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## Energy Restriction and Exercise Differentially Enhance Components of Systemic and Mucosal Immunity in Mice,<sup>1,2</sup>

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### Abstract

The prevalence of obesity, an established risk factor for several chronic diseases including cancer, has risen dramatically over the past four decades. Dietary change and/or increased physical activity are the most commonly recommended lifestyle-based strategies for preventing or reversing obesity. One of several physiological systems that may be enhanced by dietary change and exercise is the immune system. This study examines the effects of energy restriction (ER; 30% reduction relative to control energy intake) and/or EX (voluntary-wheel running) on systemic and mucosal immune function. Female C57BL/6 mice were randomized into four treatment conditions: 1) controls consuming food *ad libitum* (AL); 2) AL with access to running wheels (AL+EX); 3) 30% ER; and 4) 30% ER with access to running wheels (ER+EX). Both ER and EX reduced spleen weight and the number of splenic T and B lymphocytes ( $P < 0.05$ ). ER enhanced NK cell function, but significantly reduced Con A-induced T cell proliferation ( $P < 0.05$ ). In contrast, EX significantly enhanced Con A-induced proliferation and cytokine production from Peyer's patch cells ( $P < 0.05$ ). These data suggest that ER and EX enhance some, but not all components of the immune system, and are likely working via different biological mechanisms to regulate NK and T cell function.

### Introduction

Considerable evidence from both human and animal studies indicates that changes in energy balance can influence the risk of cancer and other chronic diseases (1,2). In humans, diverse

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epidemiological studies have found that obesity and sedentary behavior increase the risk of cancer at numerous sites, particularly in the colon (3-6). In animal studies, energy restriction (ER)<sup>8</sup> and exercise (EX) interventions delay tumorigenesis in spontaneous and chemically induced intestinal tumor models (7-10). The beneficial effects of ER and EX are likely to occur through a variety of mechanisms; however, the extent to which these overlap and the specific pathways associated with cancer prevention are poorly understood. Thus, gaining a better understanding of the effects of ER and EX, individually and combined, on a number of physiological systems is critical to elucidating the underlying biological mechanisms by which ER and EX reduce the risk of tumor formation.

One of several physiological systems that may be enhanced by ER and EX is the immune system (11-13), including both systemic and mucosal immunity. Improved systemic immune function correlates with a reduction in tumor growth in several transplantable tumor models (14,15), as well as reduced intestinal polyp number and increased survival in a spontaneous intestinal tumor model (16). The mucosal immune system provides protection along the epithelial mucosal surfaces (i.e. respiratory, urogenital, and gastrointestinal tracts). The immune cells found in the Peyer's patches, as well as other immunological sites in the gut, are in close proximity to the epithelial cells in the small intestine that become transformed during carcinogenesis. Thus, enhancement of mucosal immunity may provide selective protection from the growth and development of intestinal tumors (17). One study exploring the efficacy

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<sup>8</sup>Abbreviations used:

<b>AL</b>	consumed food <i>ad libitum</i>
<b>AL+EX</b>	consumed food <i>ad libitum</i> plus given access to voluntary running wheels
<b>Con A</b>	concanavalin A
<b>E:T</b>	effector:target
<b>ER</b>	30% energy restriction
<b>EX</b>	exercise
<b>ER+EX</b>	30% energy restriction plus given access to voluntary running wheels
<b>IL</b>	interleukin
<b>km</b>	kilometer
<b>mAb</b>	monoclonal antibodies
<b>MCP-1</b>	monocyte chemoattractant protein-1
<b>NK</b>	natural killer
<b>NKCC</b>	NK cell cytotoxicity
<b>SI</b>	stimulation index

of probiotics on gut physiology has demonstrated a correlation between enhanced mucosal cytokine production, and a reduction in chemically-induced colon carcinogenesis (18). Together, these data suggest that improved immune function, either systemic or mucosal, is beneficial in the therapy of some established tumors and more importantly, can prevent the formation of neoplastic lesions in both spontaneous and carcinogen-induced models.

Changes in energy balance, ER and moderate EX enhance some components of systemic immunity such as T cell function in aged animals (19,20) and NK cell function (21-28). However, the age of the animal at the onset, the duration and severity of ER influence immune responsiveness, reviewed in (29). Thus, additional studies are needed to fully characterize the effect of ER on immunity.

The impact of moderate, regular EX on immunity has not been well studied. Many studies have either examined the effect of an acute bout of EX or have studied the effect of high intensity, exhaustive EX on systemic immune function (30), both of which are important in understanding the physiological and immunological effects of training in athletes. However, these studies are less relevant to understanding the mechanism(s) by which moderate EX may impact immunity, and potentially serve as a cancer prevention strategy. Even less is known about the influence of ER and EX on mucosal immune function. To date, only two studies in humans have examined salivary IgA and both have demonstrated that salivary IgA is elevated with regular EX training (31,32).

The purpose of this study was to investigate the effects of ER (30% energy restriction relative to control intake) and EX (6 weeks of voluntary running) on systemic and mucosal immune function in normal, non-tumor bearing mice. We hypothesized that negative energy balance induced by ER, EX, or the combination of both would enhance systemic and mucosal immune function. We chose to initially test this hypothesis in normal mice to characterize the effects of these interventions on immune function in the absence of tumor since it is well documented that tumors produce immunosuppressive factors (33) that may mask the relationship between ER, EX and immunity. The establishment of an ER- and/or EX-induced enhancement of immunity would provide the foundation for future studies to determine the role of immune function in the anti-cancer effects of ER and/or EX.

