority of studies using this measure rarely demonstrate an *increase* in the anti-KLH Ig response with exercise training in **young healthy adults.** This is likely due to the fact that young healthy sedentary and physically active organisms already possess excellent immune responses, and elevating that response further is not necessarily a good thing. Too much immunity is just as detrimental as too little (Figure 4). In other words, the positive effects of exercise training on immune function and host defence may be most readily revealed when *in vivo* immune function is sub-optimal consequent to ageing, stress, or other factors. In fact there are several papers that demonstrate that regular physical activity reduces incidence of illness only if people report high levels of stress (26, 74).

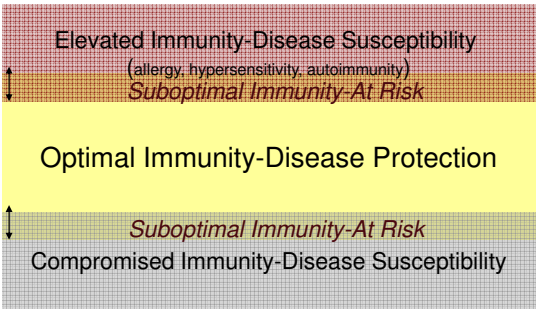

Figure 4. Exercise associated changes in immune function have greatest effects on host defence and disease susceptibility/severity, if the individual has suboptimal immune function due to ageing, stress or other factors.

A related approach that also measures *in vivo* immune function, and is reported in the exercise immunology literature is to inject **not a novel** antigen, such as KLH, but rather a mixture of antigens using influenza vaccine or tetanus vaccine that usually contain a subset of repeated antigens that have been "seen" by people before (30, 60, 61). The advantage of this approach, especially when studying humans, is that people are

willing to receive such injections because they produce useful immunity against influenza and/or tetanus. The disadvantage of this approach is that the subsequent antibody response is a mixture of primary, secondary and tertiary responses. This makes it difficult to accomplish the following: 1) measure group changes in isotypes (very little IgM is detectable in secondary and tertiary versus primary responses); 2) compare concentrations of antigen specific antibody (secondary and tertiary responses characteristically produce higher levels of IgG than primary responses); and 3) make inferences about cellular mechanisms for any detected changes (unique cellular and co-stimulatory signals are required for primary versus secondary and tertiary responses)(70). Thus the assessment of an antigen-specific immune response following vaccination yields important information about *in vivo* immune responses that are superior to measuring dynamic circulating protein or cell changes, but suffers some interpretive limitations not found after primary antigenic challenge.

An additional methodological and interpretation challenge when studying exercise-induced changes in immune responses is to determine if the measured changes in immunity are sufficient to alter host defence or disease susceptibility/severity. This is a complex challenge. It involves issues associated with immune safety net and redundancy (Figure 4) and immune response specificity relative to host disease defence. Because immune function is critical to host survival, the system has evolved a large safety net and redundancy such that it is difficult to determine how much immune function must be lost or gained to incur changes in host disease susceptibility. Studies on human immunodeficiency (HIV) patients offer insight into the issue. It is commonly reported that patients with HIV must lose at least ~50-60% of their total circulating CD4+ T cells before an increase in the incidence of opportunistic infection occurs (182). There are numerous examples of exercise altering circulating cell numbers and other measures of immunity, often by 15-25%. Whether changes of this magnitude are sufficient to alter disease susceptibility or severity likely depends on the state of the host. If, for example, immune function was optimal or functioning at 100% then ± 15-25% change may not impact host defence in a clinically significant way, because the safety net for immune function is great. If instead immune function was suboptimal due to ageing, stress or other factors placing host immunity in the "at risk zone", then a 15-25% change in immune function could have significant consequences for host defence (Figure 4). A second issue to consider when interpreting the functional significance of changes in immune measures for host defence is response specificity. That is, what specific types of pathogens or disease states could be impacted by changes in the aspects of immunity measured? For example, how would transient changes in circulating T cell numbers influence anti-viral host defence? This issue is especially challenging for human research. There are, however, several rodent disease models that establish clear links between changes in specific immune responses and corresponding changes in host defence and disease severity. Work by Shamgar Ben-Eliyahu is one example (12). Although he is not specifically testing the impact of exercise, he is exploring the impact that other stressors (i.e., surgery, drugs etc.) have on immune function and host defence. A strength of his model is that he both demonstrates stress-associated suppression in NK cell tumour killing *ex vivo* and stress-associ-

ated increases in tumour load *in vivo* (14). Furthermore he has verified that the tumour tested in these studies is primarily killed by NK cells and **not** CD8+ T cells (13). Thus using this type of approach one can measure immune function and verify relevance for host defence and disease susceptibility/severity.

A second approach used in immunology research involves challenging animals with pathogens that require specific and well-characterized immunological responses for survival. *Leishmania major*, for example, requires a Th1 dominant response for effective host defence (43). If one blocks the development of Th1 responses, the animal will die. This is a useful experimental model, because one can link changes in specifically Th1 responses (cytokines, clonal expansion, Th1 differentiation or activation, etc.) with corresponding changes in *Leishmania* disease susceptibility, severity and host survival. This type of model could be implemented in exercise immunology studies.

Controversies and future directions

Most studies in exercise immunology are conducted in humans and are usually limited to immune measures derived from the blood, such as soluble immune proteins, cell numbers, *in vitro* cellular responses to mitogen and total Ig concentrations. As previously discussed, it is difficult to determine how such changes could impact host defence, disease susceptibility or severity. Although persistent or chronic elevations in blood concentrations of inflammatory proteins may be reflective of changes in inflammatory processes, it is possible that dynamic, short-lived changes in blood borne immune factors offer little insight into how the *in vivo* immune function and/or host defence is altered. In addition, increases in concentrations of blood borne soluble proteins such as IL1β, IL8, and TNF-α that classically play a role during local tissue inflammation, likely are not related to tissue inflammation. There is no evidence that the acute increases in circulating concentrations of these proteins produced by stressors or exercise function to modulate any inflammatory process, especially in an otherwise healthy host. More likely, the acute elevations in IL-6 and IL1-β found after exercise may be more important for the *metabolic* rather than the *immunological*, responses to exercise.

Given the pleiotropic and context dependent nature of cytokines/chemokines, perhaps we should revise our thinking when trying to interpret acute and dynamic effects of exercise. Firstly, we need to consider any change in cytokine concentration within the context of the cytokine network (180). In other words, the contextual dependence of cytokines cannot be ignored. A nice immunological example of contextual dependence is the effect of transforming growth factor (TGF)-β on CD4+ T cell differentiation. Based on the 3-signal model of T cell activation and differentiation (45), cytokines play a pivotal role in CD4+ T cell differentiation after activation from Th0 (non-polarized) to Th1, Th2, Treg etc. TGF-β plus IL6, for example, drives the differentiation of the Th0 toward a Th17 cell. In contrast, TGF-β in the absence of IL-6 drives the differentiation of the Th0 toward a Treg cell. A second example of cytokine networks and context dependence can be found in the exercise immunology literature, where increases in circulating IL-6 in the presence of TNF-α is indicative of inflammation, whereas increases in cir-

culating IL-6 in the absence of TNF-α may be indicative of increased energy demand (217, 219)(Figure 6).

In conclusion, there are clear effects of both acute exercise and exercise training on measures of immune products and function. Exercise training effects on immune function and host defence are especially demonstrable when immune function is not optimal due to ageing, stress or other factors. Exercise immunology researchers are faced with challenges associated with both the immune measures and the interpretation of changes in such measures. *In vivo* antigen specific immune function can be measured by injecting subjects (both people and animals) with novel antigens and vaccination antigens; assessment of antigen specific immunoglobulin and T cell (by DTH tests) responses is a strong approach. The ability to predict if any change in antibody titre or T cell function is sufficient to alter host defence, specific disease susceptibility or disease severity however, remains debatable.

ANTI-INFLAMMATORY EFFECTS OF PHYSICAL ACTIVITY

Chronic inflammation is involved in the pathogenesis of insulin resistance, atherosclerosis, neurodegeneration, and tumour growth. Evidence suggests that the protective effect of exercise may, to some extent, be ascribed to the anti-inflam-

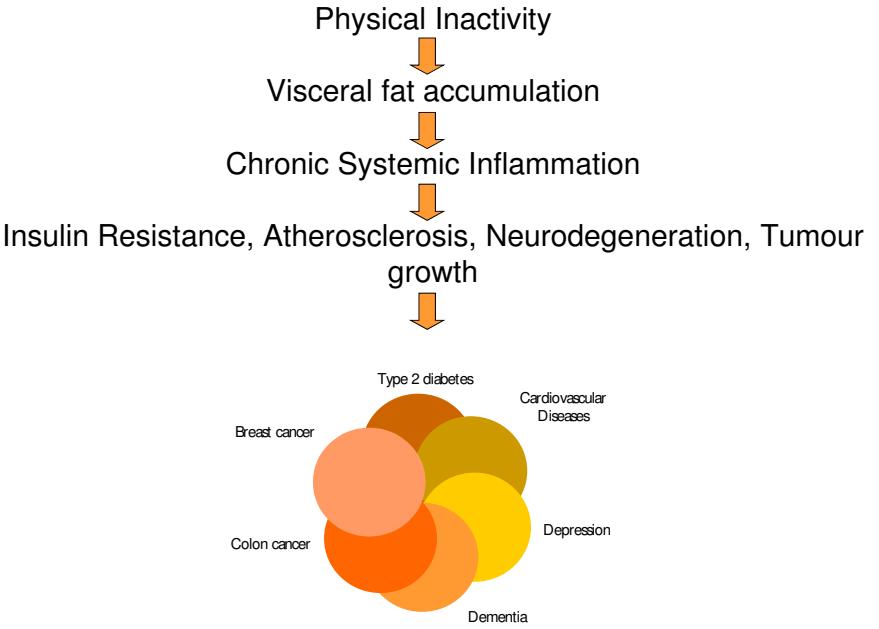

Figure 5. Hypothesis: Physical inactivity leads to accumulation of visceral fat and consequently to the activation of a network of inflammatory pathways, which promotes development of insulin resistance, atherosclerosis, neurodegeneration, and tumour growth, leading to the development of "the diseasome of physical inactivity".

matory effect of regular exercise, mediated via a reduction in visceral fat mass and/or by induction of an anti-inflammatory environment with each bout of exercise.

Background

It is well-established that physical inactivity increases the risk of type 2 diabetes (305), cardiovascular diseases (204), colon cancer (322), breast cancer (175), dementia (253) and depression (211). Physical inactivity leads to the accumulation of visceral fat and consequently the activation of a network of inflammatory pathways. Chronic inflammation promotes the development of insulin resistance, atherosclerosis, neurodegeneration, and tumour growth (104), and subsequently the development of a number of diseases associated with physical inactivity (218) (Figure 5).

The protective effect of exercise against chronic inflammation associated diseases may, to some extent, be ascribed to an anti-inflammatory effect of regular exercise. Several studies show that markers of inflammation are reduced following longer-term behavioural changes involving reduced energy intake and increased physical activity (reviewed in (225)). We suggest that the long-term anti-inflammatory effects of exercise may be mediated both via a reduction in visceral fat mass and the establishment of an anti-inflammatory environment with each bout of exercise.

Consensus

We have suggested that cytokines and other peptides that are produced, expressed, and released by muscle fibres and exert paracrine or endocrine effects should be classified as "myokines" (218). Such myokines may exert a direct effect on fat metabolism and thereby result in indirect anti-inflammatory effects. Moreover, myokines may exert direct anti-inflammatory effects or stimulate the production of anti-inflammatory components.

It is suggested that contracting skeletal muscles release myokines, which work in a hormone-like fashion, exerting specific endocrine effects on visceral fat and other ectopic fat deposits. Other myokines work locally within the muscle via paracrine mechanisms, exerting their effects on signalling pathways involved in fat oxidation.

The first identified and most studied myokine is the gp130 receptor cytokine, interleukin (IL)-6. A number of studies during the past decade have revealed that both type I and type II muscle fibres express the myokine IL-6 in response to muscle contractions. Subsequently IL-6 exerts its effects both locally within the muscle (e.g. through activation of 5' adenosine monophosphate activated protein kinase, AMPK) and, when released into the circulation, in a hormone-like fashion in a number of organs. Within skeletal muscle, IL-6 acts locally to signal through a gp130Rβ/IL-6Rα homodimer resulting in activation of AMPK and/or phosphatidylinositol-3-kinase (PI3K) to increase fat oxidation and glucose uptake (219). Although it has not been demonstrated that IL-6 has specific effects on visceral fat mass, it does appear to play an important role in lipid metabolism. IL-15 is expressed in human skeletal muscle and has been identified as an anabol-

ic factor in muscle growth. In addition to its anabolic effects on skeletal muscle *in vitro* and *in vivo*, IL-15 appears to play a role in lipid metabolism (191). Therefore, IL-15 has been suggested to be involved in muscle – fat cross talk. IL-15 mRNA levels are upregulated in human skeletal muscle following a bout of strength training (190), suggesting that regular training may lead to IL-15 accumulation within muscle. Interestingly, we demonstrated a decrease in visceral fat mass, but not subcutaneous fat mass, when IL-15 was overexpressed in murine muscle (189).

The cytokine response to exercise differs from that elicited by severe infections (Figure 6). Classical pro-inflammatory cytokines, tumour necrosis factor alpha (TNF-α) and IL-1β, in general do not increase with exercise, indicating that the cytokine cascade induced by exercise is markedly different from the cytokine cascade induced by infections, (reviewed in (219)).

To study whether acute exercise induces an acute anti-inflammatory response, a model of "low grade inflammation" was established in which a low dose of *E. coli* endotoxin was administered to healthy volunteers, randomised to either rest or exercise prior to endotoxin administration. In resting subjects, endotoxin induced a 2 to 3 fold increase in circulating levels of TNF-α. In contrast, when the subjects performed 3 h of ergometer cycling and received the endotoxin bolus at 2.5 h, the TNF-α response was totally blunted (284). This study provides some evidence that acute exercise may inhibit TNF-α production.

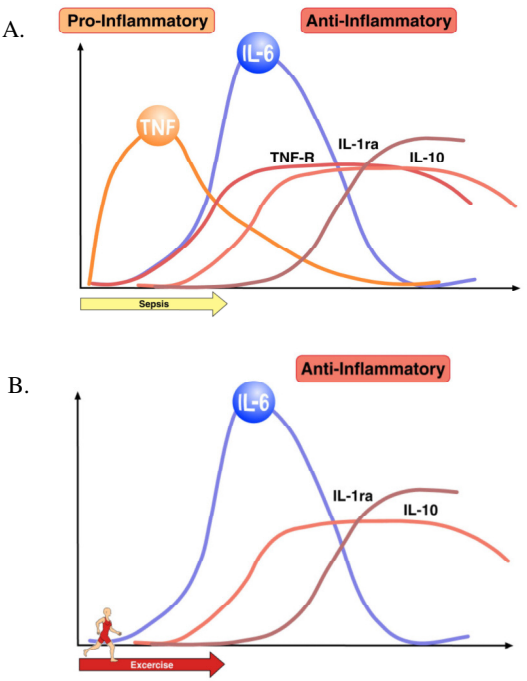

Figure 6. Comparison of sepsis-induced (A) versus exercise-induced (B) increases in circulating cytokines. During sepsis, there is a marked and rapid increase in circulating TNF-α, which is followed by an increase in IL-6. In contrast, during exercise the marked increase in IL-6 is not preceded by elevated TNF-α (220).

Typically, IL-6 is the first cytokine released into the circulation during exercise. The level of circulating IL-6 increases in an exponential fashion (up to 100 fold) in response to exercise and declines in the post-exercise period. The circulating levels of well-known anti-inflammatory cytokines such as, IL-1ra and IL-10, also increase after exercise. However, the

appearance of IL-6 in the circulation is by far the most marked and its appearance precedes that of the other cytokines. A number of studies have demonstrated that contracting skeletal muscle fibres per se produce and release IL-6. Of note, IL-6 infusion totally mimics the acute anti-inflammatory effects of a bout of exercise both with regard to induction of IL-1ra and IL-10 and with regard to suppression of endotoxin-stimulated increases in TNF-α levels. During acute exercise there is also a marked increase in adrenaline (epinephrine), cortisol, growth hormone, prolactin, and other factors that have immunomodulatory effects (104, 193). Taken together, it appears that each bout of exercise induces an anti-inflammatory environment.

