



Adaptation of macrophages to exercise training improves innate immunity

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ABSTRACT

The effects of 3-week exercise training on the functions of peritoneal macrophages from BALB/c mice were investigated. Lipopolysaccharide (LPS)-stimulated nitric oxide (NO) and proinflammatory cytokine production in macrophages from trained mice was markedly higher than those from control mice. Meanwhile, exercise training decreased the steady state level of β_2 -adrenergic receptor (β_2 AR) mRNA in macrophages. Overexpression of β_2 AR in the macrophage cell line RAW264 by transfecting with β_2 AR cDNA suppressed NO synthase (NOS) II expression but dose not influenced proinflammatory cytokine expression. When expression of transfected β_2 AR in RAWar cells was downregulated by a tetracycline repressor-regulated mammalian expression system, NOS II mRNA expression was significantly increased; this suggested that the changes in the β_2 AR expression level in macrophages associated with exercise training play a role in the regulation of NO production following LPS stimulation. These findings indicate that exercise training improves macrophage innate immune function in a β_2 AR-dependent and -independent manner.

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Physical training is associated with cardiovascular adaptations such as bradycardia at rest [1] and lower heart rate and blood pressure responses during submaximal exercise [2]. These phenomena may be explained at least in part by a decrease in the number of myocardial β_2 -adrenergic receptors (β_2 AR) causing reduced sympathomimetic effects [3]. In in vitro experiments, prolonged agonist exposure induced the loss of β_2 AR from the cell surface [4]. In addition, 24-h integrated plasma catecholamine concentrations are greater in physically trained men than in untrained men [5]. Therefore, physical training seems to induce the loss of β_2 AR and the attenuation of cellular responsiveness to sympathoadrenomedullary activity. Primary and secondary lymphoid organs, such as the thymus, spleen, and lymph nodes, receive extensive sympathetic/noradrenergic innervation, and lymphocytes, macrophages, and many other immune cells bear functional β_2 AR. β_2 AR is a member of a family of G protein-coupled receptors and is the key link to immune system regulation via the sympathetic nervous system [6]. It has been reported that the number of β_2 AR on lymphocytes also decreases during endurance training in comparison with sedentary controls, suggesting that adaptations of lympho-

cytes to the exercise-induced increase of catecholamines occur during long-term exercise training [7]. Although several investigators have demonstrated a correlation between hormone or neuropeptide levels and the immune response to acute exercise, fewer studies have attempted to evaluate the immunomodulatory role of the adaptations of immune cells to chronic exercise training.

It has been hypothesized that moderate exercise may increase the activity of various immune cell parameters and thus decrease the risk of infection, whereas intense exercise decreases the activity of the same parameters and increases the risk of infection [8]. However, the mechanisms responsible for enhanced immune response resulting from long-term exercise training have not been elucidated. Further, most studies addressing the direct effects of catecholamines and the sympathetic nervous system on immunity have examined their effects on adaptive immunity [9], but little is known about adrenergic effects on innate immunity. Alterations in susceptibility to infection have been associated with changes in various immune cell parameters, especially those of the innate immune system. Macrophages are important cellular mediators of innate immune defense and the first line of defense against microbial invaders by producing various cytokines and antimicrobial mediators.

In the current study, we observed that moderate treadmill training upregulated stimulant-induced production of nitric oxide

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(NO) and cytokines essential for antimicrobial defense in cells of the monocyte/macrophage lineage, suggesting the reduced risk of infection. On the other hand, we found that the steady state level of β_2 AR on monocytes/macrophages decreased after training. To determine whether changes in steady state levels of β_2 AR expression were involved in the regulation of NO and cytokines production, we established macrophage cell lines expressing high or low levels of β_2 AR and analyzed the effects of β_2 AR levels on macrophage functions.

Methods

Mice. Male BALB/c mice (8 weeks old) were obtained from Japan SLC Inc. (Shizuoka, Japan). The animals were cared for in accordance with the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan, based upon the Declaration of Helsinki, 1964. The mice were divided randomly into control and treadmill-trained groups of 5 mice each. Animals in both groups were reared at 25 °C with a 12 h light/dark cycle (lights off at 7 PM). Food and water were available ad libitum. In past studies, moderate exercise has been defined as brief (usually 15–60 min) bouts of treadmill running at 50–75% maximum O_2 consumption or 15–22 m/min [10]. Thus, in the current study, mice were made to run at 18 m/min, 30 min/day (as a moderate exercise), and 5 days/week for 3 weeks.

