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Exercise enhances vaccine induced antigen specific T cell responses^{1,2}

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Abstract

Regular moderate exercise has been proposed to enhance immune function, but its effects on immunity and their consequences have not been well studied. Mice without (AL) or with access (AL+EX) to voluntary running wheels were vaccinated with a model antigen (ovalbumin (OVA)) via intranasal or subcutaneous routes to target the mucosal and systemic immune compartments, respectively. EX enhanced OVA-specific CD4⁺ T cell cytokine production and proliferation in all lymphoid organs examined without changes in cell distribution in any organ. These results suggest that coupling moderate exercise with vaccination may enhance vaccine efficacy for the prevention and/or therapy of numerous diseases.

Keywords

adaptive immunity; cytokines; cell proliferation

1. Introduction

The favorable effects of a physically active lifestyle on a number of physiological processes, including cardiovascular function and insulin sensitivity, and the concomitant reduction in disease outcomes such as coronary heart disease, hypertension and diabetes are well

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documented [1, 2]. However, the effects of physical activity on immune function and the downstream consequences on disease risk have been studied to a lesser extent. The current hypothesis to explain the relationship between exercise and immune function is the Inverted J Hypothesis [3], which proposes that regular, moderate exercise enhances immune function, and in turn, may reduce the incidence of infectious disease and cancer. In contrast, physical inactivity and intense, exhaustive exercise at opposite ends of the curve both suppress immune function and may increase disease risk.

Although it has been proposed that moderate physical activity may provide protection from the incidence of infectious disease via an enhancement of immunity, few studies have addressed this question. Clinical and epidemiological studies demonstrate that the incidence of upper respiratory tract infections (URTI)⁷ [4–7] and the severity of symptoms [8–12] are significantly lower in moderately active individuals as compared to their sedentary counterparts. In animal studies, the survival rates following infection with *Salmonella typhimurium* [13] or influenza virus [14] are higher in active mice as compared to sedentary controls. However, immune function was measured in only two of the studies in which the incidence and severity of symptoms of URTI were reduced with moderate activity [6, 12]. Both studies demonstrate an increase in the mucosal antibody response (i.e. salivary IgA concentration) in moderately active individuals [6, 12]. Several other studies report an elevation in mucosal IgA in moderately active young [15, 16] and older adults [17] but did not measure URTI or other clinical endpoints. Finally, we have demonstrated that moderate exercise enhances mucosal T cell proliferation and cytokine production in response to concanavalin A (Con A) stimulation in mice [18]. The limited work in this area suggests that moderate exercise enhances antigen independent measures of immune function, e.g. total IgA and mitogen-induced T cell responses. However, no studies have examined the effect of moderate exercise on antigen-specific mucosal immunity in response to vaccination.

In addition to examining broad-based, mucosal immune endpoints, numerous studies have demonstrated a beneficial effect of moderate exercise on systemic innate immunity, in particular the phagocytic and tumoricidal activities of macrophages and the cytotoxicity of NK cells (reviewed in [19, 20]). A few studies have examined the effect of regular moderate exercise on systemic adaptive immune responses, but in most cases, in the context of an aging model. Several cross-sectional studies demonstrate that active older adults have higher antigen-specific antibody titers [21–23], higher influenza-specific *in vitro* peripheral blood mononuclear cell proliferation [22] and greater *in vivo* delayed type hypersensitivity (DTH) responses [23] as compared to sedentary individuals. Furthermore, two prospective studies in older adults reported that a 10-month exercise intervention increased influenza-and KLH-specific antibody titers [24, 25] and granzyme B activity [25]. In contrast to the exercise-induced enhancement of antigen-specific antibody titers in older adults, moderate exercise does not enhance antibody responses in young adults [23, 26, 27] or in rodent models utilizing young animals [23, 28–31]. However, one report demonstrates that DTH responses to KLH are higher in active versus sedentary young adults [23]. The studies that have been done in young animals suggest that moderate exercise may enhance cell-mediated but not humoral responses; however, additional well-designed mechanistic studies are needed to further characterize the effects of moderate activity on adaptive immune responses.

Therefore, the goals of the present study were 1) to establish a reliable model to systematically evaluate the effects of moderate physical activity on adaptive immune

⁷Abbreviations used: AL, consumed food *ad libitum*; AL+EX, consumed food *ad libitum* plus given access to voluntary running wheels; APC, antigen-presenting cell; Con A, concanavalin A; DTH, delayed type hypersensitivity; HSV-1, herpes simplex virus-1; IL, interleukin; KLH, keyhole-limpet hemocyanin; km, kilometer; LT, lymphotactin; mAb, monoclonal antibodies; MLR, mixed lymphocyte response; NK, natural killer; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell; PMA, phorbol 12-myristate 13-acetate; SI, stimulation index; TNF- α , tumor necrosis factor alpha; Treg, regulatory T cell; URTI, upper respiratory tract infection.

responses to vaccination, 2) to characterize the effect of moderate exercise on humoral and cell-mediated immune responses in the mucosal compartment using a vaccine platform that is well-documented to stimulate mucosal immunity [32] and 3) to explore the effect of the same vaccine platform on systemic immunity in an effort to compare and contrast the effect of exercise on adaptive immunity in both compartments.