Controversies
Patients with chronic inflammatory diseases such as type 2 diabetes are often prescribed exercise to improve quality of life; however, the use of exercise as a treatment for these diseases remains controversial. A systemic review has highlighted that acute and chronic exercise may elicit different responses in patients with chronic inflammatory disease when compared with healthy controls (227). For example, it has been reported that in patients with chronic obstructive pulmonary disease plasma TNF-α levels were abnormally increased compared with healthy controls following moderate-intensity exercise (236). Therefore, more needs to be understood about the nature of exercise that has anti-inflammatory effects in patients with chronic inflammatory diseases without increasing the underlying inflammatory pathology of the disease.

Future directions
To understand the mechanism of the protective, anti-inflammatory effect of exercise fully, we need to focus on the nature of exercise that is most effective at allieviating the effects of chronic inflammation in disease. The beneficial effects of endurance exercise are well known; however, the anti-inflammatory role of strength training exercises is poorly defined and remains an area for future investigation. In addition, the independent contribution of an exercise-induced reduction in visceral fat versus other exercise-induced anti-inflammatory mechanisms needs to be better understood.

EXERCISE AND CANCER

Background
Exercise can have a beneficial role in cancer prevention and therapy. Determining if regular physical activity reduces cancer risk through immunological mechanisms is of public health relevance and could lead to tailored and novel exercise prescriptions.

Consensus
The incidence of several types of cancer is reduced by regular physical activity. Comprehensive reviews by the International Agency for Research on Cancer (17) and the World Cancer Research Fund (330) identified an independent protective effect of physical activity on colon and postmenopausal breast cancer risk. Evi-

dence is also mounting that physical activity reduces risks of endometrial, lung, and pancreatic cancers.

Physical activity has a therapeutic effect in cancer patients by reducing cancer recurrence, enhancing health outcomes, and increasing survival. Women who exercised moderately prior to (81), and after a breast cancer diagnosis, had significant improvements in overall and disease-specific survival and quality of life compared to sedentary counterparts (280, 318). Protective effects of physical activity have also been observed for colorectal cancer patients (169).

There are fewer reports on exercise and neoplasia in animals with chemically-induced, transplantable, or spontaneous tumours (111). These studies describe exercise protecting against intestinal tumour incidence or number, although results with Apc^{min} mice, which develop intestinal tumours spontaneously, have been less consistent (10). A beneficial effect of exercise on mammary tumour incidence, multiplicity, growth rate and/or survival has also been reported (249).

Controversies

The biological mechanisms relating exercise and cancer are not well understood. Potential mediators include reductions in body mass and/or adiposity, decreases in reproductive hormone levels, altered growth factor milieu, enhanced antioxidant defence mechanisms, and changes in immune function, including reduced inflammation and enhanced anti-tumour immunity. Mechanisms studied in detail in humans have not been studied in animal models, and vice versa. Therefore, the relative contribution of these mechanisms in specific cancer types remains unknown. With respect to the hypothesis that exercise induces alterations in immune mediators, more is known about exercise-induced changes in inflammatory mediators than about changes in specific anti-tumour mechanisms; however, controversies exist for both hypotheses.

The association between chronic inflammation and cancer is well established (46). Human cross-sectional studies demonstrate an inverse relationship between regular physical activity and inflammatory biomarkers, including C-reactive protein (CRP), tumour necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) (123, 225). Reductions in CRP levels with exercise training have also been reported (123). Although exercise may reduce inflammatory biomarkers, clinical trials indicate variable outcomes, with an effect of exercise on CRP in some but not all studies (231). Less work has been done with IL-6 in humans, but again there are conflicting results (319). Finally, a recent randomized trial on markers of inflammation following a 12-month exercise intervention reported no change in participant colonic prostaglandin levels (1).

Animal studies demonstrate an anti-inflammatory role of exercise via multiple pathways. Exercise normalized the elevated levels of TNF-α in soluble TNF-receptor knock-out mice (126). Freewheel training lowered TNF-α expression and increased expression of antioxidant enzymes in mouse intestinal T lymphocytes (112, 113) and decreased prostaglandin E_2 level in the serum and polyps from Apc^{min} mice (121). Treadmill exercise decreased the number of

macrophages in polyps from Apc^{min} mice (8), and swimming exercise in rats reduced COX-2 positive cells in colonocytes (54). Taken together, several inflammatory pathways may be altered by exercise, but it is unclear to what extent and under what physiological conditions these changes occur.

Macrophages and natural killer (NK) cells have been studied in both tumour-bearing and healthy subjects following exercise. Collectively, animal model data show a positive effect of exercise on macrophage function, with enhanced clearance of lung metastases (324). Additionally, training results in greater *in vitro* NK cell cytotoxicity (221, 248), enhanced *in vivo* mechanisms of natural immunity and reduced pulmonary tumour metastases in mice (155, 221); however, these effects are small and modified by exercise intensity and timing. No change in NK cell cytotoxicity was observed following a 12-month walking intervention in healthy postmenopausal women (31). There are fewer studies on exercise and antigen-specific T cell functions. Moderately active older adults have higher influenza-specific *in vitro* peripheral blood mononuclear cell proliferation (132) and greater *in vivo* delayed type hypersensitivity (DTH) responses (277) compared with sedentary individuals. Moderate exercise also enhances antigen-specific T-cell mediated cytokine production and proliferation following vaccination (131, 250). Exercise improves antigen-specific T cell function, which may translate into better protection from infectious agents and greater immunosurveillance. Clinical and epidemiological studies show that the incidence of upper respiratory tract infections is lower in moderately active individuals compared with their sedentary counterparts (42). Although no T cell responses were measured, adequate adaptive immune responses play a critical role in the clearance of viral infections of the respiratory tract (323). The potential importance of adaptive immune responses in relation to exercise and virally-induced cancers cannot be overstated. For example, cervical cancer of which nearly all cases are due to human papillomavirus (HPV) is one of the leading causes of cancer death among women worldwide. However, no studies have examined the effect of exercise on the generation of HPV-specific T cells or the role of exercise in minimizing the immunosuppressive environment created by the presence of the tumour.

If an exercise-induced enhancement of anti-tumour mechanisms occurs, protection should be evident for lymphomas, due to the greater role of immune mediation. Only three studies have examined the relationship between physical activity and Hodgkin's and non-Hodgkin's lymphomas (HL, NHL, respectively). Participation in collegiate sports was associated with a trend to reduced risk of HL, although this did not reach statistical significance (212). Women who participated in strenuous physical activity at various time points in adult life had a lower risk of HL (125). Yet, a case-control study on NHL and occupational physical activity (measured as energy expenditure or sitting time) found no significant association (333).

The hypothesis that exercise-mediated changes in immunity contribute to a reduction in cancer risk is prevalent. For example, women participating in a US national sample believed the causes of breast and colon cancers were due to changes in one's immune system (60% of the sample) and lack of exercise (35-45% of the

sample) (314). Nevertheless this hypothesis is based on limited evidence (168) and many studies have significant methodological limitations (283).

Future directions

Physical activity is beneficial in preventing some cancers, and in decreasing recurrence, increasing survival, and improving quality of life for cancer patients. Multiple biological pathways may be involved, including a reduction in inflammation and an enhancement of anti-tumour immunity. Neither of the aforementioned mechanisms has been studied in adequate detail to gain a full understanding of their role in cancer prevention and therapy with respect to exercise. Inflammatory mediators have many physiological, metabolic and immunological roles and are produced in many tissues. Numerous cell types of the innate and adaptive immune system work in partnership to generate anti-tumour host responses. Additional studies will be needed to determine a) which inflammatory mediators and anti-tumour immune mechanisms are most sensitive to exercise, b) the dose, duration and frequency of exercise needed to achieve anti-inflammatory or anti-tumour effects, and c) the timing of sample collection with respect to the exercise bout to adequately capture appropriate levels of anti-inflammatory mediators and anti-tumour immune mechanisms.

Several technical limitations also need to be addressed. We suggest that the development of more sophisticated animal models is required. Although carcinogen-induced tumours have provided valuable insights, they are limited in that these carcinogens induce mutations at multiple genetic loci (117) and trigger both inflammation and immunosuppression (296). In contrast, spontaneous tumour models which 'mimic' human cancers are often limited to single mutations/pathways (i.e., ras, p53, APC, Wnt) and do not reflect complex multi-gene-environment (exercise) interactions. Additionally, many functional immunoassays require fresh cells and hours of assay preparation. Such immune readouts are difficult in epidemiological studies; while cryoprotectants allow freezing of immune cells for later analysis, viability comparisons to fresh cells are often not performed. Functional immunoassays could be conducted using lymphoid tissue harvested from animals, but relevant preclinical immunogenic tumour models would be required.

Concluding position

There is consensus that exercise training protects against some types of cancers. Training also enhances aspects of anti-tumour immunity and reduces inflammatory mediators. However, the data linking immunological and inflammatory mechanisms, physical activity, and cancer risk reduction remains tentative.

"OMICS" IN EXERCISE

Background and consensus

"Omics" is the circumspanning word for technologies which try to analyze an entire biologic field or large parts of it, using high throughput laboratory methods and correspondingly complex, high end- statistics. Accordingly, analysis by the "Omics approach" is often hypothesis free (non-targeted), and provides extremely

detailed and dense information, with a good chance of detecting unexpected responses or biological pathways. Exercise immunologists hope that "omics" will help them to gain a better understanding of mechanisms related to talent identification, exercise-induced disorders, modulation of the immune system by exercise, and prevention of diseases by exercise training. They also hope that "omics" can be used as a tool for optimizing individual training programmes.

Genomics, proteomics, and metabolomics, the classical three, appeared in this order according to the availability of high-throughput/ high-sensitivity methods. There is also diversification and refocusing into transcriptomics, spliceomics, lipoproteomics, pharmacoproteomics, interactomics, and, notably, exerciseomics. Targets of analysis are the genome itself (alleles, single nucleotide polymorphisms, methylations), gene expression (transcription), post-transcriptional regulation (microRNAs), abundance of proteins or metabolites and isomeric shifts and post-translational modifications.

Results on genome-wide screening for allotypes and single nucleotide polymorphisms associated with performance, fitness, or proneness to disease cannot be considered extensively here. Of special interest for exercise immunology are results on diabetes type-2, where at least 11 genes have been associated with the condition, including peroxisome proliferator-activated receptor delta, which is responsive to types/levels of lipids, and the fat mass and obesity associated (FTO) risk allele, which may not be responsible for reduced physical activity, but effects of which can be attenuated by exercise (see reviews (67, 241)).

To our knowledge, gene expression profiling was applied to exercise first in 2002, with work on rat muscle (39), hippocampus (174), and heart (56). A number of genes related to cell growth, signal transduction, calcium-flux, synaptic trafficking, or myosin light chains were found to be altered, some were new, some corresponding to previous findings, some were contradictory.

In humans, Mahoney et al. (158) defined a row of genes associated with muscle growth, remodeling and stress management following eccentric exercise (sterol and lipid metabolism, insulin and calcineurin pathways, c-myc and jun-D). Thalacker-Mercer et al. (297) exposed young and old adults to moderate exercise-induced muscle damage, and found vast differences in transcript activation, alluding to an undue inflammatory response in older subjects.

As first proposed by Fehrenbach et al. (66), many studies have now used peripheral blood gene expression fingerprinting/clustering for analysis of the effects of exercise. Types of exercise ranged from 30 min at 80% $\dot{V}O_2$max (44) to a half-marathon (334, 335) and heat injury in exercising military recruits (279). Time points chosen and platforms used for analysis also varied widely.

Special questions addressed by intervention or design were the effects of different workloads (29, 124), cell fractionation (183, 239), gender and age (205, 237, 238), as well as comparisons of immune suppressed patients versus healthy individuals (135), with every paper using different challenges and time kinetics.

Genes that were activated or suppressed showed remarkably little overlap between studies and between different times. Nevertheless, a number of pathways involved were identified albeit in different composition. They were related to stress genes and heat shock proteins (29, 44, 205, 279, 335), interferon (279), signal transduction (279, 334, 335), pro- and anti-inflammation (29, 44, 110, 135, 205, 237, 239, 279, 297, 334, 335), anti-oxidative system (334, 335), cell growth and wound healing (44, 237, 239, 297), apoptosis (29, 135, 237-239) and necrosis (297), neurotransmitters (124), immunity with natural killer cell activity (183, 237, 238), antigen processing and receptor signaling (239), asthma (107, 205, 237, 239) and arthritis (239).

MicroRNAs (miRNAs) are a large family of 21-22 nucleotide non-coding RNAs with presumed post-transcriptional regulatory activity. miRNA genes were formerly misperceived as junk-DNA, but are now recognized as important regulators of translation. Drummond et al. (58), Safdar et al. (254), and Radom-Aizik et al. (240) all found a number of miRNAs were increased following exercise and linked to adjustment of inflammation (240, 254). They also found dysregulation of exercise reactive miRNA (primary miRNA up, mature down) in aged subjects (58). An overview is given in Exercise Immunology Review, volume 16 (315).

Proteomics were applied to analyze the effects of exercise on rat heart (28), rat infarcted cerebellum (172), human muscle (108, 114), human plasma (332) and pig lipoproteins (244). Changes in expression of myofibrillar proteins, fatty acid metabolism, novel phosphorylation sites (28), and isoelectric species (114) were identified, shedding new light on the role of post-translational modification of proteins. Anti-inflammatory modification of serum complement through moderate exercise was shown (332), and a novel theory of lipoprotein structure including novel markers for vascular disease was proposed (244).

A rapidly increasing number of studies have analyzed the metabolome in relation to exercise - with circumstantial and limited relations to exercise immunology. Potential biomarkers of strenuous exercise and a strategy for analysis of complex data sets were proposed by Pohjanen et al. (228). Evaluating the effects of nutritive interventions in relation to exercise, subjects could be separated according to type of beverage, training, fitness stage and signs of insulin resistance (41, 142, 170, 331). Dampening of exercise-induced oxidative stress in human erythrocytes by administration of N-acetyl cysteine was shown (142). Finally, a role for endogenous medium chain acylcarnitines in lipid oxidation was proposed (143).

Consensus: "omics" in exercise

- There is a rapid activation and deactivation of genes in peripheral blood even after a short bout of exercise (44).
- Clustering is possible and cellular shifts due to exercise are reflected by the changes in the gene expression profile when using whole blood or peripheral blood mononuclear cells (66, 135, 183, 334).
- Gene expression is workload dependent; a secondary response by different genes is detected up to 24 h following exhaustive exercise only (29, 124, 208).
- Expression is influenced by age, and menstrual cycle (205, 237, 238, 297).

- Gene expression profile differences are in line with pathophysiological findings that could explain exercise-induced asthma (107).
- Immuno-suppressed (renal transplant recipient) patients can perform extensive, exhaustive exercise, showing very restricted gene expression changes (metabolism only), at the same time (135).
- Although gene expression profiling gives valuable information, the effects of miRNAs need to be evaluated (58, 315).
- Proteomics and metabolomics have started to shed new light on the role of isomeric forms and post-translational modification of proteins.
- Metabolomics can identify individuals at risk for diabetes, effects of nutrition and effects of exercise (38, 244, 331).

Controversies and future directions

The "omics" approach so far has had a major impact on knowledge about physiological and pathological processes associated with exercise. An enormous amount of new data has been generated, many pathways involved have been identified, new isoforms detected, and multiple candidates for biomarkers found.