## Materials and Methods

### Animals and treatment regimens

Forty-eight 6-week-old female C57BL/6 mice were obtained from Charles River Breeding Laboratory (Frederick, MD). Upon receipt, mice were randomized to one of four treatment groups and housed individually at the National Cancer Institute-Frederick specific pathogen-free animal facility (Frederick, MD). Animal care was provided in accordance with the procedures outlined in the "Guide for the Care and Use of Laboratory Animals." The four treatment groups included: 1) controls consuming food *ad libitum* (AL) (n=12); 2) AL-fed with access to running wheels (AL+EX) (n=11); 3) 30% ER (n=11); and 4) 30% ER with access to running wheels (ER+EX) (n=12). Mice were maintained on the ER and/or EX regimens for 6 weeks and then killed for collection of lymphoid organs. The AL control group was fed AIN-76A diet (34). The ER diet was formulated such that the reduction in calories was entirely from carbohydrates (35). All other components of the ER diet were isonutrient relative to the AL control group when administered in daily aliquots equivalent to 70% of the average daily intake of the AL control mice. Diets were manufactured by Bio-Serv, Inc. Access to running wheels was facilitated by fitting individual cages with a mouse running wheel apparatus (MiniMitter Co.). Wheel revolutions of individual mice were recorded and analyzed using the Vital View software (MiniMitter Co.). Movement was not monitored in mice that did not have access to running wheels. All mice were kept on a reverse 12 h dark (10:00-22:00)/light

(22:00-10:00) cycle and provided with access to acidified distilled water *ad libitum*. Food intake and body weights were monitored weekly, and mice were observed daily for signs of ill health.

### Body composition analysis

Mouse carcasses were scanned using a GE Lunar PIXImus Dual-Energy X-ray Absorptiometer (DEXA) to assess bone mineral density, lean mass, fat mass, and percent body fat, as previously described (35,36).

### Isolation of immune cells

Single cell suspensions of splenocytes were prepared from individual mice by mechanical dispersion as previously described (37). Peyer's patches were excised from the wall of the small intestine and the lymphoid cells were dissociated as previously described (38). The number of Peyer's patches per intestine from individual mice was counted and recorded. Single cell suspensions of splenocytes and Peyer's patch cells were counted and the viability determined via trypan blue exclusion. The viability of splenocytes and Peyer's patch cells from all treatment groups was greater than 95%. The lymphoid cells from the Peyer's patches of three mice per treatment were pooled for use in the functional assays.

### Lymphocyte proliferation assays

$1 \times 10^6$  lymphocytes from the spleen or Peyer's patches were incubated in the presence of Con A as previously described (16). To adjust for potential ER and/or EX-induced changes in the percentage of T cells, the number of T cells per well was calculated based on the total number of cells per well ( $1 \times 10^6$ ) multiplied by the percentage of CD3<sup>+</sup> cells in each tissue compartment as determined by flow cytometry in an effort to report changes in proliferation on a per T cell basis. Proliferation data are reported as stimulation indices (SI) per  $1 \times 10^6$  T cells. SI were calculated by dividing the <sup>3</sup>H-thymidine uptake in Becquerel (Bq) from lymphocytes incubated with Con A by the <sup>3</sup>H-thymidine uptake from lymphocytes incubated with media alone. The efficiency of the beta counter was 57% for <sup>3</sup>H. Data are reported as SI rather than Bq because immune experiments were performed on different experimental days and inter-assay variation by day existed. The proliferation of lymphocytes incubated with media alone ranged from 25-204 Bq. The proliferation of lymphocytes with 0.25, 0.5, 1.0, and 2.0 mg/L of Con A ranged from 0.1-2.3 kBq, 0.1-3.4 kBq, 0.6-3.8 kBq, and 0.7-3.6 kBq, respectively. Each assay was performed in triplicate.

### Cytokine production assays

$1 \times 10^6$  lymphocytes from the spleen or Peyer's patch were incubated in flat-bottomed, 96-well plates in the presence of increasing concentrations of Con A. Supernatants were harvested after 48 h of incubation with Con A. TNF $\alpha$ , IL-6, IL-10, IL-12p70, and MCP-1 were measured using the Inflammation Cytokine Cytometric Bead Array kit (BD Biosciences) as per manufacturer instructions. IFN $\gamma$ , IL-2, IL-4, and IL-5 were measured using the Th<sub>1</sub>/Th<sub>2</sub> Cytokine Cytometric Bead Array kit (BD Biosciences) as per manufacturer instructions. Cytokine concentrations were adjusted per  $5 \times 10^6$  cells in the spleen and  $2 \times 10^5$  cells in the Peyer's patches. Each assay was performed in triplicate.

### Cytotoxicity assays

NKCC was assessed in standard 4 h chromium release assay as previously described (39), using 100:1, 50:1, 25:1, 12.5:1 E:T ratios. NKCC experiments were performed using <sup>51</sup>Cr-labeled YAC-1 target cells. All NKCC measures were adjusted based on the percentage of NK cells (NK1.1<sup>+</sup>) in the spleen as quantified via flow cytometry. All experiments were performed in triplicate.

