Physical Activity Plus Energy Restriction Prevents 4T1.2 Mammary Tumor Progression, MDSC Accumulation, and an Immunosuppressive Tumor Microenvironment

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Abstract

Physical activity and the prevention of weight gain decrease breast cancer incidence and improve survival. Unraveling the biological mechanisms underlying these cancer prevention effects is difficult because activity and dietary restriction are often linked. The goal of this study was to determine whether physical activity (PA), preventing weight gain via energy restriction (ER), or the combination was most effective in delaying tumor growth, reducing metastatic progression, and improving survival in the 4T1.2 mammary tumor model. Furthermore, we determined whether any of these interventions prevented the expansion of protumor immunosuppressive cells and altered the tumor microenvironment (TME). Female BALB/c mice (n = 7–20/group) were randomized to sedentary (SED) or PA wheel cages and fed ad libitum (AL) or 90% of control food intake (ER). After 8 weeks on the interventions, mice were inoculated with 5 × 10⁴ 4T1.2 luc cells into the 4th mammary fat pad and continued on their respective intervention. PA + ER significantly delayed primary tumor growth (final tumor volume, 0.193 ± 0.042 vs. 0.369 ± 0.049 cm³, P < 0.001), reduced metastatic burden in the lungs (0.72 ± 0.36 vs. 16.27 ± 6.98, P = 0.054) and increased survival (median survival, 68 vs 40 days, P = 0.043) compared with SED + AL mice. PA + ER also reduced the expression level of metastatic and immunosuppressive genes and resulted in favorable changes in immune cell infiltrates in the tumor. These data suggest that both PA and ER are needed to reduce tumor growth, delay metastatic progression, and improve survival, and that this protection is associated with changes in immune-mediated mechanisms.

Introduction

The combination of increased body weight and physical inactivity are important lifestyle factors that contribute to the risk of breast cancer (1, 2). Elevated weight has contrasting effects on premenopausal and postmenopausal breast cancer risk (3, 4). However, being overweight or obese is a predictor of poor prognosis once breast cancer is diagnosed regardless of menopausal status (5). Data from epidemiologic studies indicate that regular physical activity (PA) significantly decreases breast cancer incidence (6) and mortality (7). This effect is observed when controlling for BMI, suggesting that exercise may be protective independent of weight status. However, the observational nature of these studies limits the ability to determine biological mechanisms mediating these effects. Because the majority of deaths from breast cancer result from metastatic disease, it is possible that the improvements in breast cancer survival observed in physically active women are due to a reduction in metastatic disease occurrence and/or progression (8, 9). Mechanistic studies are needed to support this hypothesis.

Several broad categories of host factors are identified as possible mediators underlying the relation between exercise and breast cancer risk and progression including changes in immune function (10, 11). The role of the immune system in controlling primary tumor growth is well studied (12) and involves antitumor responses...
mediated by effector cells (e.g., NK cells and CD8+ cytotoxic T cells). However, immunosuppressive cells of both lymphoid [regulatory T cells (Treg) and myeloid (myeloid-derived suppressor cells (MDSC)] lineages are also recruited to the tumor microenvironment (TME) by proinflammatory tumor-derived factors, and their abundance is positively correlated with tumor burden (13, 14). The balance between antitumor immunosurveillance mechanisms and the accumulation of tumor-driven immunosuppressive cells is critical for regulating primary tumor growth (15) and metastatic progression (16). However, the effects of alterations in energy balance on pro-versus antitumor immune responses are unknown. In non–tumor-bearing, weight-stable mice, moderate exercise significantly enhances antigen-specific T-cell responses and natural killer (NK)-cell activity (17, 18), two cell types important for mediating antitumor immunity. Thus, it is plausible that moderate PA may reduce recurrence and increase survival in patients with breast cancer by preserving or restoring mechanisms that promote immunosurveillance.

The extent to which PA, as opposed to changes in body weight, protects against primary tumor growth and metastatic progression is a critical issue. Therefore, the primary goal of this study was to determine whether PA, the prevention of weight gain achieved via mild energy restriction, or the combination prevented primary tumor growth, reduced metastatic progression, and/or improved survival in a mammary tumor model that spontaneously metastasizes. A second goal was to determine whether either single intervention, or the combination altered gene expression or immune infiltrates in the TME. We hypothesize that each single intervention may improve tumor outcomes, but that the combination of both PA and energy restriction will result in the greatest benefit on tumor outcomes and survival and this may be accompanied by beneficial changes in the TME.