2. Materials and Methods

2.1. Animals and treatment regimens

Female 6-week-old C57BL/6 mice were obtained from Charles River Breeding Laboratory (Frederick, MD). Animal care was provided in accordance with the procedures outlined in the “Guide for the Care and Use of Laboratory Animals.” Upon receipt, mice were screened for voluntary running behavior by being placed into individual cages fitted with a mouse running wheel apparatus (MiniMitter Co.; Bend, OR) for 4 days to determine the average level of running activity per mouse. Wheel revolutions of individual mice were recorded and analyzed using the VitalView software (MiniMitter Co.; Bend, OR). Mice with running activity at or above the 50th percentile (approximately 4.0 km/day) were selected for this study and randomized to either the *ad libitum* food consumption (AL) or AL plus access to voluntary running wheels (AL+EX) treatment groups. Thus, mice in both the AL and AL+EX treatment groups exhibited high running behavior. All mice were housed individually for the duration of the study. Mice were vaccinated via two different routes, intranasal (i.n.) and subcutaneous (s.c.) to target the mucosal and systemic immune compartments, respectively. Mice were assigned to one of the following treatment groups 1) AL plus mucosal vaccination (n=20); 2) AL+EX plus mucosal vaccination (n=20); 3) AL plus s.c. vaccination (n=10); and 4) AL+EX plus s.c. vaccination (n=10). All mice were fed AIN-76A diet (Research Diets, Inc.; New Brunswick, NJ). Mice were maintained on AL or AL+EX regimens for 8 weeks prior to the primary vaccination and were continued on these treatments through 3 successive weeks (weeks 9, 10, and 11 of the study). Mice were sacrificed 1 week following the last vaccination at week 12 for collection of lymphoid organs. Mice were removed from the running wheel cages and placed in standard mouse cages 24 hours prior to sacrifice to standardize the timing of lymphocyte collection with respect to the last exercise bout. Food intake, body weights and distance run were monitored weekly, and mice were observed daily for signs of ill health.

2.2. Vaccinations

Vaccinations consisted of 1 primary and 2 booster vaccinations, each separated by 1 week, with 75 µg ovalbumin, grade VI (OVA) (Sigma-Aldrich; St. Louis, MO) plus 1 µg lymphotactin (LT) (R&D Systems, Inc.; Minneapolis, MN) in PBS. Mucosal vaccinations were given intranasally (i.n.) in 10 µl and systemic vaccinations were given subcutaneously (s.c.) in 100 µl in the lumbar region of the animal.

2.3. Mixed lymphocyte response (MLR)

Single cell suspensions of splenocytes were prepared from individual C57BL/6 mice and BALB/c mice as previously described [18]. Splenocytes from experimental C57BL/6 mice and naïve BALB/c mice were irradiated (20 Gy), counted, and serially diluted in triplicate in a 96-well plate and used as antigen presenting cells (APCs). CD4⁺ T lymphocytes from experimental C57BL/6 mice and naïve BALB/c mice were isolated via Dynal® CD4 negative isolation kits (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. APCs or CD4⁺ T cells from experimental mice were incubated with BALB/c T cells or APCs, respectively, to evaluate allogeneic proliferative responses as previously described [33].

2.4. CD4⁺ T cell proliferation assay

Single cell suspensions of splenocytes and lymphoid cells from Peyer's patches and mesenteric and inguinal lymph nodes were prepared from individual mice as previously described [18]. Cells from the Peyer's patches and inguinal and mesenteric lymph nodes were pooled from 2 animals within a treatment group to generate adequate cell numbers for functional assays. CD4⁺ T cell lymphoproliferation was assessed as previously described [34].

2.5. Cytokine production assays

CD4⁺ T cells (1×10^5) from experimental animals were incubated with 5×10^5 irradiated APCs from naïve syngeneic mice in triplicate wells of a 96-well plate. Cells were stimulated with 100 µg/ml of OVA, 2.0 µg/ml of Con A, or media alone. Supernatants were harvested after 48 h of incubation with Con A and 72 h of incubation with OVA. TNF-α, IFN-γ, interleukin-2 (IL-2), IL-4, and IL-5 were measured using the Th₁/Th₂ Cytokine Cytometric Bead Array kit (BD Biosciences; San Jose, CA) according to the manufacturer's instructions.

2.6. Serum antibody responses

Antigen-specific serum antibody responses were measured 7 days following the second s.c. or i.n. booster vaccination via ELISA as previously described [33].

