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Evaluation of immune response after moderate and overtraining exercise in wistar rat

Zahra Gholamnezhad [1], Mohammad Hossein Boskabady [1]*, Mahmoud Hosseini [2], Mojtaba Sankian [3], Abolfazl Khajavi Rad [1]

[1] Neurogenic Inflammation Research Centre and Department of Physiology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[2] Neurocognitive Research Centre, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[3] Immunology Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

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ABSTRACT

Objective(s): The effect of prolonged overtraining on cytokine kinetics was compared with moderate exercise in the present study.

Materials and Methods: Male Wistar rats were randomly divided into control sedentary (C), moderate trained (MT), (V=20 m/min, 30 min/day for 6 days a week, 8 weeks), overtrained (OT) (V=25 m/min, 60min/day for 6 days a week, 11 weeks) and recovered overtrained (OR) (OT plus 2 weeks recovery) groups, (n=6 for each group). Immediately, 24 hr and 2 weeks (in OR) after last bout of exercise blood samples were obtained. The plasma concentrations of TNFα, IL-6, IL-10, IL-4 and IFNγ were measured by ELISA method.

Results: Immediately after last bout of exercise the following findings were observed; IL-6, IL-10 and TNFα concentrations increased in OT and OR groups compared with control (P<0.05–P<0.001). Serum level of IL-4 decreased (P<0.01) but IFNγ increased (P<0.05) in MT group vs. control. In addition, circulatory levels of TNFα, IL-6, IL-10 and IL-4 were higher but the IFNγ concentrations were lower in OT and OR groups than MT group (P<0.05-P<0.01). The IFN-γ/IL4 ratio was significantly increased in MT (P<0.01) while it decreased in OT group. There were not statistical differences in TNFα, IL-6, and IFNγ levels between different time intervals after exercise in MT, OT and OR groups.

Conclusion: These data confirm a positive effect of moderate exercise on immune function and a decrease in susceptibility to viral infection by inducing Th1 cytokine profile shift. However, prolonged and overtraining exercise causes numerous changes in immunity that possibly reflects physiological stress and immune suppression.

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Introduction

Changes in lifestyle and physical inactivity lead to increased incidence of many chronic non-communicable diseases which are major causes of death (36 million each year) and health problems in the world (1–3). Physical inactivity, as does smoking, increases risk for cancer, cardiovascular and chronic lung diseases and diabetes by 20%–30%, and shortens lifespan by 3–5 years (1). On the other hand regular exercise, like a miracle drug, has many beneficial effects on the body and protects against those diseases (4–6). The key recommendation of the recently published World Health Organization guidelines regarding physical activity is "adults should do at least 150 min a week of moderate intensity, or 75 min a week of vigorous intensity aerobic physical activity, or an equivalent combination of moderate and vigorous intensity aerobic activity" (7, 8). However, the research report recommends that future investigations need to evaluate the effects of physical activity intensity at fixed energy expenditure doses (9). This is due to the effects of exercise on the physiologic parameter which depends upon several factors, such as the frequency of each bout and the total duration of the exercise protocol (10).

Although new studies have shown that the site of cytokine production and action are beyond immune system; still they have been considered as secreted proteins which regulate all aspects of innate and adaptive immunity (11). There is a dynamic equilibrium between pro and anti-inflammatory cytokines. The time course of cytokine release, the local cytokine milieu, the existence of stimulating