Cell preparation and culture. About 24 h after the last session, the peritoneal cells were harvested from 5 mice by sterile lavage. In some experiments, cells from 5 mice were pooled because the number of resident macrophages in the peritoneal cavity of one mouse was not sufficient for the experiments. The cells were suspended in RPMI 1640 (Sigma–Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine (Sigma–Aldrich) and incubated in polystyrene dishes for 2 h. Adherent cells (macrophages/monocytes) were collected by incubation with phosphate-buffered saline (PBS) containing 2.5 mM EDTA at 5 °C for 15 min and washed three times.

The murine macrophage cell line RAW264 (RCB0535) was purchased from the RIKEN Cell Bank (Ibaraki, Japan) and cultured as described in our previous study [11].

Immunofluorescence staining and flow cytometry. Flow cytometric analysis was carried out as described previously [12] using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Prior to the immunofluorescence test, the peritoneal cells (1×10^6) were incubated with mouse immunoglobulin (Ig) in PBS at 5 °C for 30 min in order to avoid nonspecific binding to FcR. Thereafter, the cells were treated with phycoerythrin-conjugated anti-CD11b or anti-F4/80 monoclonal antibodies (mAbs) (Invitrogen, Carlsbad, CA) or fluorescein isothiocyanate-conjugated anti-CD36 mAbs (Becton Dickinson).

Assay of intracellular growth of *Listeria monocytogenes*. *L. monocytogenes* 104035 was cultured in brain heart infusion (BHI) broth. Adherent cells from the control ($n = 5$) or exercise-trained mice ($n = 5$) were pooled and were infected with *L. monocytogenes* at the multiplicity of infection. The cells were incubated at 37 °C for 15 min and washed 3 times with PBS to remove the suspended bacteria. RPMI 1640 supplemented with 2% FBS and 5 mg of gentamicin/ml was added to kill extracellular bacteria. After 1 h incubation at 37 °C, macrophages were lysed with 1 ml ice cold 0.1% Triton X-100. Triplicate samples were plated individually on BHI agar plates after appropriate dilution.

Cytokine assay. Adherent cells from control and treadmill-trained mice or RAW 264 cells were cultured at 37 °C for 24 h in the presence or absence of 1 μ g/ml lipopolysaccharide (LPS) from *Escherichia coli* 055 (Sigma–Aldrich, St. Louis, MO). Interferon- γ

(IFN- γ), tumor necrosis factor- α (TNF- α), and interleukin-10 (IL-10) concentrations were determined by an enzyme linked immunosorbent assay (ELISA) kit (BioSource International, Inc., Camarillo, CA) according to the manufacturer's instructions.

Determination of nitrite concentration. Nitrite in the cell culture supernatants was determined with Griess reagent [13] by using a sodium nitrite as a standard.

Western blotting analysis. Cell membrane proteins were prepared using the Plasma Membrane Protein Extraction Kit (Bio Vision, Mountain View, CA). Cytoplasmic protein extracts were prepared as described [14]. The protein concentration was determined using the Bradford reagent (Bio-Rad, Hercules, CA) and equal amounts of membrane proteins or cytoplasmic proteins were loaded. The samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride membranes (Applied Biosystems, Foster City, CA). The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS) and incubated with goat polyclonal Abs against β_2 AR (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit polyclonal Abs against NOS II (Upstate Biotechnology, Lake Placid, NY); this was followed by incubation with the appropriate secondary Abs (horseradish peroxidase-conjugated rabbit anti-goat or goat anti-rabbit IgG; DAKO, Kyoto, Japan). Immunoreactivity was visualized using an enhanced chemiluminescence reagent (ECL; GE Healthcare Bio-Science, Piscataway, NJ).

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total cellular RNA was prepared from cells using the TRIzol Reagent (Invitrogen) and 2 μ g aliquots were reverse transcribed with ReverScript I (Wako Pure Chemical, Osaka, Japan) and oligo(dT)15 primer (Roche Diagnostics, Indianapolis, IN) at 42 °C for 50 min. The reaction mixture was used directly for the polymerase chain reaction (PCR) reaction with oligonucleotide primers listed in [Supplemental table](#).

