Regular Exercise Enhances the Immune Response Against Microbial Antigens Through Up-Regulation of Toll-like Receptor Signaling Pathways

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Key Words
Regular physical exercise • Pathogenic infection • Cytokine • Toll-like receptor • Immune response

Abstract

Background/Aims: Regular physical exercise can enhance resistance to many microbial infections. However, little is known about the mechanism underlying the changes in the immune system induced by regular exercise. Methods: We recruited members of a university badminton club as the regular exercise (RE) group and healthy sedentary students as the sedentary control (SC) group. We investigated the distribution of peripheral blood mononuclear cell (PBMC) subsets and functions in the two groups. Results: There were no significant differences in plasma cytokine levels between the RE and SC groups in the true resting state. However, enhanced levels of IFN-γ, TNF-α, IL-6, IFN-α and IL-12 were secreted by PBMCs in the RE group following microbial antigen stimulation, when compared to the SC group. In contrast, the levels of TNF-α and IL-6 secreted by PBMC in the RE group were suppressed compared with those in SC group following non-microbial antigen stimulation (concanavalin A or α-galactosylceramide). Furthermore, PBMC expression of TLR2, TLR7 and MyD88 was significantly increased in the RE group in response to microbial antigen stimulation. Conclusion: Regular exercise enhances immune cell activation in response to pathogenic stimulation leading to enhanced cytokine production mediated via the TLR signaling pathways.

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Introduction

Physical activity includes exercise and other activities that involve bodily movement such as playing, working and recreational activities. Physical inactivity is related to high incidence of cancers, diabetes and ischemic heart disease. The World Health Organization (WHO) has reported that physical inactivity has been identified as the fourth leading risk factor for global mortality, causing an estimated 3.2 million deaths every year[1]. In other words, people who engage regularly in moderate exercise are often resistant to many diseases [2-5]. Some studies have shown that moderate exercise results in enhanced immunity and a decreased upper respiratory tract infection rate [6]. In contrast, exhaustive exercise can lead to reduced immunity, a high incidence of upper respiratory tract infections, and damage to erythrocytes [7-9]. Thus, any type of physical activity can influence the immune system, although these effects will often depend on the type of exercise and its duration.

Regular exhaustive exercise causes a significant reduction in the number of immune cells and disruption of leukocyte function and apoptosis. Furthermore, regular high-intensity exercise can lead to decreased secretion of cytokines, such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-1β, IL-2, IL-8 and IL-10 [10, 11]. Insufficient recovery between periods of prolonged exercise appears to exaggerate these effects [12]. These results indicate the immunosuppressive effects of excessive exercise, which, to some extent, explain the higher incidence of upper respiratory infections during the competition season. However, regular moderate exercise helps to reduce the risk of cardiovascular disease and breast cancer by boosting the immune system [8, 13]. In elderly males, those who engage regularly in moderate exercise have differences in the CD4+/CD8+ T cell ratio and the number of natural killer (NK) cells in the true resting state (i.e., more than 24 h after training) compared with those who do not exercise regularly [14]. These changes associated with moderate training may help reduce inflammation [15]. Also, aerobic exercise reduces the incidence and severity of upper respiratory tract infections [16]. Nevertheless, the mechanisms underlying the immune changes induced by regular exercise are not clear. Thus, in this study, we focused on the changes in the immune responses to a series of stimuli induced by regular exercise. We found that regular exercise improved the ability of the immune system to defend against the invasion of pathogens by producing substantial amounts of Th1-type and pro-inflammatory cytokines via the Toll-like receptor (TLR) signaling pathways. We propose that this mechanism explains, in part, why regular physical exercise enhances resistance to infection.