*Corresponding author: Mohammad Hossein Boskabady. Departemt of Physiology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. Fax +98-511-8828564; email: boskabadymh@mums.ac.ir

and inhibiting factors and their receptor densities are determinants of the net cytokine effect (12). T cell cytokines have a pivotal role in the promotion of immune responses against invading pathogens (13). There are two distinct cytokine producing T cell subtypes: $CD4^+$ T helper and $CD8^+$ T cytotoxic which have been appointed to type 1 or type 2 T lymphocytes based on their profile of cytokine production (14). Type 1 lymphocytes are essential for the cell-mediated immune and defense against intracellular pathogens by producing interferon γ (IFNγ), interleukin 2 (IL-2) and tumor necrosis factor-β cytokines. Whereas, type 2 lymphocytes produce cytokines including IL-4, IL-5, IL-6, IL-10, and IL-13 are responsible for defense against extracellular pathogens by the development of humoral immunity (15,16). These two classes of cytokines have cross-regulatory signaling; for example IL-4 and IL-10 secretion causes the inhibition of Th1-type immune responses by down-regulating macrophage-derived IL-12 production. Also IFNγ changes the balance of Th1/Th2 by suppressing the Th2-type immune responses (17).

According to hormesis theory, the responses of biological systems to stressors may be explained by the U-shaped curve. The two endpoints of this curve are inactivity and overtraining, and both of these result in decreased physiological function (18, 19). Stress may weaken host defense against external pathogens or stimuli and internal tumor development by impairing immune responses such as antibody production, natural killer (NK) activity, and lymphocyte responses to mitogens (20). It has been reported that moderate or intermittent exercise enhances immune function but prolonged and sever exercise cause numerous changes in immunity, which possibly reflects physiological stress and suppression (19, 21). Athletes tolerating more intense levels of training may be at increased risk of upper respiratory tract infection (URTI) during periods of sever exercise and for the few weeks after race events (22). Interestingly, most of the studies used applied voluntary exercise, even though the effects of enforced physical exercise, especially with different loads, are unclear. There is little information regarding whether regular exercise above a certain intensity or duration could be harmful. It would be of interest to identify the optimum exercise loading which could improve certain physiological aspects, including immune function. According to the current findings of adaptation to physical exercise, until an overtraining syndrome appears, regular exercise has beneficial effects (23). However, with overtraining, which is still a poorly understood process, the homeostatic balance involving a wide range of hormonal, metabolic, and immunologic factors is altered (24).

Many studies had evaluated post exercise immunomodulation in human, especially Olympic and marathon race athletes. However, the study of forced sever exercise and overtraining syndrome in human has clear ethical limits that are unquestioned in the literature (21, 25, 26). Different kinds of exercise may have various effects on immune parameters based on the nature, intensity and time delay between exercise bouts and immune parameter. In addition, it is not clear what part of this multifactorial stress (psychological or physical) in those human studies effects components of the immune system (27). Therefore, more evidence is needed to explain the nature and clinical feature of this immunomodulation. Consequently, in the present study we examined the effect of moderate and sever treadmill running on plasma concentrations of TNFα, IL-6, IL-10, IL-4 and IFNγ cytokines immediately, 24 hr and 2 weeks after the last bout of exercise.

Materials and Methods

Animals

Thirty adult (6–8 weeks old) male Wistar rats, (Animal House of School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran) weighing 150–200 g were used. Animals were housed under environmentally controlled conditions (12 hr light/dark cycle; 22–24°C) and food and water were available *ad libitum* throughout the experiment. Animals were allowed to adjust to new condition for two weeks. The protocols used conformed to guidelines of animal studies and were approved by the committee on the ethics of animal experiments in Mashhad University of Medical Sciences.

Training protocol

A motorized treadmill with 4 individual lanes was used. A shock grid at the back of the treadmill provided a mild shock (0.5 mA, 1 HZ) if the rat's pace went below the treadmill rate. The animals under-took a one week familiarizing period prior to the beginning of the experiments. They were placed on the treadmill 10 min/day for 5 days at a speed of 12 m/min at 0% degree inclination. Then they were scored 1–5 depending on running quality; rats that ran voluntarily with a mean rating of 3 or higher (n=24) were separated from those which refused (n=6) and those with a 3 or higher score were chosen for the study. This procedure was used to exclude possible different levels of stress between animals (25).

Twenty four rats were randomly divided into four equal groups including: control sedentary (C), moderate trained (MT), overtrained (OT) and recovered overtrained (OR).