β_2 AR plasmid constructs. Full length murine β_2 AR (β_2ar) cDNA was obtained by PCR using the primers, 5'-GCTGAATGAAGCTTC CAGGA-3' (sense) and 5'-GAGTAGAAAGCCTGTATTACAGTGGC GAGT-3' (antisense). The amplified β_2 AR fragments were subcloned in the pGEM-T Easy vector (Promega, Madison, WI) and then in the NotI-digested pcDNA4/TO/myc-His B vector (Invitrogen). The amplified PCR products were sequenced with an automatic DNA sequencer (Applied Biosystems). Plasmid DNA used for transfection was prepared using an EndoFree Plasmid Kit (Qiagen, Hilden, Germany).

Stable transfection of β_2 AR. RAW264 cells were transfected with pcDNA4/TO/myc-HIS B vector alone or pcDNA4/TO/myc-HIS B- β_2ar with the Lipofectamine reagent (Invitrogen). Selection was initiated in medium containing 500 μ g/ml zeocine (Invitrogen). RAWar cells were transfected with the pcDNA6/TR vector (Invitrogen) and selection was initiated in medium containing 1.25 μ g/ml blasticidin (Invitrogen).

Statistical analysis. The results were expressed as means \pm SEM. When 2 means were compared, Student's *t*-test for unpaired samples was used. For more than 2 groups, the statistical significance of the data was assessed by analysis of variance (ANOVA). When significant differences were found, individual comparisons were made between groups by using the *t*-statistic and adjusting the critical value according to the Bonferroni method. Differences were considered significant at $P < 0.05$.

Results and discussion

Effects of exercise training on peritoneal macrophages

Effects of exercise training on the peritoneal cell population were analyzed by flow cytometric analysis. Profiles of immunoflu-

orescence staining with mAbs specific to CD11b, F4/80, or CD36 on peritoneal cells from control and exercise-trained mice were compared (Supplemental figure). The expression patterns of cell-surface molecules stained with mAbs against F4/80 or CD36 appeared to be unaffected by exercise training. On the other hand, two distinct cell populations (CD11b^{high} and CD11b^{low}) could be seen in both the control and exercise-trained mice; the proportion of CD11b^{high} cells among the peritoneal cells from exercise-trained mice being lower than those from the control mice (control: $62.7 \pm 3.9\%$, trained: $50.6 \pm 1.6\%$). In addition, the mean density of the CD11b molecule in the CD11b^{high} cells was significantly decreased in exercise-trained mice (control: 900 ± 56 , trained: 524 ± 69). These findings suggested that exercise training influences the peritoneal macrophage phenotype and that peritoneal macrophages adapt to exercise training during 3 weeks of moderate treadmill training.

Macrophages are increasingly implicated as essential players in defense against a range of microbial pathogens. Innate immunity is rapidly triggered following infection, and this results in restriction of microbial growth in vivo. To examine the effect of exercise on microbicidal activities of peritoneal macrophages, listericidal activities were analyzed. The number of viable *Listeria monocytogenes* (LM) cells found within macrophages from exercise-trained mice decreased significantly, whereas that within macrophages from control mice did not decrease (Fig. 1), suggesting that moderate levels of exercise training enhances bactericidal activities.

In mice infected with LM, proinflammatory cytokines are produced and contribute to the host defense at an early stage of infection [15–17]. IFN- γ is well known as the most important cytokine for host defense against LM since antilisterial resistance was enhanced by the in vivo administration of recombinant IFN- γ [18]. This defense was eliminated in mice that lacked IFN- γ [19] or the IFN- γ receptor [20]. Likewise, TNF- α is required for normal resistance against LM since neutralization of TNF- α decreases macrophage activation and increases of listerial growth [21]. In addition, administration of the NOS II inhibitor aminoguanidine and genetic deficiency in NOS II render mice more susceptible to LM [22,23], indicating that NO plays an important role in bacterial clearance. As shown in Fig. 2A, IFN- γ and TNF- α secretion and NO production following LPS stimulation were significantly increased in macrophages from exercise-trained mice. Further, addition of exogenous IFN- γ and TNF- α markedly enhanced NO production by macrophages from control mice stimulated with LPS (Fig. 2B). These findings suggest that the moderate exercise training-associated increase in IFN- γ and

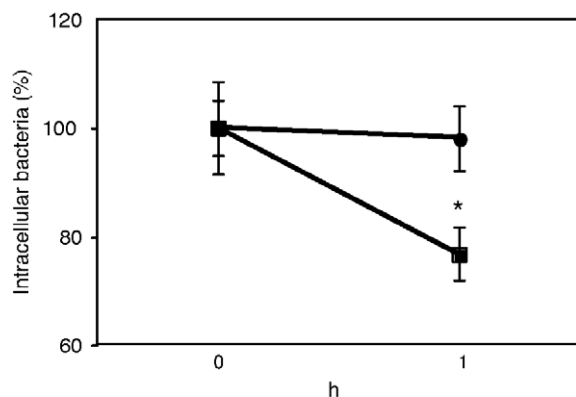


Fig. 1. Effects of exercise training on the populations of peritoneal cells. Expression of CD11b, F4/80, and CD36 on peritoneal adherent cells from control ($n = 5$) or exercise-trained mice ($n = 5$) was analyzed by flow cytometry.