Considering the vast amount of data and the high complexity of analysis applied, it is astonishing and potentially disappointing how little- if any- practical application of "omics" technology exists. There is no doubt that "omics" is generating huge steps in scientific advancement (for example detection of new proteins and metabolites, including isoforms related to lipid metabolism, diabetes type-2, and lipoprotein structure, as well as new biological pathways and gender/menstrual phase dependent gene expression). Practical applications will arise from this, but direct application of "omics" technologies for routine practical purposes (e.g., optimization of individual training/treatment programmes) will require one or more further quantum leaps of technology and yet further increased complexity of analysis. These advances need to be such that they re-simplify proceedings, and analysis will have to integrate knowledge from different levels.

In terms of genome screening for talent and for susceptibility to injury, advances may result from technological developments that will allow easier methods of purification or whole genome sequencing. These technological advances will facilitate access to instructive and sensitive personal data. It is unclear so far how the enormous danger of misuse will be handled. Determination of single factors like alpha actinin (ACTN3) variants – even if used commercially – is largely inefficient. Interaction of many different genes in optimal composition is probably required to make an athletic talent, and at this point, research is only starting. So far, it seems highly unlikely that genomics alone will have the predictive power to screen for gifted athletes (321).

At the level of gene expression, an enormous amount of knowledge about new pathways and marker molecules involved in adaption to exercise has been generated – but as yet there is no assay to answer practical questions (concerning type, intensity and duration of activity for adaptation to specific exercise) during training. Although the technology of gene expression profiling is quite advanced and can be handled in many places, practical application of these technologies is not

thinkable without rigorous standardization procedures and further technological advances (e.g. isothermic amplification). The flow of up- and down-regulation of genes in relation to exercise is so dependent on type, intensity, and duration of exercise and nutritional and conditional factors including gender, that it is highly doubtful if any experiment can ever be repeated by a different lab with identical results – even when using the same platform. So, hotspots and time lines have to be identified in order to make reliable predictions from such data, including integration of, and validation by regulatory mechanisms (miRNA) and post-translational modification, thus requiring proteomics and metabolomics.

The latter two technologies, as powerful as they already seem to be, are only just now starting to explore the potential they really have. At present, exceptionally well-equipped laboratories and highly specialized and experienced experts must meet to enable meaningful proteomics and metabolomics studies. But as the power and potential of this approach emerges, advancements of technologies can be expected in the very near future. They will be combined with genomic and gene expression data and resulting networks will then open new levels of meta-analysis for interpretation. First steps are underway (108), although up to now, a handy little tool for talent search or for individually optimized forms of training, using "omics" type analysis, is not available.

Finally, the "omics" approach on all three classical levels will probably be helpful in identifying misuse of substances or genetic interventions for doping purposes, even though direct or specific detection procedures are often preferred in the fight against doping (11). Work paving the way for "dopeomics" is underway (83, 337).

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Exercise Immunology Meets MiRNAs

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ABSTRACT

A large body of evidence indicates modified expression of protein-coding genes in response to different kinds of physical activity. Recent years have exposed another level of regulation of cellular processes mediated by non-coding RNAs. MicroRNAs (miRNAs) are one of the largest families of non-coding RNAs. MiRNAs mediate post-transcriptional regulation of gene expression. The amount of data supporting the key role of miRNAs in the adaptation of the immune and other body systems to exercise steadily grows. MiRNAs change their expression profiles after exercise and seem to be involved in regulation of exercise-responsive genes in immune and other cell types. Here we discuss existing data and future directions in the field.

Key words: exercise, immunology, miRNA, non-coding RNA, inflammation

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INTRODUCTION

The adaptation to exercise affects virtually all body systems. The immune system is among the systems most responsive to exercise. The regulation of a plethora of physiological processes is now known to be mediated by non-coding RNAs, in particular, miRNAs. These small ~22 nt RNAs are involved in post-transcriptional regulation of gene expression. Estimates are, that more than half of the human protein-coding genes are under miRNA regulation (40), which means that miRNAs are involved in nearly all major mechanisms controlling body processes.

A large amount of recent data demonstrates that miRNAs are essential for the normal activity and development of the immune system (reviewed in (15, 69, 83, 90, 119); see below). Likewise, rapidly increasing evidence indicates a role of miRNAs in the function of skeletal muscle, cardiovascular system, and other body systems. These findings promoted the research on the role of miRNAs in the adaptation to exercise. The field is young: 69 out of 76 publications on "miRNAs and exercise" found in Pubmed in August 2013 were published since 2010. However, the data obtained in this recently started trend suggest that miRNAs play a key role in mechanisms controlling the adaptation to exercise. Exercise rapidly changes the cellular levels of many miRNAs in the immune system, skeletal muscle, and cardiovascular system. These changes modulate the expression of target genes, driving short-term and long-term adaptations.

This review discusses current data on the role of miRNAs in the body adaptation to exercise with a particular attention to the immune system and miRNAs in the bloodstream since they are likely involved in post-transcriptional regulation of gene expression in the cells of the immune system.

MiRNA Biogenesis and Function

MiRNAs are key factors of gene expression that regulate a variety of processes including development, cell proliferation, differentiation, apoptosis, and different metabolic pathways. The number of described miRNAs steadily grows, and the MiRBase currently includes more than 2500 human miRNAs (release 20;(58)). MiRNA genes are located in introns of protein-coding genes, introns and exons of non-coding RNA genes, and occasionally in exons of protein-coding genes (19, 38). They are transcribed by RNA polymerase II (67, 137), although certain miRNA genes downstream of Alu repeats are transcribed by RNA polymerase III (17). The primary transcripts (called pri-miRNAs) contain a ~70 nt hairpin (called pre-miRNA), which includes the miRNA sequence. Pri-miRNA is recognized by the 'microprocessor'complex composed of RNase III Drosha and RNA-binding protein DGCR8, which processes it into pre-miRNA (38, 66, 92). Certain pre-miRNAs localized within short introns are processed by splicing machinery, where the spliceosome and debranching enzyme function as the microprocessor. Such miRNAs are known as miRtrons (107, 136). Surprisingly, miRNAs can be derived from other small RNAs, e.g. tRNAs, Y RNAs, and small nucleolar RNAs (snoRNAs) (30, 78, 97, 115). The transcription and processing of miRNAs are controlled by a variety of cofactors (38, 59).

Exportin 5 mediates pre-miRNA export to the cytoplasm (145), where they are further processed by another RNase III, Dicer, in a complex with the RNA-

binding protein TRBP. The resulting ~22 nt double-stranded molecules associate with one of the Ago family proteins, the main component of the RNA-induced silencing complex (RISC). One of the duplex strands is degraded and the mature miRNA in the RISC complex can interact with the complementary target mRNA (38). Such interaction represses the translation or initiates mRNA deadenylation and degradation (47). MiRNAs also have non-canonical functions: upregulation of mRNA translation (44, 93, 130), transcriptional gene silencing (13, 53), and even signal transduction through the binding to Toll-like receptors, which makes miRNAs a kind of hormones ((31, 32, 68), see below, c-miRNAs.). The biogenesis, functions, and regulation of miRNA activity have been reviewed in detail elsewhere (38, 47, 59, 92, 94).

Short length and imperfect complementarity with mRNA allow each miRNA to have hundreds of targets (10). MiRNA binding sites can be found in the 3'-UTR and occasionally in the 5'-UTR (65, 77, 93) as well as in the coding regions of mRNAs (39). An mRNA can have several binding sites for the same or different miRNAs, which allows complex translational regulation (40).

The majority of miRNAs are expressed in a broad range of tissues; however, some miRNAs demonstrate pronounced tissue-specific profiles (62). At the same time, each cell type has a specific miRNA expression profile. For instance, different leukocyte lineages demonstrate different miRNA expression profiles (11, 63, 98, 134); moreover, several miRNAs specific for the skeletal muscle are even considered as a special subset of miRNAs termed 'myomiRs' (54).

MiRNAs can be found not only in the cell but also in various body fluids such as plasma/serum, milk, saliva, and urine. Such miRNAs proved resistant to nucleases and were called circulating miRNAs (c-miRNAs). They have been identified in apoptotic bodies, shedding vesicles, and exosomes as well as in complex with high-density lipoprotein (57, 126). However, a big part of c-miRNAs can be found in the vesicle-free fraction in complex with Ago proteins (7, 127). There is conflicting information on the proportion of free versus vesicle-bound miRNAs (41, 127). The mechanisms of c-miRNAs emergence and their possible physiological role still remain matters of speculation (16). *In vitro* experiments demonstrated that exosomes secreted by one cell type can be absorbed by other cell types, where the exosomal miRNAs can modulate gene expression (126). This proposes c-miRNAs as a new type of messengers that allow distant cells to communicate in the body. This is particularly significant for immune cells since most of them continuously interact with body fluids.

C-miRNAs proved to be good markers for many pathologies and physiological conditions (16). In particular, plasma levels of certain c-miRNAs change after physical activity (8, 9, 110, 128). This is of both, fundamental and practical interest and can be used for functional assessment and injury diagnosis of organs after exercise of different intensities (see below, c-miRNAs). Thus, c-miRNA monitoring can be widely used in sports medicine.

MiRNAs and Immune System

The amount of data on the role of miRNAs in immune functions rapidly grows (1199 out of 1549 publications on "miRNAs and immune system" found in Pubmed in August 2013 were published since 2010). To date, the function of dozens of miRNAs has been determined and their key role in the regulation of

immune system development and function has been established. Below we describe the major progress in the field, while the interested reader can consult specific reviews (15, 37, 69, 83, 90, 119).

The critical importance of miRNAs for the immune function was demonstrated in a series of works in which miRNA biogenesis was affected. For instance, a specific deletion of Dicer in a T-cell lineage impaired T cell development and T helper differentiation and induced autoimmune diseases (71, 85, 148). The ablation of Dicer in early B cell progenitors suppressed the transition from pro-B to pre-B cells (56). Specific deletion of Dicer in activated B cells induced multiple abnormalities in the immune response to pathogen and impaired germinal center B-cell formation (141). Natural killer (NK) cells with Dicer deletion demonstrated impaired maturation, survival, turnover, and other defects (14, 120). Thus, miRNAs are required for normal development and function of the immune system.

To date, miRNA expression profiles are available for many cell types of innate and adaptive immune system: monocytes/macrophages, dendritic cells, neutrophils, eosinophils, NK cells, and different T and B cell types (2, 11, 33, 34, 63, 74, 82, 98, 106) Out of more than 2500 described human miRNAs (58), 150-600 species can be usually identified in each cell type of the immune system, 20 or 30 of which are the most abundant (11, 74, 82, 135). Immune cells have specific miRNA expression profiles, which can change during cellular development (11, 82, 106); and the concentrations of the same miRNA species at different differentiation stages can vary by a factor of 1000 (55). Interestingly, new miRNAs are still being described in the immune system (11, 34, 98).

Simultaneously with the large-scale transcriptome studies, individual miRNA species are being actively explored with focus on their function in specific cell types of the immune system (reviewed in (15, 72, 117)). MiRNAs proved to be involved in a variety of cellular pathways so that they seem to mediate all significant events in immune cells. For instance, miRNAs control the differentiation of naive T-helper cells into Th1, Th2, Th17, T-regulatory, and follicular helper T cells (T_{fh} cells) (114). In particular, the normal differentiation of T_{fh} cells requires the miRNA cluster miR-17-92 (12, 51). MiRNAs also mediate the maintenance of naive T-helper cells in undifferentiated state. MiR-125b is more abundant in naive T-helper cells than in other T cells and suppresses the expression of genes underlying the differentiation of naive T-helper cells (namely, interferon-γ , interleukin (IL)-2 receptor-β, IL-10 receptor-α, and transcriptional repressor Blimp-1), and thus maintains the undifferentiated state of these cells (106).

Interestingly, miRNA processing can change as the immune cells develop. For instance, the sequences of mature miRNAs can be shifted by one or two nucleotides at different stages of T cell development. In particular, the nucleotide sequence of miR-17 expressed in DN3 thymocytes corresponds to the canonical one, while the same miRNA expressed in DN4 and DP thymocytes is shifted 3' by one nucleotide. Cells of all other stages expressed miR-17 that was shifted 3' by two nucleotides. These shifts indicate different processing of pre-miR-17 in the course of T cell development (55). Since the seed region (the region completely complementary to the mRNA target nucleotides 2 to 7/8) (10) is short, even a single-nucleotide shift changes two thirds of the predicted miR-17 targets and the seed sequence becomes identical to that of miR-302a or miR-106a (55).

Thus, alternative miRNA processing during T cell development contributes extra variation to the regulation of miRNA targets.

The range of biochemical pathways controlled by miRNAs in immune cells is particularly wide. For instance, miRNAs control antigen presentation (e.g., miR-148/152 (75)), T-cell receptor signaling (e.g., miR-181a (70)), Toll-like receptor signaling (e.g., let-7e (4)) and cytokine production (e.g., miR-146a (43)). MiRNAs are required for normal proliferation of activated lymphocytes; for instance, miR-182 promotes clonal expansion of activated T helper cells (118) and miR-181b decreases excessive DNA damage accompanying somatic hypermutation and class switch recombination in activated B cells, thus preventing their malignant transformation (25). MiRNAs are involved in both inflammatory and anti-inflammatory responses (90).

Notably, cellular miRNAs can have an effect on the viral life cycle through the regulation of viral genome replication, while viral miRNAs in turn can have an effect on the host cell (95). Different immune abnormalities, in particular, malignant and autoimmune diseases, demonstrate altered miRNA profiles, which can point to the contribution of miRNAs to the development of these diseases (26, 96).

MiRNAs and Response of Peripheral Blood Leukocytes to Exercise

Publications from two groups have demonstrated that acute exercise changes the expression profile of many miRNAs in circulating leukocytes (100, 101, 102, 125). In a series of studies Dr. Dan Cooper and coworkers used miRNA microarrays to study the changes in miRNA expression in untrained subjects immediately after brief bouts of heavy exercise in circulating neutrophils (102), peripheral blood mononuclear cells (PMBCs: T, B, and NK cells and monocytes) (101), and circulating NK cells alone (100). In all cases, expression profiles changed for 20-40 miRNAs (Table S1). Considering that leukocytes express several hundreds of miRNA species (11, 74, 82, 135), this finding supports the specificity of the observed response. Note that most of these miRNAs are not among the most abundant species in neutrophils and PMBCs (88, 131, 134, 135). The authors also linked their miRNA data to corresponding exercise-induced mRNAs by identifying potential mRNA targets for each miRNA and selecting those which were also changed. Resulting biochemical pathways are considered candidates for being under the control of exercise-induced miRNAs. In neutrophils they include ubiquitin-mediated proteolysis, Jak-STAT signaling, and Hedgehog signaling. All these pathways mediate inflammatory response (102). Twelve pathways have been identified in PBMCs including TGF-β signaling and MAPK signaling (101). Exercise-activated pathways in NK cells are predominantly associated with cancer and cell communication: p53 signaling, melanoma, glioma, and prostate cancer, as well as adherens junction and focal adhesion (100).

Unfortunately, parallel expression data on miRNA and mRNA were only available in the NK cell study, while the data for neutrophils and PBMCs were obtained in separate experiments, which can compromise the authenticity of the identified miRNA-mRNA pairs. Moreover, different experimental procedures (ten 2-minute bouts of cycle ergometer exercise for miRNA assay and 30 minutes of constant cycle ergometer exercise for mRNA assay) were used in the study on neutrophils, and the experiments with PBMCs were carried out on individuals of

different age (men with a mean age of 22 years and late pubertal boys with a mean age of 17 years). Nevertheless, in sum, these findings are at least compatible with the assumption that many exercise-induced mRNA changes are under the control of miRNAs.