The animals of the control group were handled and placed on the treadmill with the aim of experiencing the stress of treadmill environment. Exercised groups undertook a progressive load training 6 days a week to enhance cardiorespiratory fitness and a 5 minute warm up and cool down were included in each session. MT group underwent 8

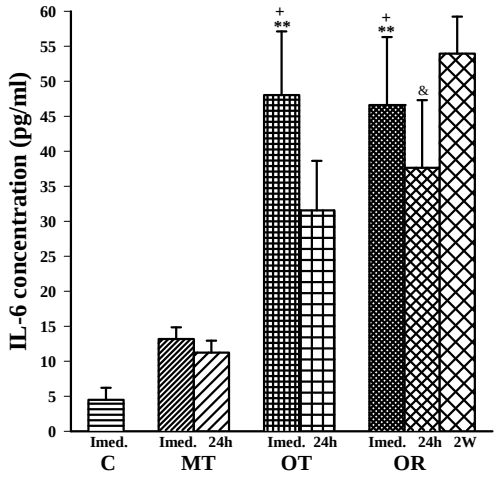

Figure 1. The levels of serum IL-6 in control sedentary rats (C), moderate trained (MT), overtrained (OT), recovered overtrained (OR), (for each group, n=6).
Statistical differences between control and other groups (immediately after last bout of exercise): **; P <0.01.
Statistical differences between MT *vs* OT and OR groups (immediately after last bout of exercise): +; P <0.05.
Statistical differences between MT *vs* OR group (24 hr after last bout of exercise): &; P <0.05.
There were not statistical differences between different times in MT, OT and OR groups

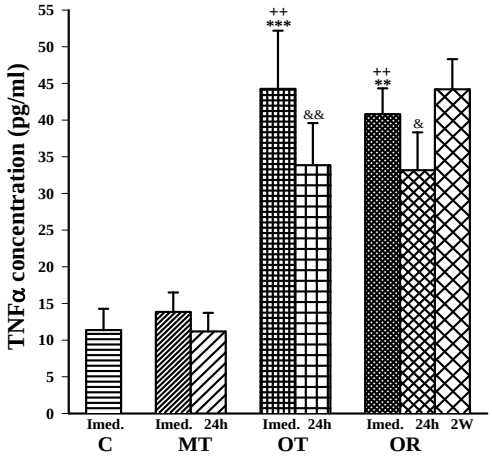

Figure 2. The levels of serum TNFα in control sedentary rats (C), moderate trained (MT), overtrained (OT), recovered overtrained (OR), (for each group, n=6).
Statistical differences between control and other groups (immediately after last bout of exercise): **; P <0.01, ***; P <0.001.
Statistical differences between MT *vs* OT and OR groups (immediately after last bout of exercise): ++; P <0.01, +++; P <0.001.
Statistical differences between MT *vs* OR group (24 hr after last bout of exercise): &; P <0.05; &&; P <0.01.
There were not statistical differences between different times in MT, OT and OR groups

weeks exercise at a speed of 15 m/min for 20 min, 6 days/week but the intensity of exercise was increased to 20 m/min for 30 min at the onset of the second week (28). OT and OR groups were submitted into the 3 phase program. In the first 4 weeks (phase I) training speed increased from 15 to 25 m/min and training time from 20 min to 60 min. In the second 4 weeks (phase II) training load was kept constant. During last 3 weeks (phase III) running intensity and training duration remained unchanged but recovery time between training sessions was reduced (from 24 hr to 4, 3 and 2 hr). The OR group had a 2 weeks recovery period after the last exercise session (25). The training program was evaluated by a performance test at the end of each phase.