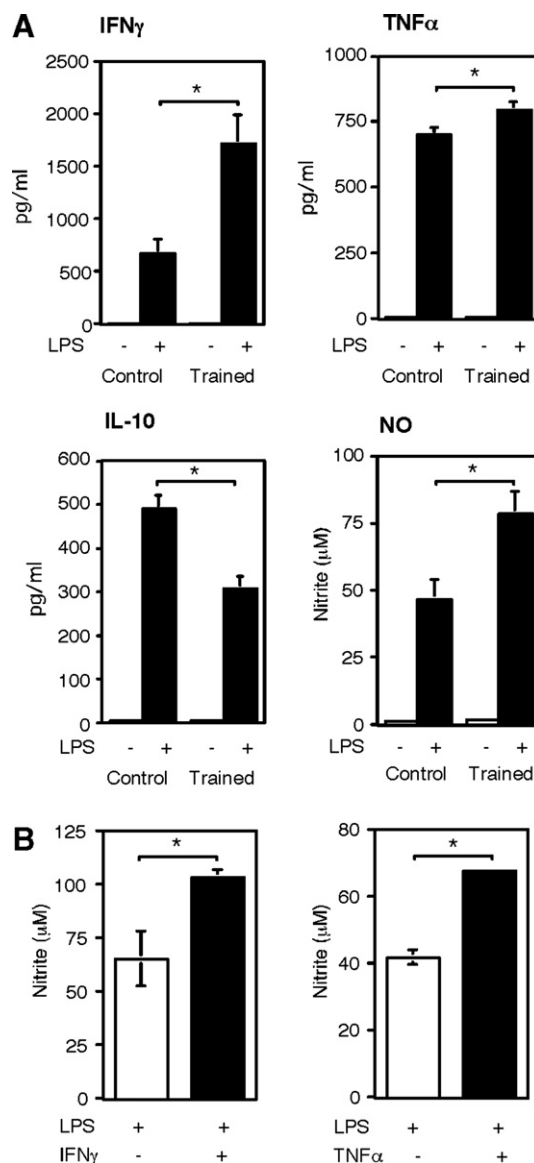


Fig. 2. Effects of exercise training on cytokine production by peritoneal macrophages stimulated with LPS. (A) Adherent cells from control ($n = 5$) and exercise-trained ($n = 5$) mice were cultured for 24 h in the presence or absence of 1 μ g/ml LPS. Concentrations of IFN- γ , TNF- α and IL-10 in cell-culture supernatants were determined by an ELISA. The nitrite concentration in cell-culture supernatants was determined with the Griess reagent. Results were expressed as means \pm SEM. *Significant difference compared to control ($P < 0.05$). (B) Peritoneal adherent cells from control mice were stimulated with LPS for 24 h in the presence or absence of IFN- γ or TNF- α . Results were expressed as means \pm SEM. *Significant difference compared to LPS alone ($P < 0.01$).

TNF- α production in response to LPS leads to sufficient expression of NOS II gene and an improvement in the NO-mediated innate immune response to microbial infection.

Host defense against microbial infection is dependent upon the coordination between innate and adaptive immune responses. The immune system defense against intracellular infection is mediated by the helper function of type 1 helper T cells (Th1). Early IFN- γ release contributes to the differentiation of T cells to Th1 cells [24]. Thus, the initial production of IFN- γ is important for generating adaptive immunity as well as for innate defense against LM infection. On the contrary, IL-10, a type of Th2 cytokine, promotes a Th1 to Th2 shift and suppresses antilisterial resistance [25,26], suggesting that the downregulation of IL-10 production seen in the peritoneal cells from exercise-

trained mice (Fig. 2A) may contribute to Th1-type adaptive immune responses against LM infection.