Materials and Methods

Subjects

Twenty college student volunteers (10 males and 10 females) belonging to the badminton club of Zhejiang University (China) and exercising three times a week (2 h, with a 5 min break after each 30 min of exercise on each occasion), were recruited as the regular exercise (RE) group (Table 1). Twenty-five healthy sedentary college students (12 males and 13 females) were recruited as the regular exercise (RE) group (Table 1). All volunteers satisfied the following requirements: no exercise in the previous 3 days, no smoking, no previous medical record of cardiovascular or metabolic diseases, no recent symptoms of upper respiratory tract infection, and abstinence from any medication for at least 1 month before the study. The females all menstruated regularly. The duration of their periods ranged from 26 to 32 days. Blood was obtained from them in the follicular phase of their cycles (i.e., days 7-12). We measured plasma titers of Anti-streptolysin O (ASO) and Hepatitis B core antibody (HBcAb) in both groups (Table 1). The protocol was approved by the Ethics Committee of the First Affiliated Hospital of Medical College, Zhejiang University (China) (Reference number 2014-273). Written informed consent was received from all participants. Anticoagulant-treated blood samples were collected from volunteers after obtaining their informed consent.
Isolation and culture of peripheral blood mononuclear cells

PBMCs were isolated by density centrifugation on Ficoll according to the manufacturer’s instructions. Cells \((2 \times 10^6/ml)\) were then cultured with ConA \((10 \mu g/ml; \text{ Vector Laboratories, Burlingame, CA, USA})\), α-GC \((0.2 \mu g/ml; \text{ Pharmaceutical Research Laboratories, Kirin Brewery, Gunma, Japan})\), heat-inactivated \(S. pyogenes\) \((2 \times 10^7 \text{ CFU/ml}; \text{ a clinical isolate (type M12/T12) from a patient with pharyngitis in Hokkaido University Hospital, Sapporo, Japan})[17]\), or hepatitis B core antigen \((\text{HBcAg}; \text{ HBV core 1–186 a.a, 2 µg/ml, PerSpec, NJ, USA})\) for 24 h. Supernatants were collected and stored at -80°C. Harvested cells were frozen at -80°C in TRIzol reagent \((\text{Invitrogen, Carlsbad, CA, USA})\) for total mRNA extraction.

Flow cytometry

The following monoclonal antibodies were used in the present study: fluorescein isothiocyanate \((\text{FITC})\)-anti-CD4 and phycoerythrin \((\text{PE})\)-anti-CD8/PerCP-anti-CD3, and PE-anti-iNKT \((\text{BD Pharmingen, CA, USA})\); FITC-anti-CD4, PE-anti-CD25, and FITC-anti-CD3/PE-anti-CD \((16+56)\) \((\text{Invitrogen})\). PE-BDCA-1, PE-BDCA-2 and PE-BDCA-3 \((\text{Miltenyi, Germany})\). Cells were stained with the appropriate antibodies and incubated in 4°C for 30 min according to standard procedures. Flow cytometry was performed on the Beckman Coulter FC500 MPL \((\text{Beckman Coulter, Brea, CA, USA})\) , and CXP software \((\text{Beckman coulter, CA, USA})\) was used for analysis.

ASO and HBcAb detection

The titers of ASO \((\text{Beckman coulter, CA})\) and HBcAb \((\text{Abbott, Abbott Park, Illinois})\) were measured using a standard clinical automatic analyzer.

ELISA

The levels of IFN-γ, TNF-α, IL-6, IFN-α and IL-12 were detected by ELISA kits \((\text{eBioscience, San Diego, CA, USA})\) following the manufacturer’s instructions.