Sample collection

At the end of the study, animals were anesthetized with diethyl ether; peripheral blood was collected from the retro-orbital sinus in the control group, immediately and 24 hr after the last session of exercise in MT and OT groups and immediately, 24 hr and 2 weeks after the last session of exercise in OR group. After allowing blood to clot on ice, serum samples were separated by centrifuging at 3000 rpm for 10 min. Serum was collected and stored at -20 °C for cytokine analysis.

Cytokine assays

Cytokine determination was performed with commercially available platinum ELISA kits (Bender Med system, Austria) according to manufacturer's instruction. They were carefully checked for specify, sensitivity and reliability. Plasma concentration of IL-4, IL-6, IL-10, TNFα and IFNγ were measured using rat ELISA kits: BMS628, BMS625, BMS629, BMS622 and BMS621 respectively. The absorbance was measured using a spectrophotometer and a microplate reader (Biotek, USA); concentration of each cytokine was calculated by a comparison curve established in the same measurement using prism 5 Graphpad. Each cytokine assay was performed in duplicate each time.

Statistical analysis

The results were presented as means ± SEM. Group-data comparisons were performed using one way analysis of variance (ANOVA) with Tukey-Kramer post-test. The impact of time on each group data was evaluated by paired t-test for MT and OT groups, and by repeated measurement of ANOVA for OR group. Significance was accepted at P<0.05.

Results

The effect of exercise on serum concentration of cytokines

Immediately after last bout of exercise the following finding was observed; IL-6 concentration was significantly increased in OT and OR groups compared with control (F (3, 20)=10.82, P<0.01 for both groups), (Figure 1). In addition, TNFα and IL-10 concentrations were significantly increased in OT and OR groups compared with control (F(3, 20) =13.32, P<0.001 for TNFα and F(3, 20)=6.02, P<0.01 for IL-10 in both groups), (Figure 2 and 3).

Serum level of IL-4 was significantly decreased

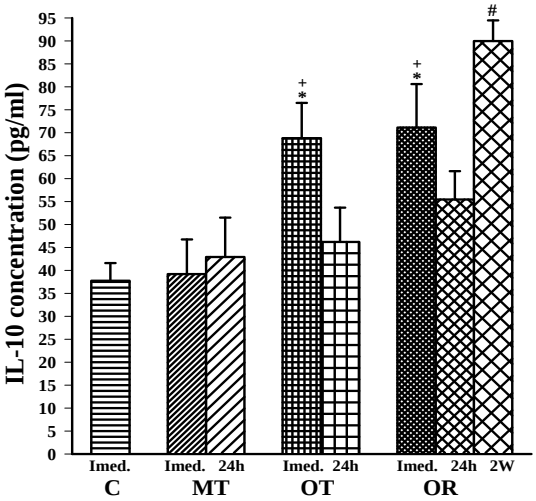

Figure 3. The levels of serum IL-10 in control sedentary rats (C), moderate trained (MT), overtrained (OT), recovered overtrained (OR), (for each group, n=6).
Statistical differences between control and other groups (immediately after last bout of exercise): *; $P<0.05$.
Statistical differences between MT *vs* OT and OR groups (immediately after last bout of exercise): +; $P<0.05$.
Statistical differences between different times: 24 hr and 2 weeks post exercise: #; $P<0.05$
There were not statistical differences between different times in MT and OT