Materials and Methods

Tumor cell line and cell culture

The 4T1.2 metastatic cell line was derived from a spontaneously arising mammary tumor in a BALB/cfC3H mouse (19). When implanted orthotopically, the 4T1.2 model mimics the metastatic progression of human breast cancer with a predilection to metastasize to lung and bone (20). 4T1.2 cells stably expressing luciferase (4T1.2luc) were provided by Dr. Shoukat Dedhar (Department of Cancer Genetics, BC Cancer Research Centre, Vancouver, BC, Canada), in 2010 with a negative test for infectious agents, including Mycoplasma. Upon receipt, cells were thawed, expanded to create a stock, and passaged less than 15 times before use. No universal standards are available for authentication of the murine cell lines. 4T1.2luc cells were maintained in DMEM (Life Technologies) containing 10% FBS (Gemini Bio-Products), 2 mmol/L glutamine (Mediatech), 1 x nonessential amino acid (Mediatech), and 8 μg/ml puromycin (Mediatech).

Animal model

Six-week-old female BALB/c mice were obtained from Jackson Laboratory. All mice were acclimated and screened for voluntary running behavior as described previously (18). One cohort of female BALB/c mice was inoculated with 5 × 10⁴ 4T1.2luc cells into the 4th mammary fat pad and sacrificed at day 7, 14, 21, 28, or 35 posttumor implantation (n = 4–8/time point) to characterize splenic and plasma cytokine changes in response to the 4T1.2 tumor (in the absence of dietary or PA interventions). Additional cohorts of mice were randomized to sedentary (SED) or PA wheel cages and fed ad libitum (AL) or 90% of control food intake [10% energy restriction (ER)]. This 2 × 2 factorial design was used to investigate the effects of PA alone, ER alone, and the combination of PA+ER on primary tumor growth, metastatic outcomes, and survival. All mice were fed AIN-76A diet (Research Diets). After eight weeks on the interventions, mice were inoculated with 5 × 10⁴ 4T1.2luc cells into the 4th mammary fat pad and continued on their respective energy balance intervention for varying lengths of time posttumor implantation. In one experiment, mice were sacrificed at day 35 posttumor implantation to determine the effect of the interventions on primary tumor growth, metastatic burden, pro- vs. antitumor immune responses and TME gene expression. These experiments were completed in a cohort of young, 10-week-old mice (n = 8–12/group) and in middle-aged, 38-week-old mice (n = 5–8/group) to determine whether age impacted the effect of the interventions on tumor outcomes. In another experiment, mice were sacrificed when tumor volumes were equal (0.05–0.20 cm³). Immune outcomes and TME gene expression were assessed to determine the effect of the interventions on immune outcomes, controlling for tumor size (n = 7–11/group). In a third experiment, the effect of the interventions on survival was determined (n = 7–8/group). Mice were placed on the interventions, and removed from the study when the primary tumor volume exceeded 1.0 cm³ or animals were moribund. Food intake, body weight, health status, and tumor size (v = (short2 × long)/2) were monitored as previously reported (21). All mice were maintained on a 12-hour light/dark cycle with free access to water. The Institutional Animal Care and Use Committee of the Pennsylvania State University (University Park, PA) approved all animal experiments.

Quantification of metastatic burden

At sacrifice, lung and femur were collected, flash-frozen, and stored at −80°C. Tissues were homogenized and
genomic DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen) per manufacturer’s instructions. Metastatic tumor burden was quantified using the Taqman system performed on a Step-One Plus (Life Technologies) to quantify the luciferase gene. The reference gene used for normalization was mouse telomerase reverse transcriptase (TERT; Life Technologies). The standard curve included five 10-fold serial dilutions (200 ng to 20 pg) of DNA extracted from cultured 4T1.2 luc cells. Luciferase data was normalized to the quantitative values for TERT in each sample to correct for fluctuations in DNA amount, quality, and reaction efficiency.