2.7. Flow cytometric analyses

Single cell suspensions of cells from the spleen, Peyer's patches, inguinal and mesenteric lymph nodes were prepared for flow cytometric analyses and analyzed on a Becton Dickinson FACScan flow cytometer (BD Biosciences; San Jose, CA) as previously described [18].

2.8. Serum cytokine measures

Serum was collected at the time of sacrifice, frozen and stored at -80°C until analyzed. LINCOplex mouse cytokine kits (Millipore; St. Charles, MO) were used for the quantification of serum leptin and IL-6. Samples were run in duplicate. The intra-assay coefficients of variation for leptin and IL-6 were <10%.

2.9. Body composition analysis

Mouse carcasses were scanned using a GE Lunar PIXImus Dual-Energy X-ray Absorptiometer (DEXA) to assess lean mass, fat mass, and percent body fat, as previously described [18].

2.10. Statistical analyses

All data are presented as the mean plus or minus the standard error of the mean. Differences in average kilometers run per day, body weight, lean mass, fat mass, percent body fat, leptin, IL-6, cytokine production and flow cytometric analyses were determined using two-tailed, paired *t*-tests assuming unequal variances. Differences in lymphocyte proliferation were examined using two-way analysis of variance (ANOVA) (treatment X concentration of APC or OVA), followed by Fisher Least Significant Difference post-hoc tests and a Bonferroni correction for multiple comparisons where appropriate. All analyses were conducted using STATA software (version 7.0 Stata Corp; College Station, TX) and statistical significance was accepted at the $P = 0.05$ level.

3. Results

3.1. Establishment of a reliable model of moderate voluntary running in mice

Previous studies in our laboratory demonstrated that running behavior is heterogeneous in C57BL/6 mice given access to voluntary running wheels [18]. Thus, to study the effects of moderate voluntary exercise on immune function we selected animals that would choose to run over the course of the 12-week experiment. In a pilot study, we compared the average running activity of mice (km/day) after 1, 4, 10, and 14 days of access to running wheels to the average distance/day run over 6 weeks. We found a significant correlation between the average km run per day over 4, 10, and 14 days and the average km run per day over 6 weeks ($\rho=0.606$, $\rho=0.696$, $\rho=0.772$, respectively; $P<0.05$). Thus, we measured running activity in all mice for 4 days prior to the onset of the study to select mice with high running activity for enrollment in the current study. Individual histograms of running activity for animals that exhibited low and high running behavior over a 4-day test period are shown in Fig. 1a and Fig. 1b, respectively. The average distance run per day among mice selected for the study ranged from 3.5–7.3 km/day over the course of the 12-week study with an overall average of 5.5 km/day (Fig. 1c). Using speed to estimate the percent of maximal oxygen consumption (% $\text{VO}_{2\text{max}}$) or relative intensity of exercise [35], we determined that mice were exercising at 60–70% of $\text{VO}_{2\text{max}}$, which is indicative of moderate exercise training. The average distance run was not altered following the administration of the primary and booster vaccinations (designated by arrows) given in weeks 9, 10, and 11 of the study (Fig. 1c). Access to running wheels in the AL+EX group prevented weight gain over the course of the 12-wk study (Fig. 1d). After 12 weeks of training, AL+EX mice had significantly lower total body weight, fat mass, and percent body fat (Table 1; $P<0.05$), but had similar lean mass as compared to AL animals. Additionally, AL+EX mice had lower serum leptin and higher serum IL-6 levels than the AL controls (Table 1; $P<0.05$). The average distance run and the exercise-induced changes in body weight and body composition did not differ between mice that were vaccinated i.n. or s.c.; therefore, data from all vaccinated animals ($n=26$) are shown in Fig. 1 and Table 1.

3.2. Exercise enhanced CD4^+ T cell proliferation in response to allogeneic stimulation

The proliferation of T cells from BALB/c mice following stimulation with irradiated splenocytes from C57BL/6 mice was used as a measure of antigen presenting capability of AL and AL+EX mice. Exercise did not increase the antigen-presenting capabilities of splenocytes (Fig. 2a; $F_{2,70} = 1.89$, $P = 0.191$). The proliferation of CD4^+ T lymphocytes from C57BL/6 mice following stimulation with irradiated splenocytes from allogeneic BALB/c mice was used as a measure of CD4^+ T cell proliferation in AL and AL+EX mice. Exercise significantly enhanced CD4^+ T cell proliferation in response to stimulation with allogeneic APCs (Fig. 2b; $F_{1,84} = 8.14$, $P = 0.006$). Significant differences between AL and AL+EX groups at each concentration of APCs were determined using Bonferroni's correction for multiple comparisons.