Effects of β_2 AR overexpression on cytokines and NO production in RAW264 cells

In agreement with earlier reports showing that β_2 AR on mononuclear lymphocytes was downregulated after endurance training [27,28] or after long-term infusion of an adrenergic agonist [29], the level of β_2 AR mRNA in peritoneal macrophages from 3-week-treadmill-trained mice was lower than that in those from control mice (Fig. 3A). To determine whether changes in β_2 AR level influence the macrophage function, we established a stable β_2 AR transfectant (RAWar) and a vector control (RAWvec). The levels of β_2 AR mRNA

and protein in RAWar cells were markedly higher than those in RAWvec cells (Fig. 3B).

We then examined whether the difference in β_2 AR expression was responsible for the exercise-associated modulation of cytokines and NO production. Although the mRNA expression levels of IFN- γ , TNF- α and IL-10 were not influenced by overexpression of β_2 AR, NOS II mRNA expression following LPS stimulation was significantly lower in RAWar cells than that in RAWvec cells (Fig. 3C). NO production and NOS II protein expression were also lower in RAWar cells than in RAWvec cells (Fig. 3D). These findings suggest that overexpression of β_2 AR decreases NOS II gene transcription, resulting in decreased NO accumulation in the culture supernatants following LPS stimulation.

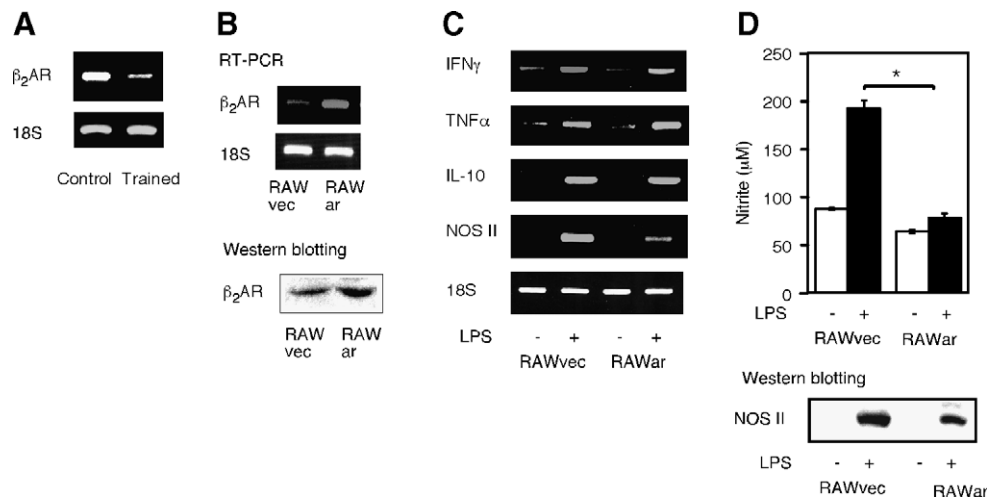


Fig. 3. Effects of the β_2 AR expression level on cytokine and NO production. (A) Adherent cells from control ($n = 5$) or exercise-trained mice ($n = 5$) were pooled. Total cellular RNA was prepared from the adherent cells of the control and exercise-trained mice, and β_2 AR mRNA expression was analyzed by RT-PCR. For normalization, 18S RNA was used. (B) Expression of β_2 AR mRNA in RAWvec or RAWar cells was analyzed by RT-PCR. For normalization, 18S RNA was used. (C) RAWvec or RAWar cells were stimulated with LPS for 24 h and cytokines and NOS II mRNA expression were analyzed by RT-PCR. For normalization, 18S RNA was used. (D) RAWvec or RAWar cells were stimulated with LPS for 24 h and nitrite concentrations in the culture supernatants were measured with the Griess reagent (upper panel). Results are expressed as means \pm SEM of triplicate cultures. * $P < 0.01$. Expression of NOS II protein was analyzed by Western blotting using anti-NOS II antibodies (lower panel).