Isolation of splenic and tumor-infiltrating immune cells

Spleens were harvested and splenocytes were prepared from individual mice as described previously (22). Primary tumors were harvested during dissection, weighed, minced, and incubated with 0.03 mg/mL Liberase (Roche) and 12.5 U/mL DNase I (Sigma-Aldrich) for 45 minutes at 37°C. Following digestion, remaining pieces were mechanically disrupted, passed through a 70-μm nylon mesh strainer (BD Biosciences), layered over Lympholyte-M cell Separation Media (Cedarlane), and centrifuged at room temperature for 20 minutes at 1,200 × g. Isolated cells were washed in cold PBS (Mediatech) and cell counts and viability were determined via Trypan Blue exclusion.

Splenic CD4+ T-cell proliferation assay

Splenic CD4+ T cells were isolated via Dynabeads Untouched Mouse CD4 Cells Kit following the manufacturer's instructions (Life Technologies). Splenic CD4+ T cells (1 × 10^5) plus 1.0 μg/mL unlabeled anti-CD28 (BD Biosciences) were incubated in the presence of anti-CD3 antibody (BD Biosciences) for 72 hours (17). Proliferation data were analyzed by tritiated (H3) thymidine (PerkinElmer) incorporation and quantified with a Microbeta Plate Reader (PerkinElmer). Each assay was performed in triplicate.

Systemic plasma cytokine analysis

Fasting blood was collected at sacrifice (day 35), centrifuged, and, plasma was stored at −80°C. G-CSF and IL6 were measured using a Milliplex MAP Multiplex Assay (EMD Millipore) and quantified on a Bio-plex 200 System (Bio-Rad) using Luminex-200 Software (Luminex), per manufacturer's instructions. Each assay was performed in duplicate.

Flow cytometric analyses

Single-cell suspensions of splenocytes and tumor immune infiltrates were washed, prepared, and stained with saturating concentrations of conjugated antibodies as described previously (18). In addition, splenic Tregs were quantified using the Mouse Regulatory T Cell Staining Kit (eBioscience) per manufacturer’s instructions. Flow cytometric analyses were performed on a BD LSR-Fortessa (BD Bioscience) cytometer. Flow cytometric analyses were plotted and analyzed using Flow Jo Software (Tree Star).

Gene expression in the TME

At sacrifice, whole tumors (n = 3–4/group) were harvested and incubated overnight in RNA later (Qiagen). RNA was isolated from individual tumors using Qiashredder columns followed by RNeasy Mini Plus Kit (Qiagen). RNA sample was reverse-transcribed using RT2 First Strand Kit (Qiagen). Samples were loaded onto qPCR plates (Mouse Cancer Inflammation & Immunity Crosstalk PCR Array, Qiagen) and cycled according to the manufacturer's instructions. Array data was analyzed using Qiagen's GeneGlobe Data Analysis Center. Threshold cycle values (CT) values were normalized via manual selection to Actb and Hsp90ab1 housekeeping genes and fold change (2^ΔΔCt) was calculated as described previously (23). Data visualization was achieved via supervised hierarchical clustering of the entire dataset (n = 3–4/group) to generate a clustergram of genes across groups with SED+AL mice as the reference group.

qRT-PCR

qRT-PCR was performed to validate the gene array results (n = 4/group). Total RNA was reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies). qRT-PCR was performed by using TaqMan Real Time PCR Reagents (Life Technologies). The relative level of gene expression was measured and normalized to 18S RNA. Primer sequences (Applied Biosciences) were selected on the basis of published studies (24). Relative differences in gene expression were analyzed using the 2^-ΔΔCt method, as described previously (23).

Statistical analysis

All data were assessed for normality and equal variances, and either parametric or nonparametric analyses were used to detect differences between treatment groups. If data were skewed, transformation (log or square root) was done prior to statistical analysis. Differences in tumor weight, metastatic burden, distribution of cells in the spleen and tumor, and plasma mediators were assessed between groups via a one-way ANOVA or Kruskal–Wallis test (depending on normality and variance) followed by a Bonferroni correction or Dunn test for multiple comparisons where appropriate. Differences in body weight and running wheel activity over time by age (pooled data) or treatment group; primary tumor volume over time by age (pooled data), treatment group, or running wheel activity level; and CD4+ T cell proliferation by concentration of antigen were examined using a two-way ANOVA. Spearman rank correlation was used to determine the relationship between cytokines and tumor volume. Survival estimates were
generated using Kaplan–Meier plots and the log-rank test was used to test for group differences. All data are presented as the mean plus or minus the SEM. All analyses were conducted using GraphPad Prism 5 Software (GraphPad Software) and statistical significance was accepted at the \( P < 0.05 \) level.