Analysis of mRNA expression

Total RNA was isolated using TRIzol \((\text{Life Technologies, Gaithersburg, MD, USA})\) according to the manufacturer’s instructions. The levels of TLR2, TLR4, TLR7, and MyD88 mRNA expression in different groups was determined by real-time quantitative PCR \((\text{qPCR})\) with an ABI Prism 7000 Sequence Detection System \((\text{Applied Biosystems-Life Technologies, Foster City, CA, USA})\) using GAPDH as an internal standard. The specific primers used were as follows: GAPDH, 5’-ATC CCA TCA CCA TCT TCC AGG-3’ (sense), 5’-GAG CCC CAG CCT TCT CCATG-3’ (anti-sense); TLR-2, 5’-TCA CTC AGG AGC AGC AAGCA-3’ (sense), 5’-TGT GAC ATT CCG ACA CCG AGA-3’ (anti-sense); TLR-4, 5’-AAT CCC CTC AGG CAT TTAGG-3’ (sense), 5’-CAG GCC TAA ACT CTC GATGG-3’ (anti-sense); TLR-7, 5’-AGC TTT AAC CTC TCG CCA TTACA-3’ (sense), 5’-TTG AGC AGA AGC CAA CT T CACT-3’ (anti-sense); MyD88, 5’-GGA CAT GGG CAC ATA CAGAC-3’ (sense), 5’-GAC ATG GTT AGG CTC CCTCA-3’ (anti-sense); STAT1, 5’-ATC AGG CTC AGT CCG GGA ATA-3’ (sense), 5’-TGG TCT GGT GTT CTC TGT TCT-3’ (anti-sense); STAT6, 5’-GTT CCG CCA CTT GCC AATG-3’ (sense), 5’-TGG ATC TCC CCT ACT CGGTG-3’ (anti-sense). Values for TLR2, TLR7, MyD88, STAT1 and STAT6 were normalized against those for GAPDH.

Data analysis

Data are presented as mean ± SD and are representative of at least two independent \textit{in vitro} experiments. The unpaired Student’s t-test or Fisher’s exact test, were used, as appropriate, for statistical analysis. Statistical significance was set at \(P < 0.05\).
Results

No significant difference in plasma cytokines between the regular exercise (RE) and the sedentary control (SC) groups

Cytokines are essential components of the immune system and play an important role in initiating and regulating immune responses. IFN-γ, TNF-α and IL-6 expression plays a central role in host defense. Thus, we first detected plasma levels of IFN-γ, TNF-α and IL-6. There were no significant differences in plasma levels of IFN-γ, TNF-α and IL-6 between the RE and SC groups (Fig. 1A–C).

Reduced frequencies of invariant natural killer T cells and NK cells in peripheral blood in the RE group

It has been reported that exercise may induce changes in the proportion of peripheral blood mononuclear cell (PBMC) subsets [18, 19]; thus we detected the frequencies of PBMC subsets to investigate the influence of regular exercise on immune cells. Flow cytometric analysis of T lymphocyte subpopulations including CD3+ T cells, CD4+ T cells, CD8+ T cells and Treg cells (CD4+CD25+hi) showed that there were no significant differences between the RE and SC groups (Fig. 2A–D). However, the frequency of invariant natural killer T (iNKT) cells in PBMC was significantly reduced in the RE group compared to that in the SC group (Fig. 2E). The frequency of NK cells in PBMC presented a similar trend to that for iNKT cells (Fig. 2F).

Increased levels of Th1 and Th2 cytokines secreted by PBMC in the RE group after HBcAg and S. pyogenes stimulation

To explore the immune responses against pathogen invasion, we determined the cytokine levels in the culture supernatants of hepatitis B core antigen (HBcAg) or heat-inactivated Streptococcus pyogenes (S. pyogenes) stimulated PBMCs. Levels of Th1 (IFN-γ and TNF-α) and Th2 (IL-6) cytokines were increased in the RE group after HBcAg and heat-inactivated S. pyogenes stimulation compared with those in the SC group (Fig. 3A and B). These results suggested that a more effective immune response was induced to prevent pathogen invasion in the RE group.