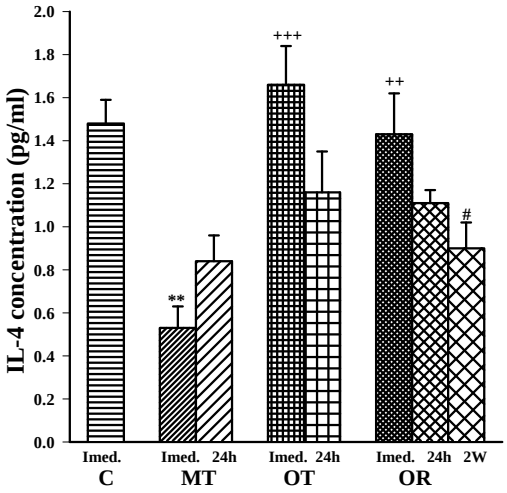

Figure 4. The levels of serum IL-4 in control sedentary rats (C), moderate trained (MT), overtrained (OT), recovered overtrained (OR), (for each group, n=6)
Statistical differences between control and other groups (immediately after last bout of exercise): **; $P<0.01$.
Statistical differences between between MT *vs* OT and OR groups (immediately after last bout of exercise): ++; $P<0.01$, +++; $P<0.001$.
Statistical differences between different times: immediately and 2 weeks post exercise: #; $P<0.05$
There were not statistical differences between different times in MT and OT

(F=11.65, $P<0.001$), (Figure 4); serum level of IFNγ was significantly increased only in MT group compared with control group (F(3,20)=5.44, $P<0.05$), (Figure 5). There were no significant differences in serum levels of IFNγ and IL-4 in OT and OR groups compared with control (Figure 5).

The effect of exercise intensity on serum concentration of cytokines

In this study, immediately after last bout of exercise serum levels of IL-6, TNFα, IL-10 and IL-4 were higher in OT and OR groups than MT group

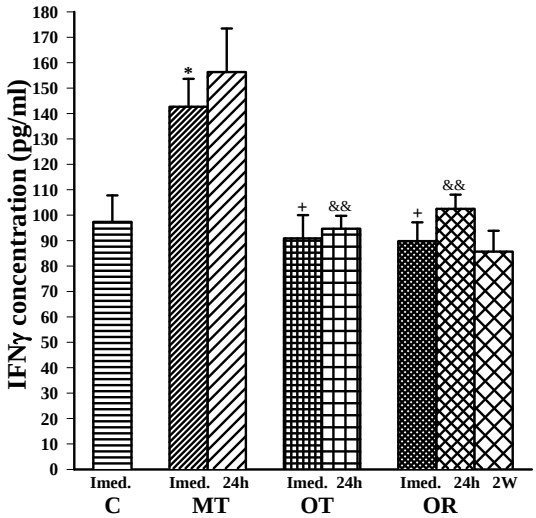

Figure 5. The levels of serum IFNγ in control sedentary rats (C), moderate trained (MT), overtrained (OT), recovered overtrained (OR), (for each group, n=6).
Statistical differences between control and other groups (immediately after last bout of exercise): *; $P<0.05$, **; $P<0.01$.
Statistical differences between MT *vs* OT and OR groups (immediately after last bout of exercise): +; $P<0.05$.
Statistical differences between MT *vs* OR group (24 hr after last bout of exercise): &&; $P<0.01$.
There were not statistical differences between different times in MT, OT and OR groups

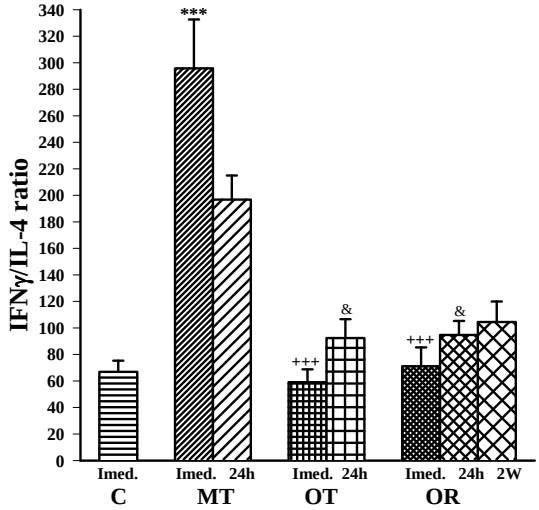

Figure 6. The ratio of serum INF-γ/IL-4 (Th1/Th2 balance) in control sedentary rats (C), moderate trained (MT), overtrained (OT), recovered overtrained (OR), (for each group, n=6).
Statistical differences between control and other groups (immediately after last bout of exercise): ***; $P<0.001$.
Statistical differences between MT *vs* OT and OR groups (immediately after last bout of exercise): +++; $P<0.001$.
Statistical differences between MT *vs* OR group (24 hr after last bout of exercise): &; $P<0.05$.
There were not statistical differences between different times in MT, OT and OR groups