3.3. Exercise had no effect on the distribution of cells in any lymphoid compartment

Exercise reduced the total number of cells in the spleen in mice receiving either i.n. or s.c. vaccinations, although this did not reach statistical significance (Table 2; $P = 0.08$ and $P=0.07$, respectively). In contrast, the total numbers of cells in the Peyer's patches, mesenteric and inguinal lymph nodes were not different between AL+EX and AL mice. Additionally, we examined the number of CD3^+ , $\text{CD3}^+\text{CD4}^+$, $\text{CD3}^+\text{CD8}^+$, CD19^+ or B220^+ , NK1.1^+ , $\text{CD11b}^+\text{I-Ab}^+$, and $\text{CD11c}^+\text{I-Ab}^+$ cells in all lymphoid organs of mice in both treatment groups in an effort to determine if exercise altered the distribution of cells post-vaccination (Table 2). Exercise did not alter the number of any cell type in the spleen, Peyer's patches, or mesenteric or inguinal lymph nodes.

3.4. Exercise had no effect on antigen-specific humoral responses

AL and AL+EX mice were vaccinated i.n. or s.c. with a primary and 2 booster vaccinations of OVA+LT. Serum β -galactosidase IgG titers (negative control antigen) in mice that were vaccinated i.n. (Fig. 3a) or s.c. (Fig. 3b) were similar in AL and AL+EX mice. There was over a 3-fold greater humoral response, i.e. OVA-specific total IgG response, to s.c. vaccination with OVA+LT than to i.n. vaccination with OVA+LT (Fig. 3, panel b vs. panel a). However, exercise had no effect on serum IgG titers to OVA in mice that were vaccinated i.n. (Fig. 3a) or s.c. (Fig. 3b).

3.5. Exercise enhanced antigen-specific cytokine production

Splenic CD4⁺ T cells were stimulated *in vitro* with 100 μ g/ml of OVA or 2 μ g/ml of Con A (as a positive control) 7 days after the last booster vaccination. Exercise significantly enhanced OVA-specific TNF- α production in mice that received either i.n. or s.c. vaccinations and OVA-specific IL-5 production in mice that received s.c. vaccination (Table 3; $P < 0.05$). AL+EX mice receiving i.n. vaccination had higher OVA-induced IL-5 levels than AL mice, although this did not reach statistical significance. Similarly, IFN- γ and IL-2 levels produced by OVA-stimulated CD4⁺ T cells from AL+EX were higher than AL animals in response to both i.n. and s.c. vaccination, although these did not reach statistical significance. IL-4 levels were below the limit of detection of the assay in all OVA- and Con A- stimulated T cell cultures.

3.6. Exercise enhanced antigen-specific CD4⁺ T cell production

CD4⁺ T cells collected from the spleen, Peyer's patches, inguinal and mesenteric lymph nodes were stimulated *in vitro* with 3.125-100 μ g/ml of OVA or 2 μ g/ml of Con A 7 days the last booster vaccination. Exercise significantly enhanced *in vitro* OVA-specific CD4⁺ T cell proliferation following i.n. vaccination in cells collected from the spleen (Fig. 4a; $F_{1,108} = 25.95$, $P < 0.001$); mesenteric lymph nodes (Fig. 4b; $F_{1,56} = 45.43$, $P < 0.001$) and Peyer's patches (Fig. 4c; $F_{1,56} = 6.75$, $P = 0.012$) with the exercise-induced enhancement of CD4⁺ T cell proliferation most pronounced in cells collected from the Peyer's patches (Fig. 4c). Exercise did not increase Con A-induced CD4⁺ T cell proliferation in cells collected from the spleen (Fig. 4a insert graph) or mesenteric lymph nodes (Fig. 4b insert graph). However, exercise did increase Con A-induced CD4⁺ T cell proliferation in cells collected from the Peyer's patches (Fig. 4c insert graph; $P < 0.05$).

Exercise also significantly enhanced *in vitro* OVA-specific CD4⁺ T cell proliferation following s.c. vaccination in cells collected from the spleen (Fig. 5a; $F_{1,60} = 12.74$, $P < 0.001$) and inguinal lymph nodes (Fig. 5b; $F_{1,80} = 10.64$, $P = 0.002$). Exercise did not increase Con A-induced CD4⁺ T cell proliferation in cells collected from the spleen (Fig. 5a insert graph) or inguinal lymph nodes (Fig. 5b insert graph). Significant differences between AL and AL+EX groups at each concentration of APCs in Figs. 4 & 5 were determined using Bonferroni's correction for multiple comparisons.