Decreased cytokine production by PBMC after non-microbial exogenous antigen stimulation in the RE group

To determine the effects of regular exercise on the immune response to a series of antigen stimuli, we also investigated cytokine production following non-microbial antigen stimulation. In this study, we used iNKT cell activation (α-galactosylceramide, α-GC) and pan-T cell stimulation (concanavalin A, ConA) as non-microbial antigen stimulation. The

![Fig. 1. Plasma cytokine levels in the RE and SC groups. Plasma levels of (A) IFN-γ, (B) TNF-α, and (C) IL-6 in the RE and SC groups were analyzed by ELISA. Data represent the mean ± SD.](image-url)
production of TNF-α and IL-6 in response to α-GC stimulation was reduced in the RE group compared with that in the SC group (Fig. 3C). However, there was no significant difference in the levels of IFN-γ between the two groups (Fig. 3C). ConA was administered in PBMC cultures as a general stimulus of T cells [20]. After ConA stimulation, the levels of TNF-α and IL-6 were decreased in the RE group compared to those in the SC group, while no changes in IFN-γ levels were observed (Fig. 3D).

Moreover, cytokine secretion by PBMC was observed in the absence of any stimulation. Consistent with the cytokine profiles after non-microbial antigen stimulation, in the absence of stimulation, the levels of TNF-α and IL-6 were reduced in the RE group compared with those in the SC group, while there was no difference in the levels of IFN-γ between the two groups (Fig. 3E). In addition, in both groups there was an upward trend in the levels of IFN-γ, TNF-α and IL-6 after α-GC stimulation. However, this did not reach statistical significance (Fig. 4A-C). These data suggest that, in the absence of microbial invasion, and therefore not at the stage of infectious burden, the cytokine production ability of the RE group is reduced compared with that of the SC group, which may account for the absence of excessive inflammatory reactions in the RE group.
**Dendritic cell function in RE and SC groups**

Antigen presentation by antigen-presenting cells (APC) stimulates T cells to produce Th1 and Th2 cytokines. As dendritic cells (DC) are important APCs, we examined the frequency of this cell subset. There were no significant differences in the frequencies of DC subpopulations (BDCA-1⁺DCs, BDCA-2⁺DCs, and BDCA-3⁺DCs) between the RE and SC groups (Fig. 5A).

To determine whether the function of DCs is changed in the RE group, we detected the production of IFN-α and IL-12 which are mainly produced by DCs. The levels of IFN-α and IL-12 secreted by PBMC after HBcAg or *S. pyogenes* stimulation were increased in the RE group compared with those in the SC group (Fig. 5B), while there were no significant differences between the two groups in the secretion of IFN-α and IL-12 after ConA and α-GC stimulation (Fig. 5C).

**Increased expressions of TLRs in the RE group**

APCs recognize pathogenic antigens by interacting with pathogen recognition receptors (PRR), which are highly expressed on immune cells [21]. Some antigens from Gram-positive
bacteria activate TLR2 signaling and induce antimicrobial peptide expression [22, 23]. Furthermore, the viral HBCag activates TLR7 signaling and recruits heterologous T cells [24]. Thus, we next analyzed TLR2, TLR7, and MyD88 expression. We found that TLR2, TLR7 and MyD88 expression was significantly increased in the RE group compared with that in the SC group without any stimulation (Fig. 6A). Moreover, TLR2, TLR7 and MyD88 expression was elevated in the RE group compared with that in the SC group after HBcAg stimulation (Fig. 6B). Following *S. pyogenes* stimulation, TLR2 and MyD88 expression was significantly increased, while TLR7 expression was decreased in the RE group compared with that in the SC group (Fig. 6C). Furthermore, TLR7 expression was elevated in the RE group compared with that in the SC group, while no obvious differences were detected in the expression of TLR2 and MyD88 between the two groups after ConA or α-GC stimulation (Fig. 6D and E, TLR signaling pathway molecule expression in the RE group following ConA or α-GC stimulation.). In addition, analysis of the expression of TLR4, which is associated with Gram-negative bacterial invasion, revealed no significant differences between the RE and SC groups following stimulation with HBcAg, *S. pyogenes*, ConA or α-GC (Fig. 6F, TLR4 expression in the RE group with or without stimulation with microbial antigens).