## Flow cytometric analyses

Single cell suspensions of splenocytes and Peyer's patch cells were washed once in PBS at 4°C.  $1 \times 10^6$  cells were stained with saturating concentrations of conjugated antibodies for 30 min at 4°C as previously described (37). Following incubation with the conjugated antibodies, cells were washed twice in PBS and then fixed in 1% paraformaldehyde for flow cytometric analyses. Lymphoid and myeloid cells were gated on forward vs. side scatter and a total of 10,000 events were analyzed on a Becton Dickinson FACScan. Histograms of flow cytometric analyses were plotted and analyzed using Cell Quest software (BD Biosciences).

## Statistical analyses

All data are presented as the mean  $\pm$  SEM. Differences in the mean kilometers run per day between AL and ER mice were tested using Student's t-test. Effects of ER and EX on body composition (e.g., body weight, lean mass, fat mass, percent body fat, and bone mineral density); lymphocyte proliferation; cytokine production and flow cytometric analyses were examined using two-way ANOVA. Body weight was included as a covariate in the analysis of bone mineral density and spleen weight; lean mass was included as a covariate for fat mass; and Con A level was included as a covariate for SI in the proliferation assays. The variances were unequal for fat mass and body weight. These data were log transformed which eliminated the unequal variances. Using the log transformed data and the untransformed data resulted in qualitatively identical results where diet had as strong effect and exercise had a moderate effect on fat mass and body weight. Therefore the untransformed data were presented to be consistent with the other 8 variables in Tables 1 and 2. Following determination of the ER and/or EX effects using two-way ANOVA, Tukey's HSD post-hoc test was used to compare individual means among treatment groups. Statistical analyses were performed using SAS JMP. Statistical significance was accepted at the  $P \leq 0.05$  level.

## Results

### Energy restriction impacts body composition to a greater extent than EX

ER significantly reduced body weight, fat mass, lean mass, percent body fat (Table 1;  $P < 0.001$ ), and bone mineral density ( $P = 0.002$ ). In contrast, EX only significantly increased bone mineral density ( $P = 0.004$ ). There were no interactive effects of ER and EX on any of the body composition measures shown in Table 1. There was heterogeneity in running activity among mice in both the AL+EX and ER+EX groups, with the distance run by individual mice ranging from 1.1 to 7.7 km/day in the AL+EX group and 0 to 5.2 km/day the ER + EX group.

### Energy restriction and EX reduce spleen weight and cellularity

Both ER and EX significantly reduced spleen weight (Table 2;  $P < 0.001$  and  $P = 0.009$ , respectively) and total splenocyte number (Table 2;  $P < 0.001$  and  $P = 0.001$ , respectively). Body weight was significantly reduced by ER but not EX (Table 1); therefore, spleen weight was divided by body weight to adjust for differences in body size among mice in each of the treatment groups (Table 2). EX reduced spleen weight as a percentage of body weight ( $P = 0.029$ ), whereas ER was close but did not reach statistical significance ( $P = 0.070$ ). Although there were robust, statistically significant effects of either ER or EX on splenic weight and splenocyte number, there were no interactive effects of ER plus EX on these parameters ( $P = 0.237$  and  $P = 0.478$ , respectively). In contrast to the splenic measurements, neither ER nor EX altered the number of Peyer's patches per small intestine or the total number of cells in the Peyer's patches (Table 2).

Since the total number of splenocytes was significantly reduced by both ER and EX (Table 2), we explored the distribution of leukocytes in the spleen among mice on each of the four

treatment groups in an effort to identify the cell types most affected by each treatment (Table 3). ER significantly reduced the number of cells in the lymphoid compartment, including a reduction in the total number of T cells (CD3<sup>+</sup>) and B cells (CD19<sup>+</sup>) (P<0.001 and P=0.003, respectively). Within the T cell compartment, ER significantly reduced both the number of CD3<sup>+</sup>CD4<sup>+</sup> (helper) and CD3<sup>+</sup>CD8<sup>+</sup> (cytolytic) T cells (P<0.001 and P=0.019, respectively). However, ER did not alter the number of NK cells (NK1.1<sup>+</sup>), macrophages (CD11b<sup>+</sup>I-Ab<sup>+</sup>), or dendritic cells (CD11c<sup>+</sup>I-Ab<sup>+</sup>). In contrast, EX reduced the number of T cells (P=0.022), and in particular the number of CD3<sup>+</sup>CD8<sup>+</sup> cytolytic T cells (P=0.028); but had no effect on the number of CD3<sup>+</sup>CD4<sup>+</sup> helper T cells (P=0.133). There was a reduction in B cell number with EX; however, this effect did not reach statistical significance (P=0.069). EX reduced the number of macrophages in the spleen (P=0.019), but not the number of NK or dendritic cells.

Unlike the robust effects of both ER and EX on the distribution of leukocytes in the spleen, neither ER nor EX significantly altered the number of T cells, B cells or macrophages in the Peyer's patches (Table 4). ER, but not EX, significantly reduced the number of dendritic cells in the Peyer's patches (P=0.021). Finally, there were no interactive effects of ER or EX on the distribution of leukocytes in the spleen (Table 3) or Peyer's patches (Table 4).