4. Discussion

To our knowledge, this study is the first to evaluate the effects of moderate exercise on antigen specific mucosal immunity in response to vaccination, and the first to compare and contrast the effect of moderate exercise on antigen specific immune responses in mucosal and systemic compartments. Furthermore, we evaluated both humoral and cell-mediated immune responses to vaccination in young, healthy animals. We have demonstrated that by selecting mice that voluntarily choose to run, all mice maintain running wheel activity (average 5.5 km/day) throughout the duration of the experiment. Mice with access to running wheels for 12 weeks had significantly lower body weights and fat mass as compared

to sedentary mice, indicative of a training effect. Mice that were active had significantly higher CD4⁺ T cell proliferation in response to stimulation with allogeneic APCs but did not have enhanced APC function, suggesting that exercise selectively enhances T cell responsiveness. Furthermore, active mice that received either i.n. or s.c. vaccinations with OVA+LT had greater OVA-specific cytokine production from splenic CD4⁺ T cells and greater OVA-specific CD4⁺ T cell proliferation in all lymphoid compartments examined. Together, these results suggest that moderate activity enhances cell-mediated immune responses in young, healthy mice after mucosal or systemic vaccination with OVA+ LT.

In the current study our goal was to determine if regular moderate activity (i.e. exercise training) enhances adaptive immune responses following vaccination. Evaluation of the relationship between exercise training and vaccine response requires that mice run consistently for the duration of the experiment. Previous reports that quantified running wheel activity in mice have documented a wide range of running behavior among individual animals [18, 36, 37]. Thus, we evaluated running activity in animals for 4 days prior to the assignment of mice to our study and selected animals that voluntarily chose to run consistently over the 4-day test period. Several studies have documented that when high running behavior is selected in rodents over the course of many genetic crosses (>10 generations), other physiological traits emerge that differ between low and high runners, such as aerobic capacity [38–40], oxidative capabilities of muscle tissue [41, 42], and cardiovascular function [43]. However, none of these studies demonstrated any difference in the physiological traits between low and high runners in the first generation. Furthermore, we have evaluated the immune response to both mucosal and systemic vaccination in animals with low and high running behavior, and animals with low running behavior have no decrements in their immune responses (data not shown). These findings, as well as the fact that we used animals that exhibited high running behavior in both the AL and the AL+EX groups, argue against the possibility that we introduced a selection bias by using only animals that voluntarily chose to run for these immune-based experiments. Additionally, the significantly lower body weight and, in particular, body fat in AL+EX mice (Table 1), in conjunction with reports of exercise-induced changes in skeletal muscle oxidative enzyme activity in other voluntary running wheel studies in mice [44], suggest that exposure to voluntary running wheels for 12 weeks is a valid form of aerobic endurance training. In addition to the changes in body composition, the differences in serum leptin and IL-6 between the AL and the AL+EX mice (Table 1) correspond with the well-documented positive association between leptin and fat mass [45] and the increase in serum IL-6 in response to exercise training [46, 47]. However, while utilizing only animals that exhibited high running behavior in this study is experimentally sound, we recognize that this design feature may narrow the applicability of our findings. Including animals with both high and low running behavior in future studies may broaden the scope of the results and allow us to further examine the relationship between running wheel activity and immune responses.

Although there were no differences in the number of cells in any lymphoid organs examined in AL and AL+EX mice, significant exercise-induced functional changes were observed. We have shown that moderate exercise enhances CD4⁺ T cell proliferation in C57BL/6 mice in response to allogeneic stimulation with APCs from BALB/c mice; however, splenic antigen presenting capabilities were not enhanced by moderate activity. Furthermore, in animals that received either a mucosal or systemic vaccination, OVA-specific *in vitro* cytokine production, specifically TNF- α and IL-5, was higher in splenic CD4⁺ T cells collected from AL+EX as compared to AL mice (Table 3). CD4⁺ T cell proliferation was also significantly higher in AL+EX mice as compared to AL mice when CD4⁺ T cells were collected from the spleen, mesenteric and inguinal lymph nodes, and Peyer's patches (Figs. 4 and 5). In contrast to the stimulatory effect on antigen-specific CD4⁺ T cell function, exercise had no effect on the generation of OVA-specific serum total IgA or IgG levels (Fig. 3). In prior

animal studies, moderate activity enhanced herpes simplex virus-1 (HSV-1)-specific cytokine production (IL-2 and IFN- γ) in older mice [48]. Furthermore, higher DTH responses to KLH were reported in active as compared to sedentary young adults [23]. Taken together, these data suggest that moderate exercise training does not universally activate immune processes, but rather it selectively enhances antigen-specific cell-mediated immunity, e.g. cytokine production and proliferation by CD4⁺ T cells, in young hosts.

The enhancement of an *in vitro* recall response found in our study could be due to either quantitative or qualitative difference in the T cells. First, exercise training could increase the number of antigen-specific cells following vaccination, which would result in a greater percentage of the total cells that were antigen specific in both the cytokine and proliferation assays in AL+EX animals. The enhancement of the T cell memory response could also be due to an enhancement of the quality of the antigen-specific T cell, i.e. cells from AL+EX mice were functionally different. Preliminary work from our laboratory suggests that the number of antigen-specific T cells in the spleens of vaccinated animals is not different in AL and AL+EX mice (data not shown), suggesting that a qualitative difference exists in the function of T cells from AL and AL+EX mice.