Th1 cells differentiation is dependent on STAT1-mediated signaling [25, 26], while STAT6 is required for development of Th2 cells [27]; therefore, we analyzed the expression of STAT1 and STAT6 in PBMC in response to different stimuli. STAT1 expression was significantly increased in the RE group compared with that in the SC group after both *S. pyogenes* and
HBcAg stimulation, while there were no obvious differences in STAT1 expression between
the two groups without stimulation or after ConA or α-GC stimulation (Fig. 7A-E, STAT1
signaling pathway molecule expression in the RE group following ConA or α-GC stimulation).

Fig. 6. TLR signaling pathway molecule expression with HBcAg, S. pyogenes, ConA and α-GC stimulation. Re-
al-time quantitative PCR analysis of TLR2, TLR7 and MyD88 mRNA expression levels in the RE group versus
the SC group (A) without stimulation and stimulated with (B) HBcAg or (C) S. pyogenes, (D) ConA and (E)
α-GC. (F) The mRNA expression of TLR4 were detected in the RE group versus the SC group with or without
stimulation. Data represent the mean ± SD.*, P < 0.05, **, P < 0.01, ***,***, P < 0.001.

Fig. 7. STAT signaling pathway molecule expression with HBcAg, S. pyogenes, ConA and α-GC stimulation.
The mRNA expression of STAT1 and STAT6 was detected in the RE group versus the SC group (A) without
stimulation and stimulated with (B) HBcAg, (C) S. pyogenes, (D) ConA and (E) α-GC stimulation. Data repre-
sent the mean ± SD.*, P<0.05, **, P < 0.01.

HBCAg stimulation, while there were no obvious differences in STAT1 expression between
the two groups without stimulation or after ConA or α-GC stimulation (Fig. 7A-E, STAT1
signaling pathway molecule expression in the RE group following ConA or α-GC stimulation).
There were no significant differences in STAT6 expression between the two groups with or without stimulation (Fig. 7A-E, STAT6 signaling pathway molecule expression in the RE group following ConA or α-GC stimulation).

**Discussion**

Some studies have indicated that endurance exercise provides an "open window" of increased susceptibility to infections, especially during the competition season [7, 28]. Recently, a series of studies have demonstrated that regular moderate exercise can decrease the rate of upper respiratory tract infections [6] and that regular exercise can induce slight changes in the immune system [14, 29]; thus, indicating an immunomodulatory role for exercise [15, 30]. However, the immune changes induced by regular exercise remain to be clarified. In this study, we recruited members of a university badminton club to investigate the effects of regular exercise on the immune responses induced by microbial stimuli.

First, we found that not only the proportions of iNKT and NK cells were reduced in the peripheral blood, but also cytokine secretion by PBMC was suppressed after α-GC and ConA stimulation in the RE group compared with the SC group. Cytokines are essential components of the immune system and play an important role in initiating and regulating immune responses. In the early phase of infectious inflammation, IL-6 is produced by monocytes and macrophages immediately after the stimulation of TLRs by distinct pathogen-associated molecular patterns (PAMPs) [31]. This acute IL-6 expression plays a central role in host defense by stimulating various cell populations. TNF-α is an important Th1-type cytokine required to eliminate antigens [32]. Low levels of TNF-α and IL-6 after α-GC and ConA stimulation indicated that regular moderate exercise induced the suppression of iNKT cell and T cell activation. Meanwhile, we compared the levels of cytokines secreted by PBMC with or without stimulation and found that, with the exception of stimulation with α-GC, the levels of IFN-γ, TNF-α and IL-6 were significantly increased after stimulation in both groups (Fig. 4). As α-GC is a prototypical activator of iNKT cells, we speculate that the low levels of cytokines secreted by PBMCs after α-GC stimulation was a consequence of low numbers of iNKT cells in PBMCs. These observations provide evidence that regular exercise ameliorates inflammatory reactions by suppressing lymphocyte activation in a generalized state and not at the stage of infection. It can be speculated that this effect reduces the inflammation associated with hyper immune reactions, such as allergy and autoimmune disease.