(F(3,20)=10.82, *P*<0.05 for IL-6; F(3,20)=13.32, *P*<0.01 for TNFα; F(3,20)=6.02, *P*<0.05 for IL-10; for both groups and F(3,20)=11.65, *P*<0.001 in OT and *P6*<0.01 in OR group for IL-4), (Figure 1, 2, 3 and 4). However, IFNγ concentration was lower in OT and OR groups (F(3,20)=5.44, *P*<0.05 in both cases) than MT group (Figure 5).

The acute and chronic effect of exercise on serum concentration of cytokines

Twenty four hours after last bout of exercise the concentrations of IL-6 in OR was higher than MT group (F(2,15)=3.93, *P*<0.05), (Figure 1). TNFα concentration in OT and OR was also higher than MT group (F(2,15)=7.59, *P*<0.01 for both groups), (Figure 2). In addition, IFNγ concentration was lower in OT and OR groups than MT group (F (2, 15) =9.65, *P*<0.01 for both groups), (Figure 5). However, there were no significant differences in IL-10 and IL-4 concentrations between OT, OR and MT groups (Figure 3-4). Twenty four hours after last bout of exercise all measured cytokines concentrations were non-significantly decreased compared with immediately after exercise (Figure 1-5). The concentration of IL-10 was significantly higher in 2 weeks than 24 hr after exercise in OR group (F(2,15)=5.11, *P*<0.05), (Figure 3). However, IL-4 concentration was decreased after 2 weeks compared with 24 hr after exercise in this group (F(2,15)=3.89, *P*<0.05 respectively), (Figure 4). IFNγ concentration did not show remarkable time dependent changes (Figure 5).

Exercise intensity and Th1/Th2 balance

Moderate exercise significantly increased (F(3,20)=22.86, *P*<0.001) IFN-γ/IL4 ratio (Th1/Th2 balance) compared with control. However, overtraining caused a non-significant decrement in IFN-γ/IL4 ratio which increased after 2 weeks recovery in comparison to control (Figure 6).

Discussion

Physiological basis of overtraining as a physical stressor remains poorly understood (25). To our knowledge, this is the first animal study to investigate the cytokine kinetics after prolonged overtraining endurance treadmill exercise in comparison to the moderate intensity one. We used here a standard training protocol for overtraining with various time periods between exercise bouts and rest (25–29). This protocol caused a decline in performance (data not shown), which is the only parameter stated in the literature to be obligatorily associated with overtraining (25, 30). Also we saw marked post exercise changes in circulatory cytokines in the OT group even after 24 hr. Therefore, to examine the reproducibility of our findings in OT group and sustainability of them, the OR group was also formed.

In this study different sampling times after overtraining showed a chronic increase in pro-inflammatory cytokine TNFα and inflammation responsive cytokine IL-6, which remained elevated at least two weeks after recovery. In addition, this load of exercise caused no change in acute IL- 4 (Th2 cytokine) concentration but it was significantly decreased after 24 hr and 2 weeks recovery. However, IL-10 (anti-inflammatory cytokine) concentration showed significant increase immediately post exercise, with a reduction after 24 hr; it increased again after 2 weeks recovery. There was no significant change in IFNγ (Th1 cytokine) at different time points after exercise. This marked post exercise elevation in IL-6 and IL-10 concentrations is supported by previous observations (11, 27, 31–32).