The exercise-induced enhancement of T cell function could be mediated via a number of cellular mechanisms. First, exercise training reduces the susceptibility of CD4⁺ T cells to apoptotic stimuli. Splenic [49, 50] and intestinal CD4⁺ T cells [51] from trained mice are less susceptible to the *in vitro* administration of apoptosis-inducing agents (e.g. hydrogen peroxide, dexamethasone, TNF- α , or anti-CD3) and *in vivo* physical stressors (e.g. exhaustive exercise). Perhaps the training effect on anti-apoptotic mechanisms in CD4⁺ T cells confers protection from environmental stimuli *in vivo* that may lead to CD4⁺ T cell death, thus allowing for greater *in vitro* CD4⁺ T cell proliferation in AL+EX animals.

Second, we (Table 1) and others [46] demonstrate an exercise-induced increase in serum IL-6. IL-6 has many physiological, metabolic and immunological roles and is produced by a myriad of immune cells [52, 53] and skeletal muscle [46]. Of interest to our study, IL-6 has been shown to enhance the proliferation of CD4⁺CD25⁻ T cells by inhibiting CD4⁺CD25⁺ regulatory T cell (Treg) function in two different autoimmunity models [54, 55], suggesting that IL-6 enables CD4⁺CD25⁻ T cells to escape from Treg-mediated inhibition. In several reports, dendritic cells are the source of IL-6 which mediates the inhibition of Treg function [55, 56]. These findings suggest that local IL-6 may be important in regulating Treg function and consequently CD4⁺ T cell proliferation. It is unknown if elevated levels of serum IL-6 mediate a similar inhibition of Treg function. Additionally, since the effect of IL-6 on CD4⁺ T cell proliferation (via its effect on Treg function) has been studied only in the context of autoimmunity in which pathological dysregulation of CD4⁺ T cell proliferation has occurred, it is not clear if IL-6 in healthy mice in the absence of other inflammatory cytokines and genetic factors would mediate inhibitory effects on Treg function and subsequent enhancement of CD4⁺ T cell proliferation. Additional studies are required to determine if the exercise-induced increase in serum IL-6 alters the function of regulatory T cells and CD4⁺CD25⁻ T cell proliferation.

In summary, we have established a reliable animal model of moderate physical activity that can be used to evaluate the effect of exercise on immune, as well as other physiological endpoints in mice. We have demonstrated that regular moderate exercise selectively enhances antigen-specific cell-mediated responses following vaccination via either a mucosal or systemic route of antigen delivery. These results suggest that regular moderate exercise may enhance vaccine efficacy and thus, may be an important lifestyle intervention in humans to couple with immunization against infectious diseases such as influenza. These findings may prove to have important public health significance. If regular physical activity

can enhance cell-mediated adaptive immune responses in young, healthy animals, perhaps participation in regular physical activity would benefit other populations in which the immune system is compromised to a greater extent.

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Fig. 1.

Individual histograms from 2 representative female C57BL/6 mice demonstrate the heterogeneity in running activity. (a) Running activity in 1 mouse that exhibited (a) low running activity and (b) high running activity over the 4-day test period. The average km/day run by each mouse in (a & b) are shown above the histogram plots. (c) Among mice selected for the study, the average km/day run was 5.5 km/day (dashed line) during the 12-week study ($n=26$). (d) Average weekly body weights of AL (●) or AL+EX (■) mice ($n=26/\text{group}$). Arrows indicate the administration of primary and booster vaccinations. Asterisks indicate significantly different body weights between AL and AL+EX groups ($P < 0.05$).

Fig. 2.

Exercise had no effect on APC function but enhanced CD4⁺ T cell proliferation assessed via mixed lymphocyte response. (a) BALB/c CD4⁺ T cell proliferation induced by increasing concentrations of allogeneic APC from AL (●) or AL+EX (■) C57BL/6 mice. Data shown are mean $\pm$ SEM (n=8/group). Two-way ANOVA revealed no significant effect of exercise on the antigen presenting capability of splenocytes ($F_{2,70} = 1.89$, $P = 0.191$) (b) C57BL/6 CD4⁺ T cell proliferation from AL (●) or AL+EX (■) induced by increasing concentrations of allogeneic BALB/c APCs. Data shown are mean $\pm$ SEM (n=8/group). Two-way ANOVA revealed a significant effect of exercise on CD4⁺ T cell proliferation in response to simulation with allogeneic APC ($F_{1,84} = 8.14$, $P = 0.006$). *Post-hoc analyses using Bonferroni's test for multiple comparisons found significant differences between AL and AL+EX groups at the designated concentration of APCs.

Fig. 3.