### **EX significantly enhances T cell proliferation and cytokine production in Peyer's patch cells**

In splenocytes, ER had a significant inhibitory effect on Con A-induced T cell proliferation (Fig. 1A; P=0.003); however, EX had no effect on T cell proliferation (Fig. 1A; P=0.352). In contrast, there were significant main effects of both ER (P<0.001) and EX (P<0.001) on Con A-induced T cell proliferation in Peyer's patch cells (Fig 1B), as well as a significant interaction between ER and EX on Peyer's patch T cell proliferation (Fig. 1B; P=0.011). EX resulted in a much greater enhancement of T cell proliferation in Peyer's patch cells in AL mice (AL+EX) than in mice that were energy restricted (ER+EX).

EX (P=0.014) but not ER (P=0.948) significantly enhanced interferon- $\gamma$  (IFN $\gamma$ ) production by splenocytes in response to Con A stimulation (Fig. 2A). However, neither ER nor EX significantly altered Con A-induced IL-6 (Fig. 2B) and IL-5 (Fig. 2C) production from cultured splenocytes. Similar to the effects of EX on splenic IFN $\gamma$  production, EX significantly enhanced IFN $\gamma$  production from Peyer's patch cells (Fig. 3A; P<0.001). In contrast, ER lowered IFN $\gamma$  production in Peyer's patch cells, although this did not reach statistical significance (Fig. 3A; P=0.064). There was a significant main effect of EX (P<0.001) on Con A-induced IL-6 production in Peyer's patch cells, as well as a significant interaction between ER and EX on Peyer's patch T cell proliferation (Fig. 3B; P=0.010), with EX having a much greater effect in AL-fed mice as compared to ER mice. Finally, there were significant main effects of both ER (P<0.001) and EX (P<0.001) on Con A-induced IL-5 production in Peyer's patch cells (Fig 3C), as well as a significant interaction between ER and EX on Peyer's patch IL-5 production (Fig. 3C; P<0.001), with EX having only a stimulatory effect on IL-5 production in AL fed mice (Fig. 3C). No differences were observed in the production of IL-2, IL-4, IL-12, MCP-1 and TNF $\alpha$  in response to ER or EX from either splenocytes or cells collected from the Peyer's patches (data not shown).

### **Energy restriction enhances NK cell function**

ER (P<0.001), but not EX (P=0.298), significantly enhanced splenic NK cell cytotoxicity over a range of effector to target (E:T) ratios (12.5:1 to 100:1) using Cr<sup>51</sup>-labeled YAC-1 target cells. There was no evidence of an interaction between ER and EX on NK cell function (P=0.368).

## Discussion

To our knowledge, these results provide the first documentation of the differential effects of ER and EX on systemic and mucosal immune function. Both ER and EX reduced the size and cellularity of the spleen, which is comprised mainly of a loss of T and B lymphocytes. Despite these phenotypic similarities, the effects of ER and EX on the functional capacity of splenic and intestinal lymphocytes markedly differed. ER significantly impaired splenic and intestinal T cell proliferation, and had an inhibitory effect on cytokine production by intestinal lymphocytes. However, ER significantly enhanced splenic NK cell function. These data suggest that ER may be an effective intervention when enhanced NK cell function is a desired outcome. However, the inhibitory effects of ER on T cell proliferation and cytokine production may limit its use as an intervention strategy since adequate T cell function is an important component of anti-tumor immunity (40). In contrast to ER, EX enhanced both mucosal T cell proliferation and cytokine production, as well as IFN $\gamma$  production in the spleen. Therefore, EX may be an effective intervention either alone, or potentially in combination with other cancer prevention strategies, to enhance the functional capabilities of lymphocytes, particularly those residing in the intestine.

Our study was designed to compare immunologic parameters in response to the two most commonly recommended weight control strategies, specifically ER and increased physical activity. ER significantly reduced body weight from both the lean and fat mass compartments; lowered percent body fat; and reduced bone mineral density, as previously reported (35,41). In contrast, 6 weeks of voluntary EX did not significantly alter body weight or composition, but did significantly increase bone mineral density, which is consistent with a moderate EX regimen (42,43). The absence of a statistically significant effect of EX on body weight or composition is likely due to a relatively short training period (6 weeks). Subsequent studies in our laboratory have shown that 12 weeks of voluntary running results in significant changes in body weight and composition (data not shown). Nevertheless, significant changes in bone mineral density in the sample of animals studied here clearly indicate that physiological changes are occurring in response to 6 weeks of EX.

Previous studies have also documented that ER and EX reduce the size and cellularity of the spleen (44-46). However, this study is the first to demonstrate that these effects are tissue specific, as the size and cellularity of the Peyer's patches were not affected by either ER or EX. The reduction in the size of the spleen with both ER and EX can be explained by a loss of T and B lymphocytes. One hypothesis to explain these findings is that ER and EX induce apoptosis of lymphocytes in the spleen. ER has been shown to increase Fas/Fas-ligand expression on lymphocytes (47) and render T cells more sensitive to apoptotic signals (48). No studies to date have examined the role of regular, moderate EX on apoptosis of splenic lymphocytes; however, an acute bout of treadmill EX has been shown to induce apoptosis of lymphocytes in the thymus, spleen and intestine (49,50). The biological mediator(s) of ER- and/or EX-mediated lymphocyte apoptosis is not known, but previous studies have shown that serum glucocorticoids, one possible candidate, are elevated in response to both ER (41,51, 52) and EX (50,53) and can induce apoptosis of T cells both *in vitro* (54) and *in vivo* (55).