Attempting to increase the depth of understanding, our group analyzed whole blood samples taken from highly trained athletes after a 30-minute treadmill test at 80% maximal oxygen uptake (moderate test, MT) (125). The whole blood approach allows fast, precise timing and minimizes artifacts. Samples were analyzed for miRNA and mRNA before and immediately after exercise, as well as 30 minutes, and 60 minutes into recovery. This allowed us to identify four dynamically regulated networks with four differentially expressed miRNAs and their validated mRNA targets. All of them displayed anti-correlated expression profiles for both, immediate post-exercise time point and recovery period. These miRNAs included miR-21 and its targets TGFBR3, PDGFD, and PPM1L; miR-24-2 and its targets MYC and KCNJ2; miR-27a and its target ST3GAL6; as well as miR-181a and its targets ROPN1L and SLC37A3 (125). All target genes are involved in processes highly relevant to exercise response including immune function, apoptosis, membrane traffic of proteins, and transcription regulation. These data are in good accordance with the findings by Cooper's group and support the assumption that miRNAs regulate key pathways of the immune response to exercise.

The number of differentially expressed miRNAs was higher in the studies by Cooper's group (several dozens) as compared to our study (five) (125). Possible reasons for this discrepancy are numerous, ranging from different numbers of subjects, different exercise procedures, different microarray systems, different cell populations and work up procedures to different fitness level of subjects. Indeed, the microarray used by our group contained four times less miRNAs, but, nevertheless, did contain two thirds of those identified by Cooper's group. Some other reasons also need to be discussed in more details.

Use of whole blood may mask mild expression changes in minor leukocyte populations like NK cells, and can also cause changes through cellular shifts, including subpopulations (125). On the other hand, analysis in isolated cell populations (100, 101, 102) is influenced by manipulation and time delay inherent to the sorting procedure. While this may still suggest that true exercise-induced miRNA changes do exist, we have to acknowledge that even within isolated cell populations, shifts in subpopulations do occur (also discussed in (100, 101, 102)) and may be responsible for a substantial part of the expression changes observed. At present this question cannot be unequivocally answered. We do however believe that the dynamic regulation of mRNA-miRNA pairs as shown in (125) would be hard to explain by a cellular shift. But finally, no matter, if shifts in leukocyte populations / subpopulations are involved or not, miRNA (and of course mRNA) expression data do mirror the actual activation status of the peripheral blood and therefore deliver valuable biological information.

While exercise procedures differed between the two groups (100, 101, 102, 125), the total duration (30 minutes) and intensity (80 vs. 76-77% of VO_2 max) were somewhat comparable. However, the highly trained athletes investigated by our group did not cross the anaerobic threshold while the less trained probands investigated by Cooper's group, did. Crossing the individual anaerobic threshold

(IAT) is associated with major physical stress, which might require the induction of more and different miRNA species than work just below the threshold. Trying to get more pertinent information on this question we decided to perform an additional ramp test to exhaustion (RTE, as described in (109); duration ~15 minutes/including 4-5 minutes above IAT) with the same athletes that had performed the 30-minute moderate test (MT) (125). Whole blood samples collected before and immediately after the test were analyzed for miRNA expression (as described in (109)). Results are given in Table 1 together with results from the MT (recalculated for 2 time points, disregarding recovery, in order to create optimal comparability between our data sets and the results described by Cooper`s group).

Table 1. MiRNAs differentially expressed in whole blood leukocytes before and after exercise tests. MiRNA species with a 1.5-fold or greater expression difference are boldfaced

MiRNA name and fold change[1]	Experiment participants	Exercise type	Time points of blood sampling
mir-24 ↑2.0 **mir-27a ↑1.5** **mir-181b ↑1.5** mir-23a ↑1.3	8 highly trained male athletes with a mean age of 21.7±2.6 years	Moderate test: 30 min treadmill running at 80% VO2 max (as described in (109, 125).	Immediately before and after exercise test
mir-181a ↑1.5 **mir-181b ↑1.5** **mir-101 ↓1.5** mir-142 ↓1.4 mir-29a ↓1.4 mir-124 ↓1.3 mir-29c ↓1.3 mir-223 ↓1.2 mir-30d ↑1.2 mir-130b ↓1.2		Ramp test to exhaustion: 15 min treadmill test with an incremental step protocol until exhaustion (as described in (109)).	

[1] Only the miRNA genes with significant differential expression are listed (false discovery rate < 0.05). The miRNA set for the MT is not identical to that published previously (125) since the samples collected 30 and 60 min after exercise were excluded.

Indeed, expression of a greater number of miRNA species was altered in RTE than in MT. To our knowledge, this is the first report that miRNAs in peripheral leukocytes can change in such a short time following any external stimulus. Numbers of expressed miRNAs in RTE were, however not excessive and clearly less than those identified in the studies by Cooper's group (which also included work above IAT). We think that, apart from crossing the anaerobic threshold, the mere fact that our group investigated highly trained athletes may also make a difference. Trained athletes are known to be able to regulate their body functions more efficiently than non-athletes, be it above or below IAT. They may therefore generally need less miRNAs to be induced than non-athletes.

One more observation we made when analyzing our results may be of interest: three of the four miRNAs found elevated after MT, namely, mir-23a, mir-24-2, and mir-27a, belong to the same cluster. They are processed from a common precursor and often coregulated (22). The members of this cluster are involved in a plethora of biological processes including haematopoiesis, angiogenesis, cell proliferation, and cardiac hypertrophy (reviewed in (22)). Analysis of their target gene set suggests the involvement of this cluster in several immune-related pathways, e.g. T-cell receptor signaling, and TGF-beta pathways (22). It will be very interesting to unveil the function of this cluster in directing the exercise response in peripheral blood leukocytes.

The sets of differentially expressed miRNAs differ between leukocyte types; however, certain miRNAs proved common for two or three sets (Table 2). Notice that the direction of changes can be opposite in some cases. For instance, miR-223 level increased in neutrophils but decreased in NK cells (Table 2). This could indicate different interpretation of the same external signal by different cell types either upregulating or downregulating the same gene; however, as in other cases, shifts in subpopulations cannot be excluded with certainty.

It is of interest that quite some exercise-induced miRNAs identified in leukocytes demonstrated a similar response in the skeletal muscle, heart, or plasma(Table 2). Moreover, only a few out of hundreds of miRNA species coincided in all microarray studies, which might indicate a critical role for them in adaptation to exercise. For instance, Keller et al. identified 21 miRNA species expression of which changed after exercise in muscle (52), and seven of them demonstrated differential expression in leukocytes, too (Table 2). Although the direction of changes was different in some cases and the sets of their targets likely differ in leukocytes and muscle, these miRNAs can be assumed to be involved in the universal adaptive response to exercise.

Data on the role of individual miRNAs in specific immune functions are increasing but still vastly fragmentary. Thus, a comprehensive discussion of possible roles of indentified miRNAs is difficult. Still, miRNAs of the miR-181 family - miR181a and miR181b - may be worthwhile discussing in more detail, since their differential expression was observed in all our tests in athletes as well as in two out of three tests in non-athletes (101, 102), (Table 2).

Since miR-181 expression was affected in different leukocyte types irrespective of the exercise intensity and training level (Table 2), its involvement in the regulation of some fundamental adaptive changes in the immune system seems likely. MiR-181a suppresses the inflammatory response induced by oxidized low-density lipoprotein in dendritic cells (139), and miR-181b suppresses the NF-kappa B-mediated inflammatory response in endothelial cells *in vivo* (121). On the other hand, Xie et al. demonstrated increased levels of miR-181a in whole blood leukocytes during the early inflammatory response, and proposed that miR-181a upregulation can compensatorily limit hyperinflammatory reactions (140). In a similar way, exercise-induced miR-181 may be interpreted as compensatory anti-inflammatory reaction to primary inflammatory stimuli caused by exercise. Indeed, exercise-induced immune reactions used to be viewed as primary inflammatory reactions followed by anti-inflammatory counter-reactions. This has recently been challenged (oral communication, ISEI meeting, Newcastle, Australia, 2013, and Asghar Abbasi, *Brain Behavior and Immunity*, in press).

Table 2. Overview of miRNAs changed in two or more cell types after exercise. Human, mouse, and rat miRNAs are prefixed with hsa-, mmu-, and rno-, respectively, following the nomenclature adopted in miRBase (58). WBL - whole blood leukocytes, FC - fold change.

MiRNA	Leukocytes, FC				Muscle	C-miRNA
	WBL	Neutr.[1]	PBMC[2]	NK[3]	FC, Muscle name, Ref.	FC, Ref.
hsa-miR-7			↑1.4	↑2.2		
hsa-miR-15a hsa-miR-15b			↑1.3		 ↓1.6 (Vastus lat.;(52))	
hsa-mir-16 rno-mir-16		↓1.23			 ↓1.36 (Soleus;(36))	
hsa-miR-21* hsa-miR-21 mmu-mir-21			↑1.5		 ↑2.3 (Gastrocn.;(5))	 ↑2.6(8)
hsa-mir-23a mmu-mir-23	↑1.3 MT				 ↓6.2 (Quadriceps;(108))	
hsa-miR-26a hsa-miR-26b			 ↑1.2		↓1.8 (Vastus lat.;(24)) ↓1.6 (Vastus lat.;(52))	
hsa-miR-27a rno-mir-27a	↑1.5 MT				 ↑2.0 (Heart;(35))	
hsa-miR-29a rno-miR-29a hsa-miR-29b hsa-miR-29c rno-mir-29c	↓1.4 RTE ↓1.3 RTE			↑2.0 ↑3.5 ↑2.3	↓2.0 (Vastus lat.;(24)) ↑1.5 (Heart;(116)) ↓1.6 (Vastus lat.;(52)) ↑2.18 (Heart;(116))	
hsa-miR-30d hsa-miR-30e	↑1.2 RTE			 ↑2.1		
hsa-mir-101	↓1.5 RTE				↓2.0 (Vastus lat.;(52))	
hsa-miR-107 mmu-mir-107		↓1.26			 ↑1.56(Quadriceps;(108))	
hsa-miR-125a		↑1.22	↓1.3		↑1.6 (Vastus lat.;(52))	
hsa-miR-126		↓1.53	↓1.3	↓3.2		↑4.0(128)
hsa-miR-130a hsa-mir-130b	 ↓1.2 RTE	↓1.61	↓1.2	↓2.9		
hsa-miR-142	↓1.4 RTE			↑2.5		
hsa-miR-145		↑1.22	↓1.3			
hsa-miR-151-5p		↓1.60	↓1.3	↓2.8		
hsa-miR-181a hsa-miR-181a2* hsa-mir-181b mmu-mir-181	↑1.5 RTE ↑1.5MT,RTE	 ↑1.64	↑1.4 ↑2.0 ↑1.7		 ↑1.37 (Quadriceps;(108)	 ↑1.5(9)
hsa-miR-199a-3p hsa-miR-199a-5p			↓1.3 ↓1.3	↓3.1 ↓2.9		
hsa-miR-221			↓1.2	↓2.1		↑5.8(8)
hsa-miR-223	↓1.2 RTE	↑1.29		↓2.9		
hsa-miR-338			↑1.4	↑2.2	↓1.6 (Vastus lat.; (52))	
hsa-miR-363		↓1.34	↑1.5	↑2.1		
hsa-mir-451			↓3.8		↓4.0(Vastus lat.; (52)) ↑4.0(Vastus lat.; (24))	
hsa-miR-652			↓1.2	↓2.2		

[1]according to (102); [2]according to (101); [3]according to (100)

It was proposed that the reaction to exercise may be more of a direct, preemptive anti-inflammatory reaction induced by IL-6 or other mediators, including miRNAs, and miR-181 is certainly a candidate for playing a critical role in such an immediate anti-inflammatory response to exercise.

MiR-181 expression also increased in muscle after acute endurance exercise (108) and during regeneration (86). MiR-181 is known to repress the repressor (Hox-A11) of MyoD and thus mediates myoblast differentiation and muscle regeneration (86). Finally, miR-181 also shows increased plasma levels following acute exercise (9). Together with its protective, anti-inflammatory impact all this prompts us to propose that miR-181 may have a central, multiple role in the adaptation to exercise.

MiRNA and Skeletal Muscle Response to Exercise

Muscle and vascular function are not the focus of this review. Nevertheless, we like to highlight some current facts and findings in this field, honouring the fact that all exercise-related gene expression probably starts in the muscle.

Although more than 150 miRNA species are expressed in the muscle (81, 122), up to 25% of muscle miRNA population correspond to just a few muscle-specific miRNAs (81) (miR-1, miR-133, miR-206, and miR-499), collectively called myomiRs; myomiRs also include less abundant miR-208 and miR-486 (54). Thus, most studies in the field are focused on the identification of changes in the expression of myomiRs (largely miR-1, miR-133, and miR-206) after various forms of exercise. Indeed, adaptation to exercise proved to affect myomiR levels. Somewhat unexpectedly, changes were, however, only moderate in general and even undetectable in some of the studies (Table S2). There is a trend towards increased levels of miR-1, and miR-133 during acute endurance exercise (89, 108) and decreased levels with chronic endurance exercise (52, 89) or with resistance exercise (27, 80, 84), (Table S2). A central function of myomiRs, in particular miR-1, is obviously long term regulation / adaptation of protein synthesis and muscle size. Validated targets of miR-1 include components of the insulin-like growth factor 1(IGF1) pathway (29), and the decrease of miR-1 with chronic endurance exercise and with both, acute and chronic resistance exercise, is therefore a plausible adaptation to the increased need for protein synthesis and muscle growth/regeneration. As mentioned above, significant changes of myomiRs were not seen in all experiments. In a study with high responders and low responders to resistance exercise (low muscle mass gain), no significant changes in expression of myomiRs was observed. Instead, some non-myomiRs (mir-451↑, miR-378↓, miR-29a↓, and miR-26a↓) were changed in low responders only, possibly in a compensatory effort (24).

Age-related loss of muscle function is accompanied by hampered transition of the miR1 precursor pri-miR-1 to miR-1 and by a failure to modify mature miR1 expression in response to a single bout of resistance exercise in elderly men (27). On the other hand, decreased miR1 expression was demonstrated in elderly men following prolonged resistance training (84). Thus, elderly men may just need more time for adaptive changes to occur. In any case, age-related changes can at least partly be relieved by training.

MyomiRs also control the expression of key myogenic transcription factors and regulators such as Pax3, and Pax7 during satellite cell proliferation and dif-

ferentiation (reviewed in (147)), and injection of myomiRs into injured rat muscle could accelerate regeneration (87). Thus myomiRs seem to have control functions in both, adaptation to exercise and regeneration of muscle.

A row of studies has investigated the consequences of enforced physical inactivity, using different experimental settings. MyomiRs miR-1, miR-133, miR206 (Table S2), mir208b and miR499 and some others were down-regulated in response to inactivity (3, 49, 81, 104), and miR208b and miR499 seem to be involved in the slow (type 1) to fast (type 2) fibre switch which accompanies muscular atrophy (81). Altogether results in this field are inconsistent and sometimes contradictory, likely due to different experimental settings. Spontaneous reinnervation following denervation and replacement of muscle fibres by fibroblasts are some of the problems encountered in those experiments.

Intriguingly, compared to myomiRs, a number of non-myomiR miRNAs showed much more pronounced differential expression in response to exercise (Table S3). Identified reactive miRNAs include both, species of low (e.g. miR-183 and miR-189 (113)) and high (e.g. miR-23 (113)) abundance. Since they are not muscle-specific, they seem to control vital functions which muscle cells share with some or all other cells. Low-level transcripts are fairly often involved in crucial processes like cell division. Thus this might also be the case for exercise modulated low-level transcripts. On the other hand, non-muscle-specific high abundance miRNAs may well be involved in fast adaptive metabolic reactions to contractile activity. Adaptation of miRNA expression to chronic endurance or resistance training shares some changes and differs in others. Three out of four miRNAs differentially expressed after resistance training, namely miR-26, miR-29, and miR-451 (24) were also changed after endurance training (52). While miR-26 and miR-29 decreased in both, miR-451 was increased after resistance and decreased after endurance exercise. Thus, there are common exercise-related and specific exercise type-related regulations of miR expression, which, together with myomiRs, can form the necessary network to govern adaptation and regeneration.