Exercise had no effect on antigen-specific serum IgG levels. Antibody titers from mice vaccinated either (a) i.n. or (b) s.c. are shown. OVA-specific serum IgG titers from AL (●) and AL+EX (■) mice are similar. β -galactosidase (control antigen)-specific serum IgG titers from AL (○) and AL+EX (□) mice are also similar. Antibody titers were measured 7 days after the second booster vaccination via ELISA. Data shown are mean $\pm$ SEM. Data are representative of two independent experiments (n=6-10/group per experiment).

Fig. 4.

Exercise enhanced antigen-specific CD4⁺ T cell proliferation following i.n. vaccination. CD4⁺ T cells were collected from the (a) spleen (n=10/group), (b) mesenteric lymph nodes (n=5 pooled groups of 2 mice/group for each treatment) and (c) Peyer's patches (n=5 pooled groups of 2 mice/group for each treatment) of AL (●) or AL+EX (■) mice. 1×10^5 experimental CD4⁺ T cells were co-incubated with 5×10^5 irradiated APCs from unvaccinated syngeneic mice in the presence of increasing concentrations of OVA *in vitro*. Insert graphs show Con A-induced CD4⁺ T cell proliferation from each lymphoid organ in AL (white bars) or AL+EX (black bars) mice. Data shown are mean $\pm$ SEM. Data are representative of two independent experiments. Two-way ANOVA revealed a significant effect of exercise on CD4⁺ T cell proliferation in response to simulation with OVA in the spleen ($F_{1,108} = 25.95$, $P < 0.001$); mesenteric lymph nodes ($F_{1,56} = 45.43$, $P < 0.001$); and Peyer's patches ($F_{1,56} = 6.75$, $P = 0.012$). *Post-hoc analyses using Bonferroni's test for multiple comparisons found significant differences between AL and AL+EX groups at the designated concentration of OVA.

Fig. 5.

Exercise enhanced antigen-specific CD4⁺ T cell proliferation following s.c. vaccination. CD4⁺ T cells were collected from the (a) spleen (n=10/group) and (b) inguinal lymph nodes (n=5 pooled groups of 2 mice/group for each treatment) of AL (●) or AL+EX (■) mice. 1×10^5 experimental CD4⁺ T cells were co-incubated with 5×10^5 irradiated APCs from unvaccinated syngeneic mice in the presence of increasing concentrations of OVA *in vitro*. Insert graphs show Con A-induced CD4⁺ T cell proliferation from each lymphoid organ in AL (white bars) or AL+EX (black bars) mice. Data shown are mean $\pm$ SEM (n=6/group). Two-way ANOVA revealed a significant effect of exercise on CD4⁺ T cell proliferation in response to simulation with OVA in the spleen ($F_{1,60} = 12.74$, $P < 0.001$) and inguinal lymph nodes ($F_{1,80} = 10.64$, $P = 0.002$). *Post-hoc analyses using Bonferroni's test for multiple comparisons found significant differences between AL and AL+EX groups at the designated concentration of OVA.

Table 1

Body composition measures and serum cytokine levels (mean $\pm$ SEM) of mice on either AL or AL+EX treatments for 12 weeks

Treatment Group	Body Weight ^a (grams)	Fat Mass ^a (grams)	Lean Mass ^a (grams)	Percent Body Fat ^a	Leptin ^b (ng/ml)	IL-6 ^b (pg/ml)
AL	29.1 $\pm$ 0.8	9.3 $\pm$ 0.7	16.6 $\pm$ 0.3	34.0 $\pm$ 1.9	2.3 $\pm$ 0.4	8.9 $\pm$ 2.9
AL+EX	24.8 $\pm$ 0.3 *	5.4 $\pm$ 0.3 *	16.2 $\pm$ 0.3	24.7 $\pm$ 1.1 *	1.6 $\pm$ 0.2 *	30.6 $\pm$ 7.9 *

^a n=26/group (both i.n. and s.c. vaccinated mice)

^b Serum leptin and IL-6 were measured in a subset of mice that did not receive any vaccinations (n=10/group).

* Significantly different from AL group (P<0.05).

Table 2

Number of cells (mean $\pm$ SEM) in each tissue compartment 7 days after the last booster vaccination among AL and AL+EX mice