ER significantly decreased Con A-induced lymphocyte proliferation of cells from both the spleen and intestine. We also found a statistically significant interaction between ER and EX on cytokine production from intestinal lymphocytes with ER inhibiting the EX-induced enhancement of IFN $\gamma$ , IL-6, and IL-5 production from intestinal lymphocytes. The effects of ER on immune function are influenced by the duration of exposure to ER, with shorter term exposure (6-8 weeks) often resulting in impaired function and longer term exposure resulting in immune enhancement, reviewed in (29). For example, short term ER has been shown to reduce splenic T cell proliferation in normal mice (56). Additionally, in several rodent

autoimmunity models, short term ER has been shown to decrease antigen specific proliferation of T cells, as well as decrease cytokine and autoantibody production (57-58). Together, these data suggest short term ER can suppress a number of T lymphocyte functions and may be beneficial in situations where T cell activation has induced a disease state. However, short term ER may not be beneficial to T cell function in young, healthy mice, as evidenced by the reduction in Con A proliferation following ER, as well as the diminution of the EX-induced enhancement of cytokine production by intestinal lymphocytes in this study.

In contrast to the inhibitory effect of ER on lymphocyte proliferation, EX significantly enhanced Con A-induced lymphocyte proliferation in intestinal lymphocytes, but had no effect on splenocyte proliferation. Previous reports in the literature have been inconsistent with some studies reporting an increase (59-62), a decrease (63), or no effect (53,64) of regular, moderate EX training on T cell proliferative responses. This heterogeneity in proliferative responses may be due to timing of lymphocyte collection with respect to the last EX bout, and varying intensity and duration of EX interventions. Finally, it appears that lymphocytes isolated from different lymphoid tissue may be differentially impacted by EX training. For example, moderate EX has been shown to enhance the Con A-induced proliferation of T cells collected from the peripheral blood but not the spleen of hamsters (53). Additionally, treadmill EX in rats resulted in an increase in Con A-induced lymphocyte proliferation in the mesenteric lymph nodes, but not the spleen (65). These results are consistent with our findings in which proliferative responses were increased following EX in intestinal lymphocytes but not splenocytes. These data suggest that EX selectively enhances T cell proliferation in some, but not all lymphoid organs.

In addition to documenting the EX-induced enhancement of intestinal T cell proliferation, this study is the first to demonstrate an EX-induced enhancement of mucosal cytokine production, which may improve cellular and humoral immune responses in the mucosa. To date, the only mucosal immune endpoint examined in response to moderate EX has been the humoral response, specifically the mucosal-associated antibody, IgA. Several studies in humans have shown that salivary secretory IgA levels were enhanced with moderate EX training (31,32). However, no studies have examined the effect of EX on cell-mediated immunity in the mucosal compartment. In the present study, the novel findings that EX led to an increase in Con A-stimulated lymphocyte proliferation in conjunction with an increase in the *in vitro* production of IL-5, IL-6, and IFN $\gamma$  from Peyer's patch cells suggest that regular, moderate EX enhances cell-mediated and potentially, downstream humoral responses in the mucosal immune system. Since the Peyer's patches are the inductive site in the mucosal immune system where immune cells first encounter antigen and initiate IgA production and mucosal T cell responses (66), an EX-induced enhancement of T cell responses in the Peyer's patches may result in improved immunosurveillance against ingested pathogens and preneoplastic and/or neoplastic cell growth in the gastrointestinal tract. However, additional studies in appropriate animal models are needed to address this question.

Finally, in our study ER significantly enhanced NK cell cytotoxicity, as previously reported (58). NK cells are important in controlling viral infections (67) and some neoplasias (39,68). The increase in NK cell cytotoxicity as a result of ER may be one mechanism by which ER may reduce tumor formation in spontaneous and chemically-induced tumor models, as it is well documented that NK cells are an important component of anti-tumor immunity (69,70). In previous studies, moderate EX has also been shown to enhance NK cell activity (21-28). The lack of a statistically significant effect of EX on NK cell cytotoxicity in these studies may be due to a relatively short training period (6 weeks). Other studies in the literature reporting an EX-induced enhancement of NK cell function have utilized a training protocol of several months.



In summary, we have documented that ER and EX, two lifestyle-based interventions known to prevent obesity and inhibit tumorigenesis in rodent models, differentially modulate components of systemic and mucosal immunity. ER enhanced NK cell function in the spleen, whereas EX mainly enhanced proliferation and cytokine production from intestinal lymphocytes. These results demonstrate that ER and EX are likely working through different mechanisms, at least with respect to regulating immune function. Additionally, these data demonstrate that moderate EX can enhance lymphocyte proliferation and cytokine production in the absence of significant changes in body composition, suggesting that the immune enhancing effects of EX in humans may be achieved in a relatively short period of time (6 weeks) without large decreases in body weight or fat mass. These findings also suggest that EX may be an important intervention to couple with ER (diet) in humans to prevent the immune inhibition that may result from short term ER. Finally, the results from this study suggest that moderate EX may be a viable intervention strategy to test in combination with other cancer prevention or therapeutic agents where an enhancement of cytokine and proliferative capabilities of lymphocytes may be beneficial. Future studies are aimed at evaluating the role of EX on antigen-specific immune function to determine if the EX-induced enhancement of immunity observed in this study impacts adaptive immune responses (i.e. response to vaccination) and influences anti-tumor immunity.