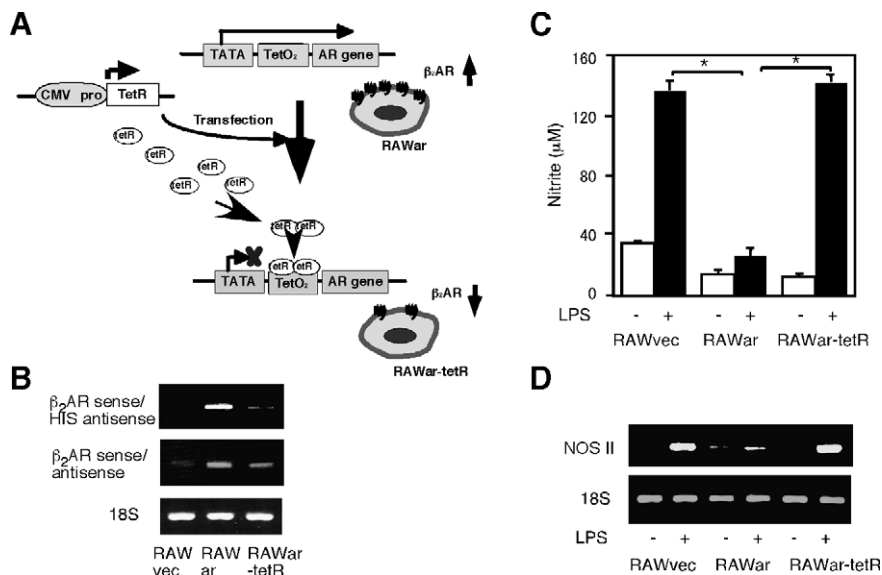


Fig. 4. Effects of downregulation of transfected β_2 AR by a tetracycline repressor-regulated mammalian expression system on NO production and NOS mRNA expression. (A) Schematic illustration of the tetracycline repressor-regulated mammalian expression system. (B) The expression of transfected and intrinsic β_2 AR mRNA in RAWvec, RAWar, and RAWar-tetR cells was analyzed by RT-PCR. For normalization, 18S RNA was used. (C) Cells were stimulated with LPS for 24 h and accumulation of nitrite in the supernatants was measured with the Griess reagent. Results are expressed as means \pm SEM from triplicate cultures. A value of * $P < 0.05$ was considered significant. (D) Cells were stimulated with LPS for 24 h, and NOS II mRNA expression was analyzed by RT-PCR (lower panel). For normalization, 18S RNA was used.

Effects of the downregulation of transfected β_2 AR by a tetracycline repressor-regulated mammalian expression system on NO production in RAW264 cells

The expression vector pcDNA4 contains a tetracycline operator region, whereas pcDNA6/TR expresses tetracycline repressor and inhibits the expression of transfected β_2 AR (Fig. 4A). To mimic the exercise training-associated downregulation of β_2 AR, RAWar cells were transfected with pcDNA6/TR, resulting in a stable transfectant, namely, RAWar-tetR cells. The expression of transfected β_2 AR, although not completely inhibited, was markedly downregulated in RAWar-tetR cells compared with RAWar cells (Fig. 4B, upper panel). As a result, the total β_2 AR mRNA was also lower in RAWar-tetR cells compared with RAWar cells (Fig. 4B, middle panel). As expected, NO production (Fig. 4C) and NOS II mRNA expression (Fig. 4D) were overtly higher in RAWar-tetR cells following stimulation with LPS than in RAWar cells. It has been reported that stimulation of β_2 AR by catecholamines increase cAMP, and PKA activation inhibits NF- κ B-induced transcription by phosphorylating the cAMP responsive element binding protein (CREB), which competes with p65 for the limited amounts of CREB-binding protein (CBP) [30]. However, it is likely that the regulation of NOS II mRNA expression observed in the current study was not determined by the catecholamine concentration but by the level of β_2 AR expression in these cells, because RAW264vec, RAWar, and RAWar-tetR cells were cultured in the same culture medium which contained only catecholamines resulted from fetal calf serum. In addition, we demonstrated that β_2 AR density influences NF- κ B activation through β -arrestin 2 [14]. Macrophages from control and exercise-trained mice were also stimulated with LPS in the same culture medium. Therefore, some immunomodulatory effects of exercise training may be attributable to the density of β_2 AR in peritoneal macrophages.

In the current study, we demonstrated that macrophage adaptation to moderate exercise training improves microbicidal activity and Th1-type responsiveness to activating signals in a β_2 AR-dependent and β_2 AR-independent manner. On the other hand, we could not elucidate the relationship between the alteration in cell population in terms of the CD11b expression and peritoneal cell functions. It has been reported that CD11b⁺ cells were able to produce NO in large quantities upon IFN- γ activation [31]. It appears, therefore, that there is DC11b-dependent regulation of NOS II expression. Further studies are required to elucidate the mechanisms underlying the exercise training-associated alteration of macrophage functions, including the role of CD11b molecules in the regulation of NOS II expression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.05.005.

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