**Results**

Body weights are lower in energy-restricted versus *ad libitum*–fed mice, and running wheel activity was maintained in tumor-bearing mice.

Mice were randomized to SED or PA wheel cages and fed *ad libitum* (AL) or 90% of control food intake (10% ER). The interventions, timing of sacrifice, and study outcomes are shown in the experimental design schema (Fig. 1A). Mice in the SED+ER and PA+ER groups (i.e., mice that were in weight maintenance, WM), weighed significantly less than mice in the SED+AL or PA+AL groups that gained weight (WG) over the course of the study (Fig. 1B; \( n = 15–20 \) /group; \( F(39,897) = 12.76; \ P < 0.001 \)). Running wheel activity of AL and ER tumor–bearing mice was similar, and did not change throughout the 13-week experiment (Fig. 1C; \( n = 20 \) /group).

The combination of PA and energy restriction (PA+ER) delayed tumor growth, reduced metastatic burden, and improved survival.

No differences were observed in the ER-induced reduction in body weight, the amount of PA, or in primary tumor

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**Figure 1.**

Body weight and running wheel activity during the study. **A,** Mice were randomized to SED or PA wheel cages and fed *ad libitum* (AL) or 90% of control food intake (10% ER) prior to tumor implantation. **B,** Body weight (\( n = 15–20 \) /group; \( P < 0.001 \)), and **C** wheel activity over time.
growth and metastatic outcomes between the cohorts of 10-week-old \((n = 7–11/\text{group})\) and 38-week-old mice \((n = 5–7/\text{group}; \text{Supplementary Table S1})\). Therefore, data from both cohorts of mice were combined for subsequent analysis of the effect of the interventions on primary tumor growth and metastatic outcomes, and are shown in Fig. 2A–D. In mice sacrificed at day 35 posttumor implantation \((n = 15–20/\text{group})\), primary tumor growth over time was significantly reduced in PA+ER mice (Fig. 2A; \(F(18,414) = 3.33; P < 0.001\)) compared with SED+AL, PA+AL, or SED+ER groups. At day 35 posttumor implantation, final tumor weight was significantly reduced in PA+ER mice compared with SED+AL mice (Fig. 2B; \(F(3,68) = 3.492; P = 0.021\)). Spontaneous lung (Fig. 2C; \(F(3,58) = 2.72; P = 0.054\)) and femur (Fig. 2D; KW = 10.06; \(P = 0.018\)) metastases were significantly lower in PA+ER and SED+ER mice. Mice in the four intervention groups \((n = 7/\text{group})\) that were sacrificed when tumor volumes were 0.05–0.20 cm\(^3\) had equivalent tumor volumes at sacrifice, as expected (Fig. 2E). However, the number of days posttumor implantation to reach an equivalent tumor size was significantly delayed by all three interventions compared with SED+AL mice (Fig. 2F; KW = 11.38; \(P = 0.010\)). Overall survival was significantly increased only by the combination of PA+ER (Fig. 2G; \(n = 7–8/\text{group}; P = 0.043\)).

The 4T1.2\textsuperscript{lac} model is characterized by splenomegaly and an increase in MDSCs