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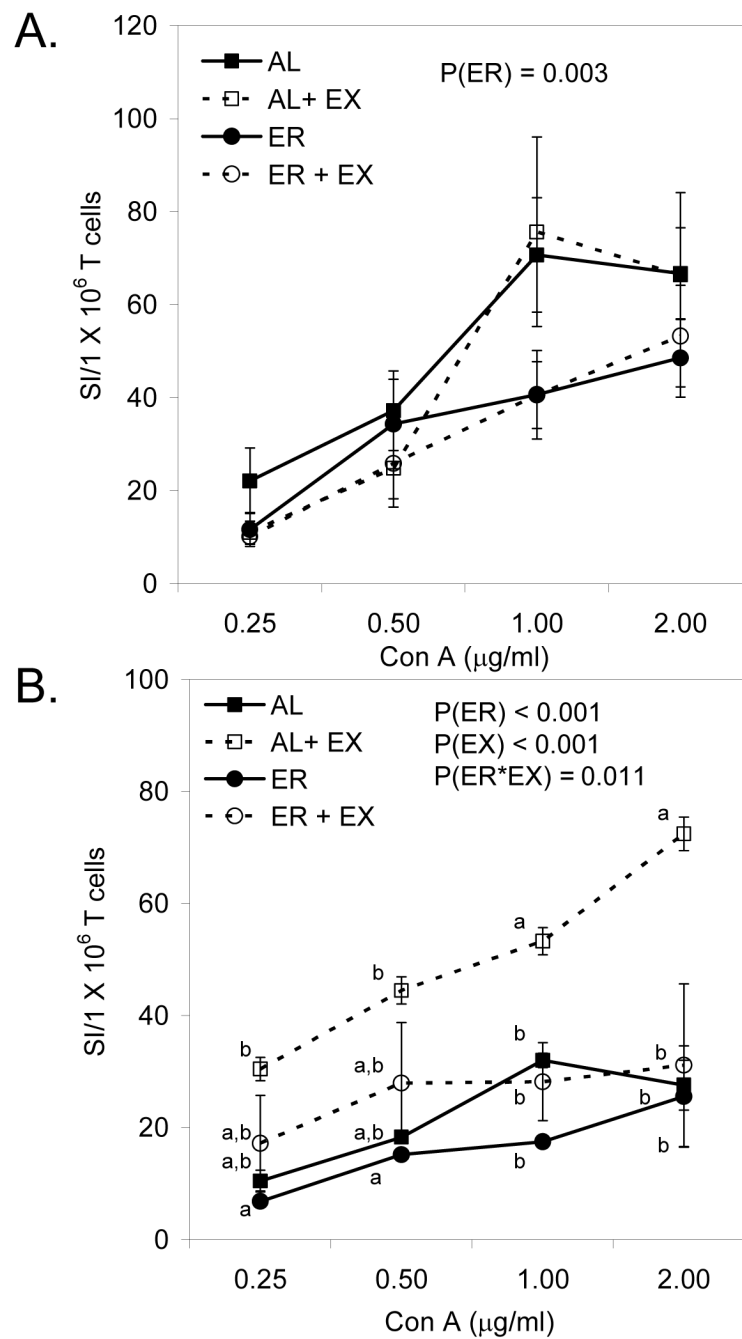
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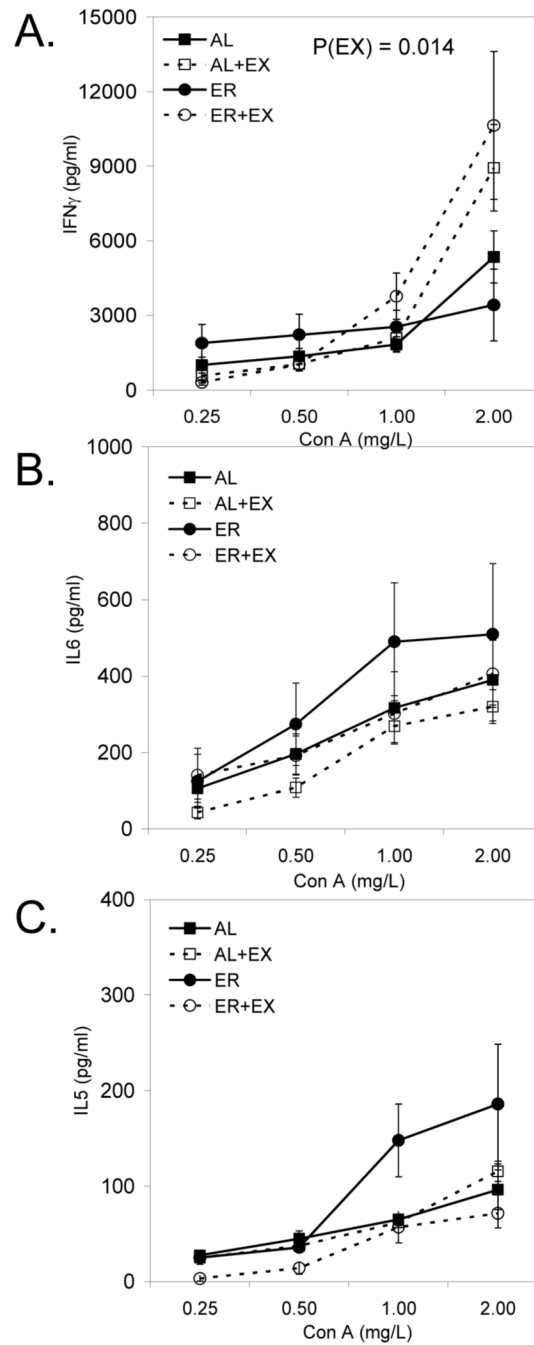
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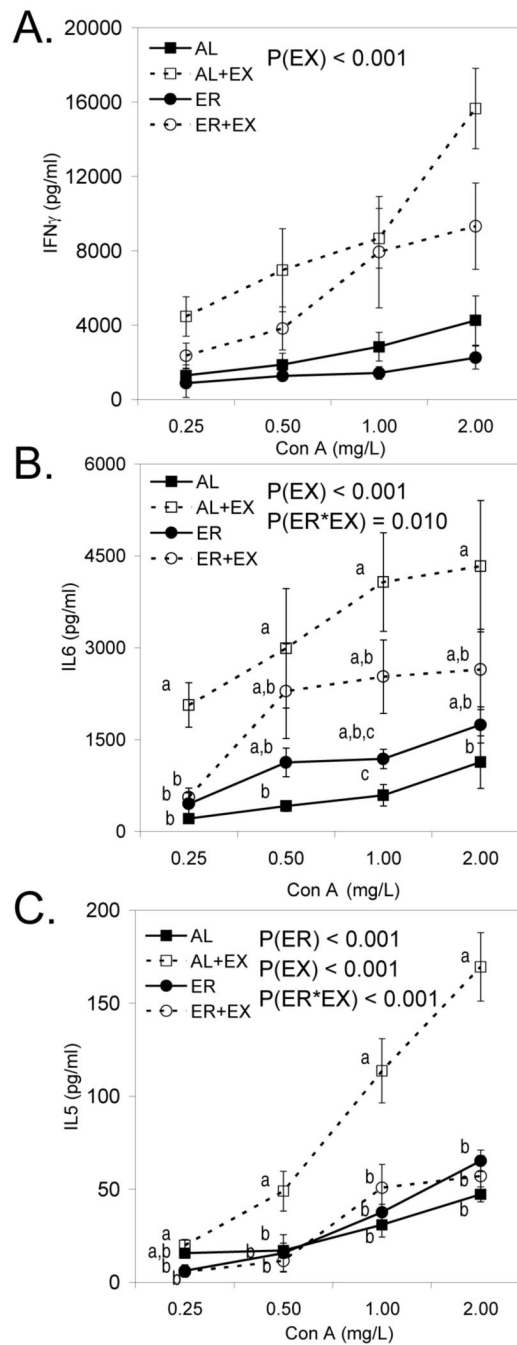
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**FIGURE 1.**