Exploration of the adaptive significance of individual miRNAs is still in its infancy. Existing data point to a role of miR-696, miR-23 and miR-494 in mitochondrial biogenesis through different pathways (5, 108, 144). PCG1 (Peroxisome proliferator-activated receptor-gamma coactivator-1 alpha) is a critical factor of mitochondrial biogenesis and a predicted target for miR-696 and miR-23 (5, 108). Its regulation by miR-696 was also confirmed by hyperexpression experiments (5). Transcription factors TFA and Foxj3 which also control mitochondrial amplification are validated targets for miR-494 (144).

When looking closer to those miRNAs which show high or very high expression changes due to exercise, we realized that they are often species-specific, taxon-specific or are synthesized via non-canonical ways (unpublished observation). MiR-616 seems to be human/primate-specific while miR-680, miR-696, miR-705, and miR-709 are mouse-specific (MirBase; http://www.mirbase.org, Table S3). MiR-720 is presumably synthesized from a tRNA (111). Recent data indicate that tRNAs can give rise to shorter miRNA-like molecules which are involved in gene silencing and most actively synthesized during stress (115). MiR-680 is encoded in the LTR of mouse retrotransposon ERVB4 (according to the annotation of the genome browser at the University of California,

http://genome.ucsc.edu). The significance of these findings is not clear at present. Possibly it has to do with the need for fast action which is associated with physical activity. It is also possible that the small size of miRNAs makes them a perfect tool of nature to gain species-specific adaptation to exercise out of a pathway which is common to all mammals. In any case, a significant fraction of exercise-associated miRNA expression changes is observed in species-specific or non-canonically synthesized miRNAs.

Chronic exercise is associated with decreased blood pressure, increased capillary numbers and physiological hypertrophy of the heart. Studies on the role of miRNAs in these adaptational processes have just started. All were conducted in rats submitted to 10 weeks of moderate training (swimming) (23, 35, 36, 116). First results indicate massive involvement of miRs in cardiac hypertrophy. 87 of 349 miRs studied were altered, among these, miR-1 and mir-133 (decreased) and members of the miR-29 family (increased) (116). These changes promote muscle fibre growth (myomiRs) and decreased collagen synthesis (miR-29). Increases in miR-27 and miR-126 and decrease in miR-16 may have critical roles in angiogenesis by targeting negative regulators of vascular endothelial growth factor (VEGF) pathway (miR126), and by reducing miR-16 dependent inhibition of angiogenesis (VEGF, its receptor VEGFR2 and FGF receptor 1 are all validated targets of miR16) (23, 36). MiR-27 probably targets angiotensin converting enzyme (ACE), and this may lead to decreased blood pressure (35). Finally, miR-27 also regulates the inflammatory response (20). Altogether, the results in this field need confirmation to prove that they are indeed an adequate mirror of vascularization.

MiRNAs Might Contribute to the Beneficial Effect of Exercise in Different Diseases

Exercise is known to be beneficial in a plethora of diseases. Recent data increasingly indicate the involvement of miRNAs in the beneficial effect of exercise. For instance, increased expression of miR-21 and decreased expression of miR-15a was observed in rats with spinal cord injury after post-injury cycling exercise. It was accompanied by the corresponding changes in the expression of their target genes: the mRNA levels of proapoptotic genes PTEN and PDCD4 decreased, while that of anti-apoptotic factor Bcl-2 increased (73).

Spontaneously hypertensive rats demonstrated increased levels of miR-16 and miR-21 and decreased level of miR-126 in the soleus muscle relative to control. Some of their targets showed anti-correlated expression. Exercise training normalized the expression of these miRNAs to levels similar to controls (36). Exercise training also normalized the expression of certain targets of these miRNAs; for instance, the levels of anti-apoptotic factor Bcl-2 and proangiogenic factor VEGF (targeted by miR-16) increased (36).

Microarray analysis of the whole blood in patients with coronary arterial disease after coronary artery bypass graft surgery and exercise rehabilitation program demonstrated increased expression of miR-92a and miR-92b. At the same time, the mRNA level of the respiratory chain component NDUFA1 and proapoptotic factor CASP3, which are predicted targets of these miRNAs, decreased (124).

Thus, miRNAs are obviously mediators of antiapoptotic and proangiogenic

effects of exercise. At present we do not know how these findings relate to exercise-induced lymphocyte apoptosis.

Circulating MiRNAs and Exercise

Emerging data indicate changes in the blood levels of c-miRNAs after exercise (see Table S4). Uhlemann et al. evaluated human plasma concentrations of miR-126 and miR-133 before and after different exercises and found elevated concentrations of these miRNAs following a marathon (miR-126 and miR-133) and after resistance exercise (miR-133) (128). Banzet et al. likewise found increased levels of miR-133 and other myomiRs (miR-1, miR-208b, and miR-499) in plasma after downhill walking (9), Table S4. Thus, increased plasma levels of these miRNAs may be used as markers for injury of muscle (miR-133) and endothelial cells (miR-126). Mir-133 may serve as a convenient replacement of creatine phosphokinase (CPK), while miR-126 could be the first available marker for endothelial damage (128).

It should be noted that not all published data on the subject are in agreement. For instance, Sawada et al. (110) observed no changes in the serum level of myomiRs in humans after acute resistance exercise, while Uhlemann et al. (128) reported myomiR differential expression after a similar exercise. Different percentage of eccentric load, different time points, and different numbers of probands can explain this inconsistency.

Apparently, c-miRNA concentrations change after exercise not only through cell damage. Changed plasma levels of miR-149*, miR-146a, and miR-221 were demonstrated three days after resistance exercise, while levels of myomiRs remained unaltered (110). Further, increased plasma levels of c-miRNAs miR-181b and miR-214 in absence of elevated myomiRs or CPK were reported immediately after uphill exercise (9). This suggests that the increased levels of these miRNAs resulted from active secretion rather than cell damage. Baggish et al. studied exhaustive exercise tests before and after a 90-day period of rowing training (8). Eight miRNAs involved in angiogenesis, inflammation, muscle contractility, and adaptation to hypoxia were analyzed. Four patterns of c-miRNA response to exercise have been revealed. (i) The levels of miR-146a and miR-222 increased after acute exhaustive exercise before and after sustained exercise training; (ii) miR-21 and miR-221 levels increased after acute exhaustive exercise only before sustained exercise training; (iii) miR-20a level increased after sustained exercise training but not after acute exhaustive exercise; and (iv) miR-133a, miR-328, and miR-220 levels remained unaltered after all tests (8), (Table S4). The unchanged levels of miR-133a likely indicate the absence of muscle damage, while the different patterns of c-miRNA response observed point to the existence of different control mechanisms not associated with cell damage.

C-miRNA quantification can be used to predict the risk of cardiovascular diseases. Microarray data show that healthy individuals with low maximal oxygen uptake have increased levels of circulating miR-21, miR-210, and miR-222 (18). Low maximal oxygen uptake is indicative of a predisposition to cardiovascular diseases (138); accordingly, the identified miRNAs can serve as convenient noninvasive markers of risk for these diseases (18).

Thus, the first studies in the field demonstrated that exercise-induced changes in plasma/serum levels of c-miRNAs can result from both, cell damage

or independent mechanisms. The contribution of cell damage seems to increase with exercise load (128). Rapid and substantial (several-fold) increase in c-miRNA levels after intense exercise in the absence of tissue damage markers in plasma (8) can indicate the peak release of previously synthesized miRNAs into the bloodstream, while stable changes in c-miRNA levels after long-term exercise training suggest modified basal expression and/or secretion of miRNAs.

Table 3. C-miRNAs described in relation to exercise and possible relevance for the immune system.

MiRNA name, fold change and source		Presumed immune-related effects[a]	Number of hits[b]
miR-1	↑4.0 (9)	Anti-inflamm., anti-astmathic, anti-prolif.(42, 76, 123)	16
miR-20a	↑ 3.0 (8)	Unknown	0
miR-21	↑ 2.6 (8)	Both pro- and anti-inflamm., oncogenic (32, 60, 91, 99, 142)	93
miR-126	↑4.0 (128)	Pro-angiogenic; anti-inflamm., pro-astmathic (pro-Th2) (6, 79, 103, 105)	29
miR-133	↑8.9 (128), ↑4,8 (9), NC (8)	Unknown; found in inflammatory vesicles (46)	2
miR-146a	↑3.0 (8), ↓2.0 (110)	Anti-inflamm.; possible counterpart of mir-21 in regul. of inflamm. (21, 50, 112, 143)	17
miR-149*	↑2.3 (110)	Unknown (rare passenger strand of anti-inflamm. mir-149)	0
miR-181b	↑1,5 (9)	Anti-inflamm., controls autoimmunity (48, 132)	4
miR-208	↑11,5 (9)	Unknown	0
miR-214	↑1,8 (9)	Prolif. stim, pro-oncogen. (105, 146)	6
miR-221	↑ 5.8 (8) ↓ 2.0 (110)	Anti-inflamm., anti-angiogenic (28, 45, 129)	13
miR-222	↑2.4 (8)	Anti-inflamm. (105)	9

[a] as judged from described effects of corresponding cellular miRs (personal interpretation of literature data)

[b] hits found in PubMed search for name of miRNA and inflammation

NC = no change

In most cases, the cell source(s) of c-miRNAs remain unclear. The bulk of miRNAs are expressed in several cell types albeit at different rates (62). For instance, miR-133 is abundant in the skeletal muscle and heart but also detectable in the brain (61) and brown adipose tissue (133). MiR-126, typical of endothelium, is also expressed in the liver, haematopoietic cells, and some other tissues (62). Accordingly, direct identification of the c-miRNA source is nearly impossible except in case of overt cell damage (64). Further studies on the mechanisms of miRNA secretion and the nature of circulating miRNA-containing complexes may help to address these questions.

To date, the effect of altered c-miRNA concentrations on body systems remains largely unclear. Possible effects can be deduced from validated targets of these miRNAs; in the case of exercise, these are largely associated with muscle function, angiogenesis, inflammation, and oxidative stress, i.e., the processes primarily affected by exercise. Here, the question, how circulating miRNAs may find and enter

their target cells, needs to be discussed. Studies mentioned above evaluated c-miRNAs without considering their carrier. The nature of the carrier (high-density lipoprotein-particles, exosomes, apoptotic bodies, shedding vesicles or vesicle-free Ago protein bound miRNAs) may dramatically influence the cell/organ-specific targeting and even biological effects.

To our knowledge, at present, no clear proof is available to show which biological effects can be attributed to exercise-induced c-miRNA expression. In spite of all the imponderabilities mentioned above, it seems, however, reasonable to assume, that they have identical or similar effects as their cellular counterparts. On this basis, Table 3 presents the presumed effects of exercise-induced c-miRNAs as judged from literature data with focus on immune related effects. The majority of exercise-induced miRNAs has predominantly anti-inflammatory effects rather than inflammatory ones. Since exercise-induced gene (mRNA) expression also has a remarkable anti-inflammatory bias (1) this concordance makes likely that the protective generalized reaction of the body to exercise is organized by help of miRNAs or co-organized together with hormones at a very early stage.

Intriguingly, it has recently been shown that c-miRNAs can also induce biological effects without directly interacting with mRNA. Tumour cells can use extracellular miRNAs as ligands to Toll-like receptors (TLR7 in mice and TLR8 in human) of macrophages and thereby modulate the immune response to their favor (31, 32, 68). Although, up to now, this new and exciting hormone-like mechanism has only been demonstrated for cancer and for neurodegenerative processes, it may well be functional under physiological conditions as well. Modulation of immune functions by exercise is one of the fields to be investigated in this context.

Altogether we are convinced that the future will see c-miRNAs as useful markers for exercise-related damage or malfunction and will also expose an important biological role for them in exercise immunology.

Concluding Remarks and Future Directions

Recent data indicate that miRNAs are an essential element in the adaptation of the immune system and other systems to exercise. Progress in the field requires flanking progress in related fields, namely, detailed data on the role of each miRNA in the function of different cell types. Such data are actively generated now, and since miRNAs are a hotspot in molecular biology, further data expansion can be expected in the nearest future. The improvement of technical approaches is also desirable, in particular, rapid sorting of blood cells and preservation of isolated RNA. RT-PCR is widely used in miRNA studies discussed here but it allows only a small number of miRNA species to be detected; in the cases when microarray technology is used, microarray data files are not always made publicly available. Wide application of large-scale screening methods and publication of comprehensive microarray data will undoubtedly accelerate the progress in exercise immunology.

As of now, only limited numbers of exercise physiology studies involving miRNAs have been carried out on human and animal groups of different size, age, and sex, using different exercise designs, all of which makes correct comparison of results and data meta-analysis largely impossible. Small sample size in the

studies and small changes in the studied parameters (often below 50%), coupled with significant individual variation in gene expression levels, open the door to misinterpretations. In this context, the development of uniform study designs by the scientific community is of primary importance.

Other classes of non-coding RNAs can also be expected to mediate the reaction of our immune system to acute or chronic exercise. For instance, well-studied tRNAs unexpectedly proved to mediate stress responses (115), and exercise is of course known to be a kind of stress. Moreover, the involvement of another group of non-coding RNAs, snoRNAs, in the reaction of leukocytes to exercise has recently been demonstrated by our group (109). We expect many more exciting discoveries about the role of miRs & Co in exercise immunology.

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Table S1. MiRNAs differentially expressed in different leukocyte types before and after exercise test. MiRNA species with a 1.5-fold or greater expression difference are boldfaced.

Leukocyte type	Neutrophils		PBMC		NK cells
MiRNA name and fold change	**hsa-miR-485-3p ↑2.92** **hsa-miR-520d-3p ↑2.82** **hsa-miR-181b ↑1.64** **hsa-miR-130a ↓1.61** **hsa-miR-151-5p ↓1.60** **hsa-miR-1238 ↑1.58** **hsa-miR-193a-3p ↑1.58** **hsa-miR-1225-5p ↑1.55** **hsa-miR-126 ↓1.53** hsa-miR-20a ↓1.24 hsa-miR-106a ↓1.39 hsa-miR-20b ↓1.29 hsa-miR-17 ↓1.40 hsa-miR-93 ↓1.24 hsa-miR-130b ↓1.41 hsa-miR-16 ↓1.23 hsa-let-7i ↓1.25 hsa-miR-107 ↓1.26 hsa-miR-185 ↓1.28 hsa-miR-18a ↓1.35 hsa-miR-18b ↓1.29 hsa-miR-194 ↓1.26 hsa-miR-22 ↓1.28	hsa-miR-363 ↓1.34 hsa-miR-660 ↓1.23 hsa-miR-96 ↓1.28 hsa-miR-125a-5p ↑1.22 hsa-miR-145 ↑1.22 hsa-miR-197 ↑1.38 hsa-miR-212 ↑1.44 hsa-miR-223 ↑1.29 hsa-miR-340* ↑1.25 hsa-miR-365 ↑1.37 hsa-miR-505 ↑1.24 hsa-miR-629* ↑1.44 hsa-miR-638 ↑1.36 hsa-miR-939 ↑1.40 hsa-miR-940 ↑1.39	**hsa-miR-451 ↓3.8** **hsa-miR-181a* ↑2.0** **hsa-miR-181b ↑1.7** **hsa-miR-486-5p ↓1.7** **hsa-miR-363 ↑1.5** **hsa-miR-1225-5p ↑1.5** **hsa-miR-21* ↑1.5** hsa-miR-181a ↑1.4 hsa-miR-181c ↑1.2 hsa-miR-338-3p ↑1.4 hsa-miR-26b ↑1.2 hsa-miR-132 ↑1.3 hsa-miR-15a ↑1.3 hsa-miR-939 ↑1.3 hsa-miR-7 ↑1.4 hsa-miR-140-5p ↑1.3 hsa-miR-940 ↑1.3 hsa-miR-125b ↓1.4 hsa-let-7e ↓1.4 hsa-miR-320 ↓1.2 hsa-miR-151-5p ↓1.3 hsa-miR-31 ↓1.3 hsa-miR-125a-5p ↓1.3	hsa-miR-99b ↓1.4 hsa-miR-652 ↓1.2 hsa-miR-151-3p ↓1.3 hsa-miR-130a ↓1.2 hsa-miR-126 ↓1.3 hsa-miR-199b-3p ↓1.3 hsa-miR-23b ↓1.2 hsa-miR-221 ↓1.2 hsa-miR-199a-5p ↓1.3 hsa-miR-584 ↓1.3 hsa-miR-145 ↓1.3	**hsa-let-7e ↓2.6** **hsa-miR-126 ↓3.2** **hsa-miR-126* ↓2.9** **hsa-miR-130a ↓2.9** **hsa-miR-151-5p ↓2.8** **hsa-miR-199a-3p ↓3.1** **hsa-miR-199a-5p ↓2.9** **hsa-miR-221 ↓2.1** **hsa-miR-223 ↓2.9** **hsa-miR-326 ↓2.3** **hsa-miR-328 ↓2.3** **hsa-miR-652 ↓2.2** **hsa-miR-142-3p ↑3.0** **hsa-miR-142-5p ↑2.5** **hsa-miR-192 ↑2.2** **hsa-miR-29a ↑2.0** **hsa-miR-29b ↑3.5** **hsa-miR-29c ↑2.3** **hsa-miR-30e ↑2.1** **hsa-miR-338-3p ↑2.2** **hsa-miR-363 ↑2.1** **hsa-miR-590-5p ↑2.6** **hsa-miR-7 ↑2.2**
Experiment participants	Eleven healthy men 19–30 years old of average fitness (non-athletes)		Twelve healthy men with a mean age of 22±1 years of average fitness (non-athletes)		Eleven healthy men 20–29 years old of average fitness (non-athletes)
Exercise type	20 min of exercise consisting of 10 2-min bouts of constant work rate cycle ergometry with a 1-min rest interval between each bout (total test time is 30 min) at 76-77% peak VO2				
Time points of blood sampling	Immediately before and after exercise test				
Reference	(102)		(101)		(100)

Table S2. Differential expression of myomiRs in the skeletal muscle after different exercise types and forced immobility.