To characterize the effect of 4T1.2 tumor growth on immune cell distribution in the spleen, cohorts of mice \((n = 4–8/\text{group})\) were sacrificed at 7, 14, 21, or 28 days after tumor implantation (with no energy balance intervention). Spleens were enlarged in tumor-bearing mice (Fig 3A) and splenocyte counts increased proportionately with tumor volume (Fig. 3B). Tumor-
Figure 3.
Splenic immune changes in the 4T1.2 model. A, The spleen from a control, non-tumor-bearing mouse versus a representative tumor-bearing mouse harvested at day 35 post-tumor implantation. B, Splenocyte cell counts (bars) and tumor volume (open circles) over time (*P < 0.001). The percent of CD3$^+$ T cells (C; *P < 0.001), CD4$^+$ T cells (D; *P < 0.001), and CD8$^+$ T cells (E; *P < 0.001) over time. The percent of total Gr-1$^+$ CD11b$^+$ MDSCs (F; *P = 0.017), Gr-1$^+$CD11b$^+$ monocytic mMDSC subset (G; *P = 0.632), and Gr-1$^+$CD11b$^+$ granulocytic gMDSC subset (H; *P = 0.002) during tumor progression. Asterisks indicate a significant difference from day 7 values (n = 4–8/group). Correlations between tumor weight at sacrifice and the number of splenic Gr-1$^+$CD11b$^+$ MDSCs (I; *P < 0.001), plasma G-CSF (J; *P < 0.001), and plasma IL6 (K; *P < 0.001).
induced splenomegaly was characterized by a significant decrease in the percent of CD3+ T cells (Fig. 3C; \( P < 0.001 \)), CD4+ T cells (Fig. 3D; \( P < 0.001 \)), and CD8+ T cells (Fig. 3E; \( P < 0.001 \)), and a significant increase in the percent of total Gr-1+CD11b+ MDSCs (Fig. 3F; \( P = 0.017 \)), which was mainly due to an increase in the percent of the Gr-1+CD11b+ granulocytic (g) MDSC subset (Fig. 3H; \( P = 0.002 \)) with no change in the percent of the Gr-1+CD11b+ monocytic (m) MDSC subset (Fig. 3G; \( P = 0.632 \)) during tumor progression. There were no significant changes in the percent of CD19+ B cells or F4/80+ macrophages in the spleen during tumor progression (Supplementary Fig. S1). Tumor weight at sacrifice was correlated with splenic Gr-1+CD11b+ MDSCs (Fig. 3I; \( P < 0.001 \)), plasma G-CSF (Fig. 3J; \( P < 0.001 \)), and IL6 (Fig. 3K; \( P < 0.001 \)).

The combination of PA+ER prevented tumor-induced splenomegaly and altered splenic immunity

In mice sacrificed at day 35 posttumor implantation, the number of splenocytes was significantly lower in PA+ER mice compared with both SED+AL and PA+AL mice (Fig. 4A; \( F(3,72) = 5.60; P = 0.002 \)). CD4+ T-cell proliferation was significantly greater in PA+ER mice compared with PA+AL mice (Fig. 4B; \( n = 15-20/\text{group}; F(3,315) = 2.99; P = 0.038 \)). The number of CD4+CD25+FoxP3+ Tregs was lower, but not statistically significant, in the PA+ER mice compared with SED+AL mice (Fig. 4C; \( n = 8-9/\text{group}; F(3,34) = 1.76; P = 0.176 \)). The number of total Gr-1+CD11b+ MDSCs (Fig. 4D; \( n = 15-20/\text{group}; F(3,72) = 5.06; P = 0.003 \)), CD11b+Ly6CD11c+Ly6G- monocytes (Fig. 4E; \( \text{KW} = 11.50; P = 0.009 \)), and CD11b+Ly6C+Ly6G+ granulocytic (g) MDSCs (Fig. 4F; \( F(3,72) = 4.38; P = 0.007 \)) was significantly lower in PA+ER mice compared with either SED+AL or PA+AL groups.

In mice that were sacrificed when tumor volumes were equal in size, the number of splenocytes was significantly lower in both the SED+ER and PA+ER groups compared with SED+AL mice (Fig. 4G; \( n = 7-11/\text{group}; F(3,32) = 4.78; P = 0.004 \)). Furthermore, the number of Gr-1+CD11b+ MDSCs (Fig. 4H; \( n = 7-11/\text{group}; F(3,30) = 4.48; P = 0.010 \)) was significantly lower in the PA+ER group compared with the PA+AL group. The distribution of other immune cell populations in the spleen at day 35 and at equal tumor volumes is shown in Supplementary Table S2A and S2B, respectively.