The effect of ER and EX on the proliferation of T cells collected from the spleen (A) and Peyer's patches (B). Lymphocytes were stimulated with Con A to induce T cell proliferation at the concentrations indicated for 72 h. Data shown are means  $\pm$  SEM (n=11-12/group). When the interaction between ER and EX was significant, Tukey's post hoc test was done to compare individual means among treatment groups. Means without a common letter differ ( $P < 0.05$ ).

**FIGURE 2.**

Con A-induced splenic IFN $\gamma$  (A), IL-6 (B), and IL-5 (C) production from mice maintained on AL, AL+EX, ER, or ER+EX treatments. Lymphocytes were stimulated with Con A to induce cytokine production at the concentrations indicated for 48 h. Data shown are means  $\pm$  SEM (n=11-12/group).

**FIGURE 3.**

Con A-induced IFN $\gamma$  (A), IL-6 (B), and IL-5 (C) production from cells collected from the Peyer's patches in mice maintained on AL, AL+EX, ER, or ER+EX treatments. Lymphocytes were stimulated with Con A to induce cytokine production at the concentrations indicated for 48 h. Data shown are means  $\pm$  SEM (n=11-12/group). When the interaction between ER and EX was significant, Tukey's post hoc test was done to compare individual means among treatment groups. Means without a common letter differ (P<0.05).

TABLE 1

Distance run and body composition measures (mean  $\pm$  SEM)<sup>1</sup> among mice exposed to AL, AL+EX, ER, or ER+EX treatment conditions for 6 weeks

Treatment Group	Mean Kilometers Run per Day	Body Weight (grams)	Fat Mass (grams) <sup>2</sup>	Lean Mass (grams)	Percent Body Fat	Bone Mineral Density <sup>3</sup> (g/cm <sup>2</sup> $\times$ 1000)
AL (n=12)	-	24.1 $\pm$ 1.1	7.9 $\pm$ 1.1	16.1 $\pm$ 0.4	33.8 $\pm$ 3.1	49.9 $\pm$ 1.6
AL+EX (n=11)	4.2 $\pm$ 0.7	23.2 $\pm$ 0.8	7.9 $\pm$ 0.9	15.9 $\pm$ 0.4	32.4 $\pm$ 2.4	51.3 $\pm$ 2.5
ER (n=11)	-	18.9 $\pm$ 0.4	4.5 $\pm$ 0.4	14.8 $\pm$ 0.5	24.8 $\pm$ 1.8	45.6 $\pm$ 1.8
ER+EX (n=12)	1.4 $\pm$ 0.5	17.6 $\pm$ 0.5	3.9 $\pm$ 0.4	13.8 $\pm$ 0.3	24.7 $\pm$ 1.7	46.4 $\pm$ 1.4
P (ER)	0.004	<0.001	<0.001	<0.001	<0.001	0.002
P (EX)	-	0.117	0.210	0.254	0.576	0.004

<sup>1</sup> Differences in mean kilometers run per day between groups tested with t-test, group differences remaining variables tested using two-way ANOVA.