Tissue	Treatment group	Total cell number	CD3 ⁺	CD3 ⁺ CD4 ⁺ ($\times 10^6$)	CD3 ⁺ CD8 ⁺	CD19 ⁺ or B220 ⁺ ^a	NK1.1 ⁺	CD11b ⁺ L-Ab ⁺ ($\times 10^5$)	CD11c ⁺ L-Ab ⁺
i.n. vaccination									
Peyer's patches ^b	AL	11.0 $\pm$ 0.3	2.6 $\pm$ 0.2	1.3 $\pm$ 0.1	0.8 $\pm$ 0.1	8.4 $\pm$ 0.2	0.3 $\pm$ 0.1	1.4 $\pm$ 0.1	2.0 $\pm$ 0.1
	AL+EX	12.0 $\pm$ 1.8	2.5 $\pm$ 0.5	1.3 $\pm$ 0.2	0.8 $\pm$ 0.1	9.1 $\pm$ 1.3	0.3 $\pm$ 0.2	2.1 $\pm$ 0.4	3.1 $\pm$ 0.6
mesenteric lymph nodes ^b	AL	26.7 $\pm$ 2.2	13.1 $\pm$ 1.1	7.5 $\pm$ 0.7	5.9 $\pm$ 0.6	12.4 $\pm$ 1.4	11.1 $\pm$ 2.8	12.2 $\pm$ 1.1	9.4 $\pm$ 0.8
	AL+EX	23.8 $\pm$ 6.6	15.0 $\pm$ 4.7	8.3 $\pm$ 2.8	7.0 $\pm$ 2.0	7.7 $\pm$ 1.8	12.7 $\pm$ 4.1	10.9 $\pm$ 2.7	7.8 $\pm$ 1.1
spleen ^c	AL	106.8 $\pm$ 7.7	37.3 $\pm$ 3.1	22.9 $\pm$ 2.1	16.8 $\pm$ 1.5	52.8 $\pm$ 5.9	74.3 $\pm$ 6.9	32.7 $\pm$ 2.5	22.1 $\pm$ 2.6
	AL+EX	86.3 $\pm$ 7.5	35.9 $\pm$ 3.8	20.7 $\pm$ 2.2	16.1 $\pm$ 1.8	49.1 $\pm$ 5.9	62.4 $\pm$ 7.6	21.3 $\pm$ 1.7	18.0 $\pm$ 1.2
s.c. vaccination									
inguinal lymph nodes ^d	AL	23.0 $\pm$ 2.9	15.5 $\pm$ 1.8	8.6 $\pm$ 1.1	6.9 $\pm$ 0.7	5.7 $\pm$ 0.9	3.0 $\pm$ 0.3	3.6 $\pm$ 0.4	3.2 $\pm$ 0.4
	AL+EX	20.3 $\pm$ 2.7	14.0 $\pm$ 1.9	7.6 $\pm$ 1.1	6.4 $\pm$ 0.8	4.6 $\pm$ 0.7	2.8 $\pm$ 0.6	3.3 $\pm$ 0.5	3.0 $\pm$ 0.5
spleen ^e	AL	131.5 $\pm$ 9.0	49.4 $\pm$ 5.2	29.1 $\pm$ 2.5	20.3 $\pm$ 2.9	64.7 $\pm$ 3.2	58.4 $\pm$ 2.3	30.8 $\pm$ 5.0	25.8 $\pm$ 5.0
	AL+EX	100.8 $\pm$ 12.5	39.9 $\pm$ 4.0	22.9 $\pm$ 2.3	16.9 $\pm$ 1.7	53.4 $\pm$ 5.1	53.7 $\pm$ 4.1	41.8 $\pm$ 5.7	23.2 $\pm$ 3.9

^a Anti-CD19 antibodies were used to identify B cells in the lymph nodes and spleen; anti-B220 antibodies were used to identify B cells in the Peyer's patches.

^b n = 5 pooled groups of 2 mice per group.

^c n = 10 individual mice per group.

^d n = 3 pooled groups of 2 mice per group.

^e n = 6 individual mice per group.

Table 3

Cytokine responses (mean $\pm$ SEM) from splenic CD4⁺ T cells stimulated *in vitro* with OVA (100 μ g/ml)^a 7 days after the administration of either a s.c. or i.n. booster vaccination with 75 μ g OVA + 1 μ g LT

Treatment Group	TNF- α	IFN- γ	IL-2	IL-5
—————(pg/ml/5 $\times$ 10 ⁵ cells)—————				
s.c. vaccination				
AL (n=6)	248.4 $\pm$ 32.4	8.4 $\pm$ 2.1	96.8 $\pm$ 27.1	187.5 $\pm$ 72.6
AL+EX (n=6)	355.6 $\pm$ 32.3^b	13.4 $\pm$ 1.7	139.3 $\pm$ 20.6	377.9 $\pm$ 89.4^b
i.n. vaccination				
AL (n=10)	811.2 $\pm$ 107.9	25.9 $\pm$ 4.9	149.5 $\pm$ 29.1	209.3 $\pm$ 79.1
AL+ EX (n=10)	1418.5 $\pm$ 115.7^b	36.1 $\pm$ 15.9	255.4 $\pm$ 57.4	453.2 $\pm$ 161.6

^aCon A stimulation (2 μ g/ml) of isolated CD4⁺ T cells was used as positive control. In all cultures the amount of each cytokine produced exceeded 0.5 ng/ml/5 $\times$ 10⁵ cells.

^bSignificantly different from respective AL group (P<0.05)