Exercise type	MiRNA name[1] and fold change			Species	Experiment participants	Muscle studied	Exercise modality	Experiment duration	Time points of expression analysis	Reference
	miR-1	mir-133a	mir-206							
Chronic endurance	↓1.5	↓1.3	↓2.0	Human	10 trained males with a mean age of 30.5 years	Vastus lateralis	Cycle ergometer	12 weeks	Before and 3-5 days after training	(89)
	↓1.6	↓1.9	NC[2]	Human	24 sedentary males with a mean age of 23 years	Vastus lateralis	Cycle ergometer	6 weeks	Before and one day after training	(52)
	NC[3]	NC	NC	Mouse	8-week-old mice	Gastrocnemius	Treadmill running	4 weeks	Experimental and control mice were analyzed one day after experiment	(5)
Acute endurance	↑ 1.3	↑1.4	NC	Human	10 trained males with a mean age of 30.5 years	Vastus lateralis	Cycle ergometer	60 min	Before and immediately after exercise	(89)
	↑1.4	NC	ND	Mouse	7 mice of 4 month old in experimental and control groups each	Quadr. femoris	Treadmill running	90 min	Experimental and control mice were analyzed 3 h after exercise	(108)
Resistanse	↓1.7	NC	NC	Human	6 sedentary males with a mean age of 29 years	Vastus lateralis	Leg extension+essential amino acid solution	8 sets of 10 repetitions	Immediately before and 3 h after exercise	(27)
	↓1.3	ND	ND	Human	14 males and 13 females with a mean age of 80.1 years	Vastus lateralis	Resistance exercise or eccentric ergometer sessions	12 weeks	Before and 2-3 days after training	(84)
	NC	NC	NC	Human	17 untrained males of18-30 years old	Vastus lateralis	Rotating split-body resistance training program	12 weeks	Before and 2 days after training	(24)
	↓2.0	↓2.0	NC	Mouse	10-week-old mice	Plantaris	*In vivo* model of muscle hypertrophy	1 week	Experimental and control mice were analyzed after experiment	(80)

Resting muscle	↓1.1	↓1.1	ND	Human	12 physically active males with a mean age of 26.2 years	Vastus lateralis	Bed rest	7 days	Before and after experiment	(104)
	NC[3]	NC	NC	Mouse	14-week-old mice	Gastrocnemius	Hind limb fixation	5 days	Fixed and free limb muscles	(5)
	NC	NC	NC	Rat	3 6-month-old rats in two experimental and one control groups, each	Soleus	Hind limb suspension	2 and 7 days	Experimental and control rats were analyzed after experiment	(81)
	NC (trend toward a decrease)	NC (trend toward a decrease)	↓2.0	Mouse	4 77-day-old mice in experimental and control groups each	Gastrocnemius	Space flight	~12 days	Experimental and control mice were analyzed ~4 h after landing	(3)
	↓~3.3	↓~3.3	NC	Rat	Adult animals	Soleus	Denervation	4 weeks	Experimental and control rats were analyzed after experiment	(49)

[1]The data on the most often studied myomiRs are presented.
[2]The data on miRNAs with a 1.5-fold or greater expression difference are reported in the original paper.
[3]The data on miRNAs with a twofold or greater expression difference are reported in the original paper.
NC, no change
ND, not determined

Table S3. MiRNA species with most pronounced expression changes in muscle after exercise. Texan-specific miRNAs are boldfaced.

MiRNA name	mir-183	mir-189	mir-432*	mir-451	mir-589	**mir-616**	mir-21	**mir-696**	**mir-709**	mir-720	mir-23	mir-451	**mir-680**	**mir-696**	**mir-705**	mir-762
Fold change	↑2.0	↑2.0	↑2.3	↓4.0	↓3.5	↑2.0	↑2.3	↓2.9	↓2.3	↓2.0	↓6.2	↑4.0	↑2.3	↑2.1	↑2.1	↑2.1
Reference	(52)						(5)				(108)	(24)	(5)			
Exercise type	Chronic endurance										Acute endurance	Resistance	Immobilization			

Table S4. Differential expression of miRNAs in human plasma and serum after exercise.

MiRNA name	Fold Change	Experiment participants	Exercise type	Time points of expression analysis and c-miRNA source	Reference
miR-1	↑4.0	9 recreationally active males of 27-36 years old	30-min downhill walking	Before and 6 h after exercise; plasma	(9)
miR-20a	↑ 3.0	10 competitive male athletes with a mean age of 19.1±0.6 years	Rowing for 90 days	Before and after 90 days of exercise training; plasma	(8)
miR-21	↑ 1.9	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance exercise (cycle ergometer) **prior** to sustained exercise training	Before and immediately after exercise; plasma	(8)
	↑ 2.6	10 competitive male athletes with a mean age of 19.1±0.6 years	Rowing for 90 days	Before and after 90 days of exercise training; plasma	(8)
miR-126	↑3.4	22 male marathon runners with a mean age of 56.8±5.2 years	Marathon race	Before and immediately after exercise; plasma	(128)
	↑2.0	13 healthy individuals (7 males and 6 females) with a mean age of 30.4±2.0 years	Single symptom-limited exercise test	Before and 5 min after exercise; plasma	(128)
	↑4.0	13 healthy well trained males with a mean age of 32.4±2.3 years	Cycle ergometer for 4 h below the anaerobic threshold	Before and immediately after exercise; plasma	(128)
	NC	11 trained subjects (4 males and 7 females) with a mean age of 37±2 years	Singular resistance training with additional eccentric loads (lat pulldown, leg press and butterfly)	Before and immediately after exercise; plasma	(128)
miR-133	↑8.9	22 male marathon runners with a mean age of 56.8±5.2 years	Marathon race	Before and immediately after exercise; plasma	(128)
	NC	13 healthy individuals (7 males and 6 females) with a mean age of 30.4±2.0 years	Single symptom-limited exercise test	Before and 5 min after test; plasma	(128)
	NC	13 healthy well trained males with a mean age of 32.4±2.3 years	Cycle ergometer for 4 h below the anaerobic threshold	Before and 1 h after exercise; plasma	(128)
	↑2.1	11 trained subjects (4 males and 7 females) with a mean age of 37±2 years	Singular resistance training with additional eccentric loads (lat pulldown, leg press and butterfly)	Before and immediately after exercise; plasma	(128)
	NC	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance and chronic endurance (90 days of rowing) exercises	Before and immediately after exercises; plasma	(8)
	↑4.8	9 recreationally active males of 27-36 years old	30-min downhill walking	Before and 6 h after exercise; plasma	(9)
miR-146a	↑3.0	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance exercise (cycle ergometer) **prior** to 90-day exercise training	Before and immediately after exercise; plasma	(8)
	↑3.0	10 competitive male athletes with a mean age of 19.1±0.6 years	Rowing for 90 days	Before and after 90 days of exercise training; plasma	(8)
	↑7.5	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance exercise (cycle ergometer) **after** 90-day exercise training	Before 90-day exercise training and immediately after acute exercise; plasma	(8)
	↓ 2.0	3 physically active males	Acute resistance exercise (bench press and bilateral leg press)	Before and 3 days after exercise; serum	(110)
miR-149*	↑2.3	3 physically active males	Acute resistance exercise (bench press and bilateral leg press)	Before and 1 day after exercise; serum	(110)

miR-181b	↑1.5	9 recreationally active males of 27-36 years old	30-min uphill walking	Before and immediately after exercise; plasma	(9)
miR-208	↑11.5	9 recreationally active males of 27-36 years old	30-min downhill walking	Before and 6 h after exercise; plasma	(9)
miR-214	↑1.8	9 recreationally active males of 27-36 years old	30-min uphill walking	Before and immediately after exercise; plasma	(9)
miR-221	↑ 3.6	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance exercise (cycle ergometer) **prior** to 90-day exercise training	Before and immediately after exercise; plasma	(8)
	↑ 5.8	10 competitive male athletes with a mean age of 19.1±0.6 years	Rowing for 90 days	Before and after 90 days of exercise training; plasma	(8)
	↓ 2.0	3 physically active males	Acute resistance exercise (bench press and bilateral leg press)	Before and 3 days after exercise; serum	(110)
miR-222	↑2.5	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance exercise (cycle ergometer) **prior** to 90-day exercise training	Before and immediately after exercise; plasma	(8)
	↑2.4	10 competitive male athletes with a mean age of 19.1±0.6 years	Rowing for 90 days	Before and after 90 days of exercise training; plasma	(8)
	↑4.0	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance exercise (cycle ergometer) **after** 90-day exercise training	Before 90-day exercise training and immediately after acute exercise; plasma	(8)

NC, no change

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Gender- and menstrual phase dependent regulation of inflammatory gene expression in response to aerobic exercise

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ABSTRACT

The immunological reaction to exercise has been investigated with increasing intensity in the last 10-20 years, with most human studies performed in male subjects. Recently, gender-specific aspects have received growing attention, but studies carefully monitoring the influence of gender, including the menstrual cycle, are rare. Here, we report gene expression patterns in response to a run at 93% of the individual anaerobic threshold of 9 women with regular menstrual cycles and no use of oral contraceptives who ran both at day 10 (follicular phase, F) and at day 25 (luteal phase, L) of their cycle. 12 male subjects (M) served as controls. The mRNA was pooled group wise and processed on a gene expression microarray encompassing 789 genes, including major genes of the inflammatory and anti-inflammatory reaction. The differences of gene expression between time points t_0 (before run) and t_1 (after run) were analyzed. Females in L showed a higher extent of regulation than females in F or men. Among those genes which were up-regulated above 1.5 fold change (log_2) pro-inflammatory genes were significantly enriched ($p=0.033$, after Bonferroni correction) in L, while this was not the case in F or M. Conversely, women in L showed a strong trend towards down-regulation of anti-inflammatory genes. Some prominent genes like IL6 (coding for interleukin-6), and IL1RN (also termed IL1RA, coding for interleukin-1 receptor antagonist) were clearly regulated in opposite directions in L as opposed to F and M.
In conclusion, women in L showed a distinctly different pattern of gene regulation in response to exercise, compared with women in F or M. The overall direction of

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gene expression changes of women in L is clearly pro-inflammatory. This finding accentuates a need for careful consideration of the female cyclic phase when investigating women in exercise immunology studies. Our results may also have implications relevant to other forms of stress in females.

Keywords: gender, inflammation, gene regulation, aerobic exercise, menstrual cycle, stress response, IL6, IL1RN, IL1RA.

INTRODUCTION

Recent studies have documented that significant gender dimorphisms exist in certain immune responses to different types of exercise (6, 15, 27-29). Gender differences in response to exercise have clear implications for understanding gender-specific adaptations to exercise for athletic performance and overall health. However, while in general the impact of exercise on immune functions has received considerable and increasing attention in recent years, it is still unclear to what extent gender and fluctuations in sex hormones influence immunological responses to exercise.

Several gender-related differences in immune function under non exercise conditions have been identified, and it has been hypothesized that at least some of these differences could be attributed to female sex hormones (7). Numerous clinical studies have demonstrated that immune responsiveness is greater in women than in men (7): women have lower incidence and mortality to several types of infections (7), higher serum concentrations of some immunoglobulins (IgM) (12), a higher absolute number of T-helper lymphocytes (1), and a differential regulation of cytokine production (12, 14). Leukocyte chemotaxis (7) is also sensitive to gender related hormones. Mitochondria from females generate smaller amounts of hydrogen peroxide than those of males and have higher levels of mitochondrial reduced glutathione and antioxidant enzymes (26). Several menstrual cycle associated effects on parameters of the immune system have been described. Compared to the follicular phase (F), the luteal phase (L) of the menstrual cycle was associated with increased concentrations of leukocytes and lymphocyte subsets (5, 9), increased prostaglandin (PG) E2 and PGI2 release by stimulated monocytes (3, 11, 25), a greater capacity of immune cells to produce cytokines (5, 9, 13), a higher plasma cytokine activity (14), but variable effects on plasma cytokine levels (2, 8, 13). In contrast, other studies associate the follicular phase with greater cytokine production from immune cells (14) and higher serum IL-6 levels (2).

The fact that the majority of exercise studies has been done in males does not really come as a surprise. However, in situations where a new hypothesis has to be proven or disproven for the first time, it may be a forgivable or even a wise concept to start off with males only to avoid unforeseeable interferences from fluctuations of sex hormones occurring in women depending on the different phases of their menstrual cycle. Even worse than that, we know that in competitively training female athletes the cycle is often disturbed or abolished. In addition many females take oral contraceptives which again can have an impact on immunological functions as well (27). Thus, it can be tedious and not very easy to find well

defined and willing groups of female volunteers to do meaningful studies. Nevertheless we think that time has come to do exactly that.
A number of studies have reported no differences in cell counts and functions (4, 16-19, 31), plasma cytokine levels (16, 30), and lymphocyte apoptosis (20) between men and women concerning the response to different kinds of exercise. However, it appears that these studies did not control for the menstrual status of the women at the time of testing. In contrast to studies reporting no differences, others have reported gender differences in the immune-related responses to treadmill running (5), cycling (8, 27-29) and eccentric exercise (15, 25). In a recent study (Fehrenbach et al. unpublished), we found out that intracellular HSP70 showed gender and menstrual cycle dependent reactions in lymphocytes and monocytes 24 h after exercise. Timmons et al. (2005) have reported gender and menstrual cycle dependent changes in leukocyte and cytokine responses to cycling (27).
In the present study we used mRNA from the above mentioned HSP study to run a microarray analysis on 789 genes, which were partly selected on the basis of their relation to inflammatory processes. The study had a group of regularly menstruating women who ran twice, once on day 10 (follicular phase) and once on day 25 (luteal phase) of their menstrual cycle and a group of males for comparison.
The first results of this investigation focusing on the differences in gene expression immediately after compared with before a 1 h run close to the individual anaerobic threshold are presented here.

MATERIAL AND METHODS

Subjects

Twelve female (W) and 12 male runners (M) gave informed consent to participate in the study. The investigation was approved by the University Ethics Committee. All were experienced athletes with normal dietary habits. They were not on any medication and they performed endurance training on a regular basis. The W included in the study had regular menstrual cycles and did not use oral contraception. Determination of the cyclic phases was based on a diary, kept by the women, beginning three months prior to the study. To confirm the cyclic phases, the hor-

Table 1: Anthropometric and physical characteristics of the subjects.