It is believed that exercise can induce a primary increase in IL-6, followed by an increase in IL-1ra and IL-10 (11, 26–27, 33). Many probable sources have been proposed for IL-6 elevation after exercise which are related to exercise intensity, duration, the mass of muscle recruited and one's endurance capacity (11, 26, 34). Recent studies have shown that contracting skeletal muscles, but not immune cells, are the main source of IL-6 in the circulation in response to exercise (35). It is shown that IL-6 mRNA is upregulated in contracting skeletal muscles (36-38) and exercise enhances the transcriptional rate of the IL-6 gene (39). Furthermore, adipose tissue may contribute to the exercise-induced augmentation of IL-6 in the circulation but at recovery time (40). It has been proposed that as muscle glycogen level decreases, IL-6 may signal the liver to increase its glucose output and prevent a sever fall in exercise induced glucose concentration which has a lipolytic effect (38, 41-44).

Previous studies most of which had been performed after marathon races or used the human endurance exercise protocol reported controversial findings for serum TNFα (35). Ostrowski *et al* reported: "the plasma level of IL-1beta, TNF alpha, sTNF-r1 and sTNF-r2 peaked in the first hour after exercise (2. 1-, 2.3-, 2.7- and 1.6-fold, respectively)" but in other studies TNF α in serum was not detected and there was no significant increase in TNF-alpha mRNA, in spite of TNF-alpha mRNA detection in resting muscle samples (45-46). It has been demonstrated that a bout of prolonged exercise had no effect on monocytes IL-6 protein expression and TNFα or IL-1β production from them (38). However, the exercise protocol (intensity, duration) and other factors like sex, race, and examiners adaptation and even sensitivity and specificity of the test should be considered as main factors of this bias.

Our data showed that overtraining treadmill running caused prolonged IL-6 elevation. This second increase may be correlated with muscle damage which caused a low-grade inflammation

with macrophage and neutrophils penetration to damaged tissue during 6–48 hr post exercise, activating macrophage release of IL-6 as part of an inflammatory response. Moreover, TNFα may stimulate IL-6 production (26) and IL-6 in turn, may activate IL-10 and cortisol production (47). It is also proposed that an acute bout of physical activity may cause physiological responses which are very similar in many aspects to those induced by infection, sepsis or trauma (48). Therefore, our findings showed that after prolonged strenuous exercise like acute exercise there is a chronic low systemic inflammation due to TNFα and IL-6 elevation which is balanced by increased IL-10 concentration as an anti-inflammatory cytokine.

Type 2 lymphocytes, monocytes and B cells are the main producers of IL-10 and together with IL-4 can inhibit type 1 T cell production (38). In this study we showed a significant reduction in IL-4 and elevation in IFNγ concentration after moderate exercise, which confirmed the hypothesis that the T1 cell responses are strengthened following moderate intensity exercises (21). The fact that moderate elevation in IL-6 was not accompanied by TNFα and IL-10 changes after moderate exercise may support its anti-inflammatory effects (26). However, the mechanism of this immunomodulatory effect is not completely known to date. It has been suggested that sever overtrained exercise may lead to tissue trauma, which would activate local cells to produce cytokines, stimulating the differentiation of Th2 cells, and also elevate circulating levels of stress hormones, including cortisol and catecholamines, which can inhibit the production of IL-12 (main inducer of Th1 cells) and would up-regulate Th2 lymphocyte responses (49-51). However, in turn, moderate exercise training has been shown to induce down-regulation of the steady state level of β2-adrenergic receptor on macrophages. This effect was associated with decrease in the suppressive effects of catecholamines on IL-12 production, thereby resulting in the up-regulation of Th1 responses (52). It seems that after regular exercise with adequate intensity and duration the body has the capability to cope with the exercise (stressor) and as a result adaptation takes place. This adaptive effect may mediate the health-beneficial effect and play an important role in protection against chronic non-communicable diseases which are associated with low-grade inflammation (53).

Conclusion

The results of the present study showed that in contrast to moderate or intermittent physical activity, prolonged and overtraining exercise causes numerous changes in immunity that possibly reflect physiological stress and immune suppression, which is in agreement with hormesis theory.

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