<sup>2</sup> Lean mass included as a covariate for fat mass.

<sup>3</sup> Total body weight included as a covariate for bone mineral density.



**TABLE 2**

Lymphoid characteristics (mean ± SEM) among mice exposed to AL, AL+EX, ER, or ER+EX treatments for 6 weeks

Experiment Group	Spleen Weight (mg)	Spleen Weight as a Percentage of Body Weight <sup>f</sup>	Total Splenocyte Cell Number (X 10 <sup>6</sup> )	Number of Peyer's Patches per Intestine <sup>2</sup>	Total Peyer's Patch Cell Number <sup>2</sup> (X 10 <sup>6</sup> )
κ	73.9 ± 2.6	0.31 ± 0.02	109.8 ± 4.9	6.8 ± 0.4	18.1 ± 4.6
κ	68.5 ± 3.5	0.29 ± 0.01	88.1 ± 5.1	6.7 ± 0.4	17.0 ± 6.7
κ	57.1 ± 3.1	0.30 ± 0.01	71.9 ± 5.5	7.0 ± 0.6	16.0 ± 3.5
κ	43.1 ± 4.4	0.26 ± 0.02	57.6 ± 5.6	6.9 ± 0.4	10.8 ± 3.1
	<0.001	0.070	<0.001	0.631	0.160
	0.009	0.029	0.001	0.908	0.260

body weight included as a covariate for spleen weight.

<sup>f</sup>data represent the mean ± SEM of 4 pooled groups of 2-3 animals per group.

TABLE 3

Distribution of leukocytes in the spleen (mean  $\pm$  SEM)<sup>1</sup> among mice exposed to AL, AL+EX, ER, or ER+EX treatments for 6 weeks

Total T cells (CD3 <sup>+</sup> )	Helper T cells (CD3 <sup>+</sup> CD4 <sup>+</sup> )	Cytolytic T cells (CD3 <sup>+</sup> CD8 <sup>+</sup> )	CD4:CD8 Ratio	B cells (CD19 <sup>+</sup> )	NK cells (NK1.1 <sup>+</sup> )	Macrophages (CD11b <sup>+</sup> T-Ab <sup>+</sup> )	Dendritic cells (CD11c <sup>+</sup> T-Ab <sup>+</sup> )
60.7 $\pm$ 4.8	32.1 $\pm$ 2.3	24.4 $\pm$ 1.9	1.3 $\pm$ 0.04	43.9 $\pm$ 4.4	5.1 $\pm$ 0.3	1.0 $\pm$ 0.1	1.2 $\pm$ 0.2
50.8 $\pm$ 2.8	30.1 $\pm$ 1.6	19.8 $\pm$ 0.9	1.5 $\pm$ 0.07	33.0 $\pm$ 5.8	4.5 $\pm$ 0.4	0.6 $\pm$ 0.1	1.1 $\pm$ 0.2
40.9 $\pm$ 4.6	19.3 $\pm$ 2.3	19.5 $\pm$ 1.9	1.0 $\pm$ 0.07	27.3 $\pm$ 4.6	4.5 $\pm$ 0.8	1.1 $\pm$ 0.2	1.2 $\pm$ 0.2
31.7 $\pm$ 3.1	15.1 $\pm$ 1.6	16.1 $\pm$ 1.8	1.0 $\pm$ 0.05	21.7 $\pm$ 2.9	4.5 $\pm$ 0.8	0.7 $\pm$ 0.2	1.0 $\pm$ 0.2
<0.001	<0.001	0.019	<0.001	0.003	0.655	0.672	0.653
0.022	0.133	0.028	0.136	0.069	0.660	0.019	0.618

total number of cells in each subset ( $\times 10^6$ ).

**TABLE 4**

Distribution of leukocytes in the Peyer's patches (mean  $\pm$  SEM)<sup>1,2</sup> among mice exposed to AL, AL+EX, ER, or ER+EX treatments for 6 weeks

Treatment Group	T cells (CD3 <sup>+</sup> )	B cells (B220 <sup>+</sup> )	Macrophages (CD11b <sup>+</sup> /I-Ab <sup>+</sup> )	Dendritic cells (CD11c <sup>+</sup> /I-Ab <sup>+</sup> )
AL	3.5 $\pm$ 0.7	13.2 $\pm$ 1.8	0.8 $\pm$ 0.2	0.7 $\pm$ 0.1
AL+EX	2.8 $\pm$ 0.7	12.6 $\pm$ 2.4	0.9 $\pm$ 0.2	0.6 $\pm$ 0.1
ER	2.9 $\pm$ 0.3	11.5 $\pm$ 0.9	0.7 $\pm$ 0.3	0.4 $\pm$ 0.1
ER+EX	1.7 $\pm$ 0.3	8.3 $\pm$ 1.5	0.9 $\pm$ 0.3	0.3 $\pm$ 0.1
P (ER)	0.141	0.112	0.820	0.021
P (EX)	0.101	0.312	0.747	0.287

<sup>1</sup>Data shown are the total number of cells in each subset ( $\times 10^6$ ).

<sup>2</sup>These data represent the mean  $\pm$  SEM of 4 pooled groups of 2-3 animals per group.