	Men (n=12)	Women (n=9)
Age (yrs)	32.6 (28.7 – 36.4)	29.68 (25.4 – 33.7)
Body mass index (kg· m^2)	21.6 (20.9 – 22.3)	20.9 (19.9 – 22.0)
Training sessions (1 · $week^{-1}$)	5.8 (5.3 – 6.2)	4.4 (3.8 – 5.1)*
Training distance (km · $week^{-1}$)	60.8 (53.9 – 67.7)	38.9 (28.6 – 49.2)*
V_{IAT} (km · h^{-1})	14.0 (13.4 – 14.5)	11.8 (11.1 – 12.5)*

V_{IAT}, running velocity at the individual anaerobic threshold. Data are presented as means (95% CI). *p<0.01, men vs. women

monal status of W was determined by measuring oestrogen, progesterone and LH using the ADVIA Centaur immunoassay system (Siemens Healthcare Diagnostics, Fernwald, Germany). After hormonal assessment, three women had to be withdrawn from the study due to luteal insufficiency. The physical characteristics of the remaining athletes are shown in Table 1.

Preliminary Testing

Before participating in the main study the athletes performed an incremental exercise test on a treadmill (Saturn, HP Cosmos, Traunstein, Germany) to determine the running velocity (V_{IAT}) at the individual anaerobic threshold (IAT). Capillary blood for lactate measurement (EBIO, Eppendorf, Hamburg, Germany) was obtained from the earlobe after every stage and heart rate was monitored continuously using a heart rate monitor (Polar Electro, Finland). V_{IAT} was calculated by the method of Dickhuth (23) using a PC-routine.

Continuous runs

The main investigation consisted of continuous runs (CR) on the treadmill with duration of 60 min and a running velocity corresponding to 93% V_{IAT}. The exercise procedure started at 09:00 a.m. The W had to perform the identical CR twice: once in the follicular phase (F) of their cycle at day 10 and once in the luteal phase (L) of their cycle at day 25. Capillary blood lactate was determined before and immediately after exercise. Venous blood samples were drawn one hour before (t_0; 8:00 a.m.) and immediately after the end of the CR (t_1; 10:00 a.m.).

PBMC isolation and RNA extraction

EDTA anti-coagulated venous blood samples were used for the isolation of peripheral blood mononuclear cells (PBMC) using the Ficoll-hypaque density gradient technique as described previously (10). After gathering the cells in RLT-buffer total RNA was extracted using an RNeasy minikit (Qiagen, Hilden Germany) in accordance with the manufacturer's protocol. The RNA from M (n=12) and W (L/F; n=9) was pooled using equal amounts of RNA for the corresponding runs for t_0 and t_1. The integrity of extracted RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California, USA).

Microarray data generation and statistical analysis

Microarray data were generated using 65mer oligonucleotide microarrays produced at the IKET, University of Tübingen as previously described (33). We used a 2,402 feature array including transcripts as well as buffers, controls and empty spots. The genes on the array were selected inter alia with a focus on inflammation and regulation of inflammatory processes. Every feature was printed at least in duplicate. The array contained 789 genes in total, while some transcripts were contained up to 12 times in duplicate. For further details of the array used in this study can be obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/) under accession number GPL5676.

An indirect reference design was used with Cy3 labeled uniRNA (Stratagene, La Jolla, California, USA) and Cy5 labeled sample RNA. Amplification of sample RNA was performed using Ambion´s Amino Allyl Message Amp II aRNA Amplification Kit (Ambion Inc., Austin, Texas, USA) together with Amersham CyDye

Post-labeling Reactive Pack (GE Healthcare, Buckinghamshire, UK) following the manufacturer`s protocols, and assessing dye incorporation using a Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). After an aRNA fragmentation using Ambion´s Fragmentation reagents (Ambion Inc., Austin, Texas, USA) hybridization was performed for 14 h at 48°C. Subsequently, the hybridized and washed slides were scanned in a microarray scanner (Affymetrix Inc. Santa Clara, California, USA). The photomultiplier tube voltage was set to 100% for both green and red channels. The resulting green and red images were overlaid using ImaGene 5.0 (Biodiscovery Inc. El Segundo, California, USA) as well as for raw data collection.

Data analysis

Further statistical and bioinformatic analysis was performed using the limma (Linear models for microarray) package for R from the bioconductor project (24). The data was normalized using printtip-loess intra-array normalization on the normexp-background corrected expression values followed by inter-array quantile normalization across groups. For further analysis, normalized expression values of duplicate features were averaged. For the different pools (F, L, and M) the fold change ($fc=t_1-t_0$) between $\log_2$ expression values of both time points was computed. On the basis of the fold changes, up-regulated genes ($fc > 1.5$) and down-regulated genes ($fc < -1.5$) were determined. 81 different genes from the array with clearly pro-inflammatory impact and 43 different genes with clearly anti-inflammatory impact were selected for a closer analysis (see addendum). For both pro-inflammatory and anti-inflammatory gene sets and each group, the number of genes exceeding the respective fold change thresholds between t_0 and t_1 was calculated. For significance testing, the same number of genes contained in the respective set was sampled 10,000 times and the fraction of genes exceeding the threshold p value was calculated. A gene set with $p < 0.05$ was considered significantly enriched. Tests were omitted if no genes of the set exceeded the threshold. The result of the analysis of the above mentioned gene sets encompassing the pro- inflammatory and anti-inflammatory genes are listed in the addendum. We are aware that due to pooling the RNA, no classical significance testing could be performed. To control the false-positive rate, we used rather conservative thresholds, requiring absolute fold changes of at least 1.5 ($\log_2$) for genes to be considered significantly regulated.

The raw microarray data is available in GEO (http://www.ncbi.nlm.nih.gov/geo/).

Table 2: Resting hormone levels in women and pre- and post-exercise lactate concentrations.

		Men (n=12)	Women, F (n=9)	Women, L (n=9)
Estrogene (pmol · l^{-1})	Pre-CR	n.d.	370 (111 – 629)	491 (296 – 687)
Progesterone (nmol · l^{-1})	Pre-CR	n.d.	4.0 (1.9 – 6.1)*	23.8 (15.0 – 32.6)
Blood lactate (mmol· l^{-1})	Pre-CR	0.9 (0.7 – 1.2)	1.0 (0.9 – 1.1)	0.9 (0.7 – 1.0)
Blood lactate (mmol· l^{-1})	Post-CR	2.1 (1.5 – 2.6)+	2.4 (1.6 – 3.3)+	2.6 (2.1 – 3.0)+

Data are presented as means (95% CI). F, follicular phase; L, luteal phase; n.d., not detected. *p<0.01, women, F vs. women, L; + p<0.01, post-CR vs. pre-CR. There were no significant differences between F, L and M.

RESULTS

The treadmill runs were performed at a speed which corresponded to 93% V_{IAT}. At the end of exercise, blood lactate concentrations were significantly increased in all groups, but still remained in a range typical for more intensive but still predominantly aerobic exercise. No significant differences were detected between M, F and L (see table 2).

Statistical analysis

The enrichment analysis yielded one enriched gene set. In group L, we found the pro-inflammatory genes enriched among the up-regulated genes (p= 0.0017, after Bonferroni-correction for 10 tests: 0.017).

In general, L showed a high degree of regulation having 129 genes up–regulated and 143 down-regulated, compared with F (48 / 32) and M (34 / 29). This was especially pronounced in the gene sets specifically selected for their strong relation to inflammation. From the 81 genes judged as pro-inflammatory, 20 stood out to be regulated above the mentioned threshold of 1.5 (log_2). Of these, 13 were up-regulated and 7 were down-regulated. 17 of the anti-inflammatory genes were regulated above the threshold, of which 6 were up-regulated and 11 down-regulated. In M and F, much lower regulation was observed (see figure 1).

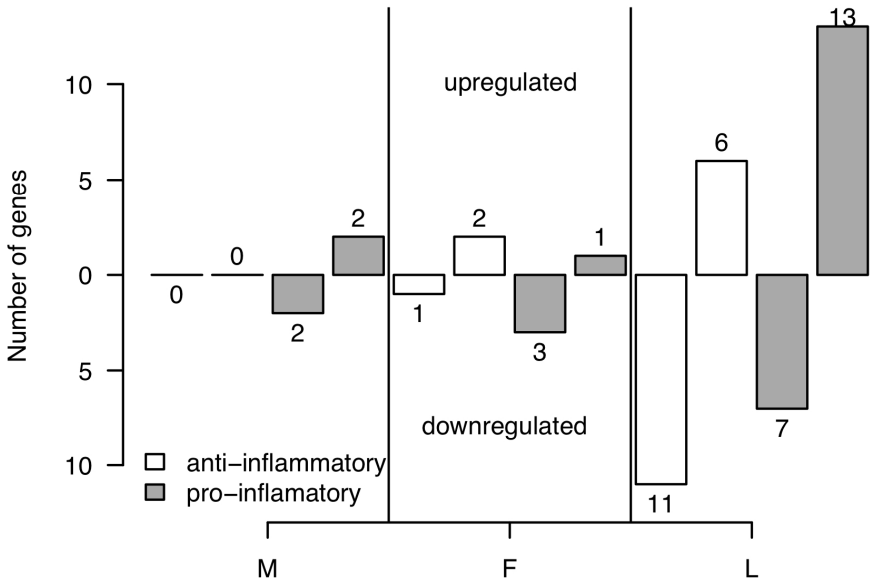

Figure 1: Major changes in expression of anti- (white) and pro (black)- inflammatory genes (see addendum) in the three groups. Bars pointing upwards denote up-regulated genes; bars pointing downwards denote down-regulated genes. A threshold of +/- 1.5 (log_2) was used (see addendum).

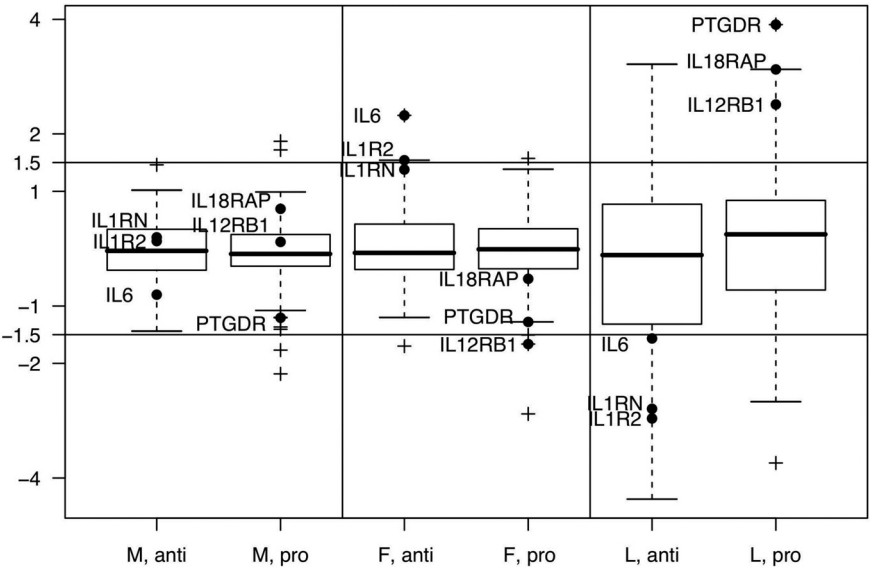

Figure 2: Box plots of $\log_2$ fold change for the selected gene lists, separately for each group. Genes of special interest were marked at their respective positions in the respective boxplot, + denotes outliers (below or above +- 1.5* interquartile range) not considered in this context. For the marked pro- and anti-inflammatory genes, we observe a strong inverse regulation. Note that the variances for both gene sets differ significantly between L and M or F (F-test p value < 10^-9).

By arbitrarily setting another cutoff at $\log_2$ 1.0 (up-regulated (*fc* > 1.0) or down-regulated (*fc* < -1.0)) in either direction, 35 genes from the pro- inflammatory and 25 genes from the anti-inflammatory subset came up in L. Little changes were detected in M (9 / 4) and F (9 / 6).

When aligning the detected genes, according to their pro-inflammatory impact on the one hand (pro-inflammatory genes up-regulated/ anti-inflammatory genes down-regulated) and to their anti-inflammatory impact on the other hand (pro-inflammatory genes down-regulated / anti-inflammatory genes up-regulated), a strong pro-inflammatory response was revealed in L (see figure 1). Neither in F nor in M was a comparable regulation observed.

For some genes of either set, a strong inverse regulation was detected. This was especially pronounced for the anti-inflammatory genes IL6, the decoy receptor interleukin 1 receptor type II (IL1R2) and IL1RN, which were up-regulated during exercise in F, while consistently down regulated in L (see figures 2 and 3). IL1RN codes for the IL-1 receptor antagonist. In the literature the expression IL1RA is used synonymously for the gene. Furthermore, we found several pro-inflammatory genes, including prostaglandin D2 receptor (PTGDR), interleukin 18 receptor accessory protein (IL18RAP) and interleukin-12 receptor beta 1 (IL12RB1) to be down-regulated in F, while strongly up-regulated in L (see figure

2). Some of the remaining genes of both sets exhibited a similar pattern of regulation. A comparable inverse regulation, into the opposite direction (pro- inflammatory impact in F, and anti-inflammatory impact in L) was exhibited by only one anti-inflammatory gene, adrenergic receptor beta 2 (ADRB2) which was down-regulated in F but up regulated in L (for further information see addendum).

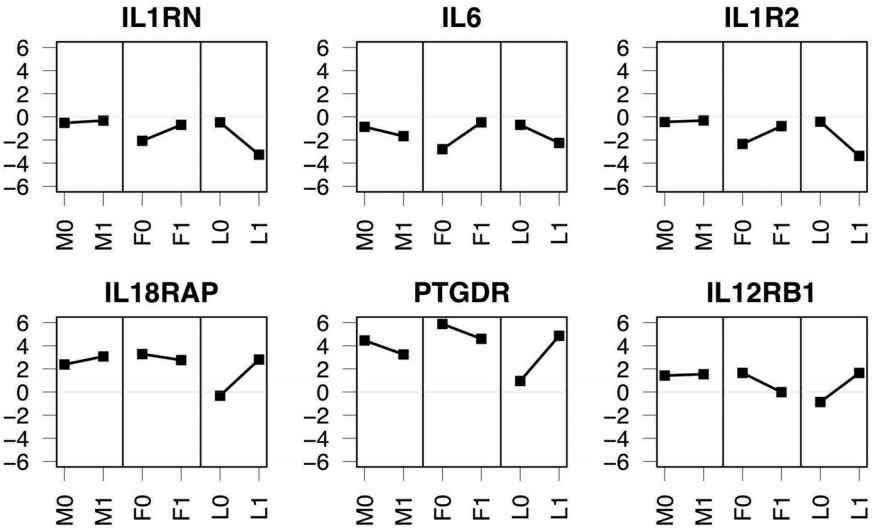

Figure 3: Profile plots for selected pro-inflammatory genes (upper row) and anti-inflammatory genes. The plots show expression values for t_0 and t_1 for each group. The abscissa shows the expression value.

DISCUSSION

Among mammals, very few things are regulated with such a high species-specificity as reproduction. Obviously there is enough flexibility built into this area of physiology to enable each species to adjust optimally to its needs. Conception susceptibility of females decides if newborns arrive all together in spring (typical for favored victims of predators) or several times during the year (like in dogs) or every few weeks (rodents). Human females are disposed to essentially all year long readiness for sexual activity with frequent and regular periods of conception susceptibility.