Tumor and splenic immune outcomes varied by activity level in energy-restricted mice

To evaluate whether the protective effect of PA is activity-level dependent, mice in both the PA+AL and PA+ER groups were stratified by running activity. Mice below the 50th percentile of running activity in each group (5.2 km/day and 5.7 km/day in the PA+AL and PA+ER groups, respectively) were designated low runners, and mice at or above the 50th percentile of running activity within each group were designated high runners. PA(LOW)+AL and PA(HIGH)+AL mice did not differ in body weight; whereas, PA(HIGH)+ER mice weighed significantly less than PA(LOW)+ER mice over the course of the study (Fig. 5A; \( n = 17/\text{group}; F(13,416) = 2.81; P = 0.001 \)). Mice in both the PA(HIGH)+AL and PA(HIGH)+ER groups averaged more km/day than mice in the PA(LOW)+AL and PA(LOW)+ER groups (Fig. 5B; \( F(1,384) = 42.33; P < 0.001 \)). Primary tumor growth was significantly reduced in PA(HIGH)+ER mice compared with PA(LOW)+AL, PA(HIGH)+AL, and PA(LOW)+ER mice (Fig. 5C; \( F(21, 350) = 3.55; P < 0.001 \)). However, no difference in tumor growth over time was detected between PA(HIGH)+AL and PA(LOW)+AL mice. In addition, the number of splenocytes (Fig. 5D; \( F(3, 50) = 7.13; P = 0.004 \)) was significantly lower in PA(HIGH)+ER mice compared with PA(LOW)+AL (\( P < 0.05 \)), PA(HIGH)+AL (\( P < 0.05 \)), and PA(LOW)+ER mice (\( P < 0.05 \)) but no difference in splenocyte count was observed between PA(HIGH)+AL and PA(LOW)+AL mice. The number of splenic Gr-1+CD11b+ MDSCs (Fig. 5E; \( F(3, 50) = 9.63; P < 0.001 \)) was significantly lower in PA(HIGH)+ER compared with PA(LOW)+AL and PA(HIGH)+AL mice.

The combination of PA+ER altered gene expression and immune cell infiltrates in the TME

Clustergrams were generated to display genes for which expression levels were altered greater than 2-fold when tumors were harvested at 0.05–0.20 cm\(^3\) (Fig. 6A) and at day 35 posttumor implantation (Fig. 6B). Both SED+ER and PA+ER interventions reduced the expression of Iacda, Pdcd1, Ifng, Foxp3, and Idol when tumor volumes were equal (Fig. 6B). At day 35 posttumor implantation, gene expression of most of the chemokines and chemokine receptors, as well as Foxp3 and Ifng, was downregulated by all of the interventions; however, Idol gene expression was lowest in the PA+ER group at day 35 posttumor implantation (Fig. 6D). Validation of specific genes altered by PA and ER interventions using qPCR when the tumors were equal in size (Supplementary Table S3A) and at day 35 posttumor implantation (Supplementary Table S3B) confirm the results from the PCR array.

In mice sacrificed when tumor volumes were equal and at day 35 posttumor implantation, the percent of tumor-infiltrating CD8+ T cells was significantly elevated in tumors from PA+ER mice compared with SED+AL mice (Fig. 6E; \( F(3,36) = 4.94; P = 0.006 \) and Fig. 6H; \( F(3,41) = 8.51; P < 0.001 \)). The percent of total Gr-1+ CD11b+ MDSCs was significantly lower in tumors from PA+ER mice compared with SED+AL mice when tumor volumes were equal (Fig. 6F; \( F(3,36) = 3.57; P = 0.023 \)) and at day 35 posttumor implantation (Fig. 6I; \( F(3,41) = 4.83; P < 0.001 \)). The ratio of CD8+ T cells: total MDSCs was higher in tumors from PA+ER mice compared with SED+AL mice when tumor volumes were equal (Fig. 6G; \( F(3,36) = 5.34; P = 0.004 \)) and at day 35 posttumor.
The combination of PA + ER prevented tumor-induced splenomegaly and altered splenic immunity. In mice sacrificed at day 35 post-tumor implantation the number of splenocytes (A, n = 15–20/group; P = 0.002), CD4⁺ T-cell proliferation (B, n = 15–20/group; P = 0.038), splenic CD4⁺CD25⁺FoxP3⁺ Tregs (C, n = 8–9/group; P = 0.016), total Gr-1⁺CD11b⁺MDSCs (D, n = 15–20/group, P = 0.003), CD11b⁺Ly6C⁺Ly6G⁻gMDSCs (E, n = 15–20/group, P = 0.009), and CD11b⁺Ly6C⁺Ly6G⁺gMDSCs (F, n = 15–20/group, P = 0.007) by treatment group. In mice sacrificed at equal tumor volumes (0.05–0.20 cm³) the splenocyte count (G, n = 7–11/group; P = 0.004) and number of MDSCs (H, n = 7–11/group; P = 0.010) by treatment group. Significantly different than SED + AL (°) and PA + AL (†).
implantation from mice in SED+ER and PA+ER mice compared with SED+AL mice (Fig. 6); \( F_{(3,41)} = 19.59; P < 0.001 \). The percent of tumor-infiltrating CD4+ T cells and MDSC subsets in mice sacrificed when tumor volumes were equal, and at day 35 posttumor implantation are shown in Supplementary Fig. S2.