In this study, we used HBCAg and *S. pyogenes* as microbial antigens to investigate the effect of physical training on the immune response to pathogenic infections. We found that the levels of IFN-γ, TNF-α and IL-6 secreted by PBMC were increased in the RE group after HBCAg and *S. pyogenes* stimulation. Responses to HBCAg and *S. pyogenes* stimulation may be influenced by previous infection by hepatitis B virus or *S. pyogenes*. In order to control for this, we measured the titers of HBcAb and ASO in our volunteers. We found that titers to HBcAb were negative of them, probably as a consequence of universal HBV vaccination in China. Two subjects in each group had positive ASO titers (P = 0.7397, Table 1). The levels of cytokines secreted by PBMCs and the mRNA expression level of the TLR signaling pathway molecules in both subjects in the RE group were higher than those in the two subjects in the SC group, except for TLR7, TLR4 and STAT6. These results were consistent with the trends in the two groups (Fig. 8). These data indicate that these antigens induce an enhanced Th1 and pro-inflammatory cytokine response. Antigen presentation by APCs is required for the activation of effector T cells by HBCAg and *S. pyogenes* [33, 34]. Previous studies have demonstrated that plasmacytoid DCs are decreased in athletes after running a marathon, and that long-term intensive training may affect the function of innate immune cells, in particular, reducing their capacity to respond to acute challenges, and possibly contributing to an increased risk of infection [35, 36]. Moreover, IFN-α and IL-12 derived from DCs play an essential role in Th1 cell differentiation and protecting against pathogen invasion [37]. Thus, in this study, we analyzed the frequencies of DC subsets in peripheral blood. Although no differences were observed between the RE and HR groups, the levels of IFN-α and IL-12 in
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Culture supernatants of PBMC stimulated in vitro with *S. pyogenes* or HBcAg were increased in the RE group compared with the SC group. However, no obvious differences were observed between the two groups following α-GC and ConA stimulation. Increased levels of IFN-α and IL-12 could significantly enhance the T cell response against pathogens.

TLRs are key sensors of bacteria, viruses, fungi and protozoa [38]. A series of studies have suggested that some antigens from Gram-positive bacteria activate TLR2 signaling and induce antimicrobial peptide expression [22, 23]. Also, HBcAg activates TLR7 signaling and recruits heterologous T cells [24]. Individual TLR signaling pathways are divergent, although the MyD88 signaling pathway is common to most TLRs. MyD88-deficient mice do not produce inflammatory cytokines such as TNF-α and IL-12p40 in response to stimulation by any of the TLR ligands [39]. Thus, in our subsequent examination of the expression of TLR2, TLR7 and MyD88, we found that expression of all three was significantly increased in the RE group with or without microbial antigen stimulation. The increased expression of TLRs could activate T cells via DC intrinsic and extrinsic mechanisms [40], which further confirms regular exercise induces a more effective immune response to prevent pathogen invasion.

In conclusion, we evaluated the changes in immune responses to microbial antigens induced by regular moderate exercise. In the absence of infection, the proportion of NKT and NK cells was decreased, and their activation was suppressed. These observations indicate a generalized suppression of inflammatory lymphocyte activation following regular exercise. However, regular exercise enhances the response to pathogen invasion through upregulation of the antigen-presenting function of DCs via the TLR signaling pathway. This process activates effector cells to produce substantial amounts of cytokines, which may enhance resistance to the invasion of infectious pathogens. We propose that this mechanism, to some extent, explains why regular exercise enhances resistance to pathogen invasion.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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