The situation as described makes animal experiments very tricky to translate to the human situation. Nevertheless, the findings of Nickerson et al. (21), that female rodents did not show elevated myocardial heat shock proteins (HSP) after exercise stress, while males did, prompted us to run a study designed to explore the reaction of HSP to exercise in controlled relation to the female menstrual cycle. To our surprise, females showed strikingly different patterns of regulation, depending on the phase of their menstrual cycle. While at d10 (F), they regulated

HSP upwards (like males), at d25 (L) they regulated downwards (unpublished data). The observation, that the human females seem to take out an important cell protective system during L in reaction to stress induced us to run a gene expression chip analysis focused on genes anyhow related to inflammation or protective anti-inflammatory regulation.

In essence we found an impressive coordinated movement of genes in the direction of a pro-inflammatory impact. It is intriguing – and also reassuring -- that this movement was a combined action of pro-inflammatory genes being up-regulated and anti-inflammatory genes being down-regulated. Although only the pro-inflammatory up-regulation was significant, the down-regulation showed at least a very strong trend and importantly encompassed some key markers which we know from numerous studies as reactive to exercise. Central markers of the protective regulations following exercise like IL6, IL1RN (coding for interleukin receptor 1 antagonist, see addendum) and IL1R2 were significantly down-regulated in L, while they were significantly or borderline significantly up-regulated in F (see figure 2). HSPB (coding for HSP 27), a central gene in the HSP system followed essentially the same pattern. Likewise, important pro-inflammatory genes like PTGDR, IL18RAP, arachidonate 5-lipoxygenase (ALOX5) or IL12 (see addendum) were highly significant up-regulated in L, while they were down-regulated in F. Concerning ALOX5, a gender specific secretion pattern of leukotrienes, governed by androgens, via regulation of extracellular signal related kinases (ERKs) has recently been found (22).

The overall number of genes which were significantly regulated following the exercise challenge underlines the exceptional state of the organism in the luteal phase with females regulating 200+ genes in L while in F only about 70 genes were regulated, similar to the number in males (60).

The question regarding what is behind these striking cycle dependent differences is not easy to answer. It seems safe to say, that, immediately after one hour of exercise, (t_0-t_1) there is a substantial change in gene expression in the direction of an increased pro-inflammatory state in women in the luteal phase. It is also highly likely, that this has to do with reproductive function of women. In the uterine endometrium of adult women a steady increase in the expression of important pro-inflammatory cytokines has already been shown starting in the mid luteal phase and continuing up to the very late luteal phase (32). However, this situation might be different in PBMCs. What we do not know is:

(a) Whether the observed effect is the same at other time points of the luteal phase or whether it is specific for the last few days of the cycle;
(b) Whether the regulation on the mRNA level is accompanied by coordinated translation into the corresponding proteins.

Concerning (a), further analysis of different time points of the cycle should show if the observed phenomenon is characteristic throughout the luteal phase. If not, the observed reaction could rather be understood as something that is related to the initiation of menstruation.

Concerning (b), further studies have to be done to find out to what extent the observed gene expression changes are accompanied by corresponding changes in protein expression. Analysis of serum proteins will be necessary and helpful, but not necessarily sufficient to clarify this point. Fast clearance by the kidney or degradation is likely to occur and might blur the picture. Experiments measuring intracellular, membrane, or ex vivo released proteins will probably be necessary.

There were some indications that a part of the pro-inflammatory genes which were up-regulated in L had quite a low level of expression at rest. Vice versa, part of the anti-inflammatory genes which were down-regulated in L, came from quite high levels of expression at rest. It is therefore possible that the gene expression changes seen in reaction to exercise in L may constitute a fast return to normal from a highly anti-inflammatory state at rest, rather than a truly pro-inflammatory response. Substantially more analysis, including generation of protein data will have to be done to clarify this point. Both possibilities, may, however, make sense.

On the one hand, the organism in L which is prepared for a pregnancy may need a highly anti-inflammatory / immunosuppressive state in order to tolerate the fertilized egg, which, from the standpoint of immunology, is a foreign intruder. A major external stressor like physical exercise might then induce a quick return of this cycle specific expression pattern back to a normal pattern to be prepared for fending off an infection. But even if the observed change of gene expression constitutes a really pro-inflammatory impulse, a second signal (e.g. danger signals) might be necessary to provoke a prolonged inflammatory reaction.

The biological significance of the observed gene expression change can thus not be clearly judged at present. Of course it seems possible that the inflammatory impulse created by substantial exercise is sufficient to induce parturition of an incumbent early pregnancy. Lynch et al. (14) showed in an elegant study that men and women regulate the IL1/ IL1RN system in a completely different way, with women showing differential regulation in F and L. These authors showed that ex vivo monocytes from women secrete high amounts of IL1 and its antagonist IL1RN in balanced amounts during F, so that no bioactivity results, while in L there is a deficit of the antagonist, resulting in bioactivity in the supernatants. They link this finding to the role of IL1 in parturition and during birth. In the light of these experiments, it seems plausible that the pro-inflammatory response of women in L may constitute a mechanism designed to end a very early pregnancy in case of major external stress input. After all, human females get a new chance to conceive in the next month and nature may prefer to destabilize a pregnancy under influence of stress rather than carry it on under high risk.

In conclusion, women in their luteal phase showed a distinctly different pattern of gene regulation in response to exercise, compared with women in their follicular phase or men. This finding accentuates a need for careful consideration of the female cyclic phase when investigating the stress response to exercise in women. Our results may also have implications relevant to other forms of stress in females.

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In memoriam Elvira Fehrenbach †

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Addendum
Anti-inflammatory genes

↑ : Up regulated, fc > 1.5

↓ : Down regulated, fc < -1.5

Gene	Accession Id	Description	M	F	L
ADRB2	NM_000024	adrenergic, beta-2-, receptor, surface			
ADRB2	NM_000024	adrenergic, beta-2-, receptor, surface			↑
ADRB2	NM_000024	adrenergic, beta-2-, receptor, surface			
ADRB2	NM_000024	adrenergic, beta-2-, receptor, surface		↓	↑
ADRBK2	NM_005160	adrenergic, beta, receptor kinase 2			
AHSA1	NM_012111	AHA1, activator of heat shock 90kDa protein			
		ATPase homolog 1 (yeast)			
CD163	NM_203416	CD163 molecule			↑
CD19	NM_001770	CD19 molecule			
CD33	NM_001772	CD33 molecule			
CSF3R	NM_172313	colony stimulating factor 3 receptor (granulocyte)			
CSF3R	M59820.1	Human granulocyte colony-stimulating factor receptor			↑
CYC1	NM_001916	cytochrome c-1			↓
GPX1	NM_201397	glutathione peroxidase 1			
GPX3	NM_002084	glutathione peroxidase 3 (plasma)			↓
GPX4	NM_002085	glutathione peroxidase 4 (phospholipid hydroperoxidase)			
GSS	NM_000178	glutathione synthetase			
GSTM3	NM_000849	glutathione S-transferase M3 (brain)			
GSTP1	NM_000852	glutathione S-transferase pi 1			
HSPB1	NM_001540	heat shock 27kDa protein 1			↓
HSPB1	NM_001540	heat shock 27kDa protein 1			↓
HSPB1	NM_001540	heat shock 27kDa protein 1			↓
IL10RB	NM_000628	interleukin 10 receptor, beta			
IL13	NM_002188	interleukin 13			
IL13RA2	NM_000640	interleukin 13 receptor, alpha 2			
IL16	NM_172217	interleukin 16 (lymphocyte chemoattractant factor)			
IL1R2	NM_173343	interleukin 1 receptor, type II		↑	↓
IL1RN	NM_173843	interleukin 1 receptor antagonist			↓
IL2RB	NM_000878	interleukin 2 receptor, beta			

Gene	Accession Id	Description	M	F	L
IL4R	NM_001008699	interleukin 4 receptor			
IL6	NM_000600	interleukin 6 (interferon, beta 2)		↑	↓
IL6R	NM_181359	interleukin 6 receptor			
IL6ST	NM_175767	interleukin 6 signal transducer (gp130, oncostatin M receptor)			
LILRA2	NM_006866	leukocyte immunoglobulin-like receptor, subfamily A, member 2			↑
MT3	NM_005954	metallothionein 3			
PPARA	BC000052.2	peroxisome proliferator-activated receptor alpha, mRNA			
PPARA	NM_005036	peroxisome proliferator-activated receptor alpha			
PPARG	BC006811.1	peroxisome proliferator-activated receptor gamma			
PRDX4	NM_006406	peroxiredoxin 4			↓
PRDX5	NM_181652	peroxiredoxin 5			↓
PROC	NM_000312	protein C (inactivator of coagulation factors Va and VIIIa)			
PROK2	NM_021935	prokineticin 2			↑
PTGIS	NM_000961	prostaglandin I2 (prostacyclin) synthase			
SOD1	NM_000454	superoxide dismutase 1, soluble			↓
SOD2	AY267901	superoxide dismutase 2, nuclear gene for mitochondrial product.			
SOD3	NM_003102	superoxide dismutase 3, extracellular			
STIP1	NM_006819	stress-induced-phosphoprotein 1			
THBD	NM_000361	thrombomodulin			
TXN	NM_003329	thioredoxin			
TXN2	NM_012473	thioredoxin 2			
TXNIP	NM_006472	thioredoxin interacting protein			

Proinflammatory genes

↑ : Up regulated, fc > 1.5

↓ : Down regulated, fc < -1.5

Gene	Accession Id	Description	M	F	L
ALOX5	NM_000698	arachidonate 5-lipoxygenase			
ALOX5	NM_000698	arachidonate 5-lipoxygenase			↑
CASP1	NM_033295	caspase 1 (interleukin 1, beta, convertase)			
CASP1	NM_033292	caspase 1, transcript variant alpha			↑
CASP1	NM_033294	caspase 1, transcript variant delta			
CASP3	NM_032991	caspase 3 transcript variant beta			
CASP3	NM_032991	caspase 3	↑		
CASP5	NM_004347	caspase 5			
CASP5	NM_004347	caspase 5			
CASP9	NM_001229	caspase 9 transcript variant alpha			
CASP9	NM_032996	caspase 9, apoptosis-related cysteine peptidase			
CCL4	NM_002984	chemokine (C-C motif) ligand 4			
CCR1	NM_001295	chemokine (C-C motif) receptor 1			
CD14	NM_000591	CD14 molecule			↑
CD160	BC014465.1	CD160 molecule			
CD1B	NM_001764	CD1b molecule			
CD1B	NM_001764	CD1b molecule			
CD2	NM_001767	CD2 molecule			
CD44	NM_001001392	CD44 molecule (Indian blood group)	↓		
CD58	NM_001779	CD58 molecule			
CD59	NM_203331	CD59 molecule, complement regulatory protein			
CD69	NM_001781	CD69 molecule			
CD80	NM_005191	CD80 molecule			
CD83	NM_004233	CD83 molecule	↑		
COX7A2	BC100852.1	cytochrome c oxidase subunit VIIa polypeptide 2 (liver)			↓
CSF1	NM_172212	colony stimulating factor 1 (macrophage)			
CSF2	NM_000758	colony stimulating factor 2 (granulocyte-macrophage)			
CX3CR1	NM_001337	chemokine (C-X3-C motif) receptor 1			
CXCL10	NM_001565	chemokine (C-X-C motif) ligand 10			
CYSLTR1	NM_006639	cysteinyl leukotriene receptor 1			↑
DAP	NM_004394	death-associated protein			↓

Gene	Accession Id	Description	M	F	L
DAPK1	NM_004938	death-associated protein kinase 1			↑
FCGR3B	NM_000570	Fc fragment of IgG, low affinity IIIb, receptor (CD16b)			
HIF1AN	NM_017902	hypoxia-inducible factor 1, alpha subunit inhibitor			
HLA-DRA	NM_019111	major histocompatibility complex, class II, DR alpha			
ICAM2	NM_000873	intercellular adhesion molecule 2			
ICAM3	NM_002162	intercellular adhesion molecule 3			↓
ID2	NM_002166	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein			
IFNAR1	NM_000629	interferon (alpha, beta and omega) receptor 1			
IFNG	NM_000619	interferon, gamma			↓
IFNG	NM_000619	interferon, gamma		↓	↑
IFNG	NM_000619	interferon, gamma			
IFNGR1	NM_000416	interferon gamma receptor 1			
IGF2	NM_000612	insulin-like growth factor 2 (somatomedin A)			
IGF2	NM_000612	insulin-like growth factor 2 (somatomedin A)		↓	
IGF2	NM_000612	insulin-like growth factor 2 (somatomedin A)			↓
IHPK3	NM_054111	inositol hexaphosphate kinase 3			↓
IL11	NM_000641	interleukin 11			
IL12RB1	NM_153701	interleukin 12 receptor, beta 1		↓	↑
IL12RB2	NM_001559	interleukin 12 receptor, beta 2			
IL15	NM_172174	interleukin 15			
IL18	NM_001562	interleukin 18 (interferon-gamma-inducing factor)			
IL18R1	NM_003855	interleukin 18 receptor 1			
IL18RAP	BC106765.2	Homo sapiens interleukin 18 receptor accessory protein			↑
IL1A	NM_000575	interleukin 1, alpha			
IL1A	NM_000575	interleukin 1, alpha			
IL1A	NM_000575	interleukin 1, alpha			
IL1B	NM_000576	interleukin 1, beta			
IL1R1	NM_000877	interleukin 1 receptor, type I			
IL21R	NM_181079	interleukin 21 receptor			
IL24	NM_181339	interleukin 24			
IL5RA	NM_175728	interleukin 5 receptor, alpha			
IL8RA	NM_000634	interleukin 8 receptor, alpha			
IL8RA	NM_000634	interleukin 8 receptor, alpha			
INDO	NM_002164	indoleamine-pyrrole 2,3 dioxygenase			
IRAK1	NM_001569	interleukin-1 receptor-associated kinase 1			↓
LBP	NM_004139	lipopolysaccharide binding protein			

Gene	Accession Id	Description	M	F	L
LTA	NM_000595	lymphotoxin alpha (TNF superfamily, member 1)			
LTB	NM_009588	lymphotoxin beta (TNF superfamily, member 3)			
MAP2K4	NM_003010	mitogen-activated protein kinase kinase 4			
MAPK14	BC031574.1	Homo sapiens mitogen-activated protein kinase 14			
MAPK14	NM_139014	mitogen-activated protein kinase 14			
MAPK8	NM_139049	mitogen-activated protein kinase 8			
MAPK8	NM_139049	mitogen-activated protein kinase 8			
MAPKAPK2	NM_032960	mitogen-activated protein kinase-activated protein kinase 2		↑	
MGST2	NM_002413	microsomal glutathione S-transferase 2			
MGST3	NM_004528	microsomal glutathione S-transferase 3			
NGFR	NM_002507	nerve growth factor receptor (TNFR superfamily, member 16)			↑
NOS1	NM_000620	nitric oxide synthase 1 (neuronal)			
NOS2	NM_000625	nitric oxide synthase 2, inducible			
NPY1R	NM_000909	neuropeptide Y receptor Y1			↑
PRKCA	NM_002737	protein kinase C, alpha			
PRKCB	BC036472.1	Homo sapiens protein kinase C, beta 1			
PRKCQ	NM_006257	protein kinase C, theta			
PRKCZ	BC014270.2	protein kinase C, zeta			
PTGDR	U31099.1	Human DP prostanoid receptor (PTGDR)			↑
PTGS1	NM_080591	prostaglandin-endoperoxide synthase 1	↓		
PTGS2	NM_000963	prostaglandin-endoperoxide synthase 2			
SELE	NM_000450	selectin E			
SELL	NM_000655	selectin L			↑
SELP	NM_003005	selectin P (granule membrane protein 140kDa, antigen CD62)			
SMAD5	NM_001001419	SMAD family member 5 (SMAD5), transcript variant 2			
TBXAS1	NM_030984	thromboxane A synthase 1 (platelet)			↑
TGFB1	NM_000660	transforming growth factor, beta 1			
TGFB1	NM_000660	transforming growth factor, beta 1			
TIAM1	NM_003253	T-cell lymphoma invasion and metastasis 1			
TIAM2	NM_012454.	T-cell lymphoma invasion and metastasis 2 transcript variant 1			
TNF	NM_000594	tumor necrosis factor (TNF superfamily, member 2)			