**Discussion**

This study examined the effects of PA, the prevention of weight gain via mild dietary restriction, or the combination of both interventions on tumor progression (primary tumor growth, metastatic spread, and survival) and gene expression and immune outcomes in the TME in a preclinical metastatic breast cancer model. We found that the combined effects of moderate PA and 10% energy restriction (PA+ER) culminated in a significant delay in primary tumor growth, a reduction in spontaneous metastases, and an improvement in survival that was not observed with either single intervention. The favorable effects of PA+ER on tumor progression and survival were accompanied by a significant reduction in splenic MDSCs, an increase in CD4+ T-cell proliferation, a reduction in the expression level of metastatic and...
The combination of PA+ER altered gene expression and immune infiltrates in the TME. Clustergram displaying genes altered >2-fold in tumors harvested at similar size (0.05–0.20 cm³; A) and at day 35 posttumor implantation (n = 3–4/group; C). Genes that were significantly altered (>2.5-fold) in tumors from mice in the intervention groups compared with tumors from SED+AL mice in tumors harvested at similar size (B) and at day 35 posttumor implantation (D; n = 3–4/group). In tumors harvested at similar size and at day 35 posttumor implantation, the percentage of tumor-infiltrating CD8⁺ T cells (E; P = 0.006) and (H; P < 0.001), respectively; the percent of total Gr-1⁺CD11b⁺MDSCs (F; P = 0.025) and (I; P < 0.001), respectively; and the ratio of CD8⁺ T cells:total MDSCs (G; P = 0.004) and (J; P < 0.001), respectively differed in the PA+ER group compared with other intervention groups. Significantly different than SED+AL (*), PA+AL (†), and SED+ER (‡).
immunosuppressive genes in the TME, and favorable changes in immune cell infiltrates into the tumor. Furthermore, the beneficial effect of the dual intervention on tumor growth and immune outcomes was related to the volume of activity, with the greatest activity conferring the most robust delay in tumor growth and MDSC accumulation. Our results suggest that there is an additive effect of the combination of PA and energy restriction on tumor progression, survival, and immune outcomes that cannot be achieved by preventing weight gain via energy restriction alone.

To our knowledge, this is the first study to show a beneficial effect of moderate PA in weight-stable mice on spontaneous metastases and survival in an aggressive mammary tumor model. Few preclinical studies have been designed to examine the effects of preventing weight gain in adulthood on metastatic progression. In two studies, mice were given an intravenous injection of mammary tumor cells and randomized into voluntary exercise or SED control groups (25, 26). In both studies, exercise following tumor cell injection did not alter the development of lung metastases. However, because tumor cells were administered intravenously, many of the biological events crucial for spontaneous metastases to occur may have been bypassed. Moderate to severe ER (25%–40% reduction in calories) in 4T1 tumor-bearing mice reduces the total number of lung metastases that originate both spontaneously from the primary tumor and experimentally from intravenous injection of tumor cells (27). In this study, we demonstrated that the dual intervention reduced spontaneous metastasis to the lung and bone and improved survival. These findings provide evidence that moderate activity in a weight-stable host may be contributing to a reduction in breast cancer mortality by preventing metastatic disease.

The activity-induced beneficial effects in weight-stable mice could be mediated by numerous biological mechanisms. We have demonstrated that a reduction in splenomegaly/accumulation of MDSCs, enhanced T-cell function, acquisition of a less immunosuppressive TME, and an increase in the CD8⁺ T cell: MDSC ratio in the tumor are occurring concurrently with the beneficial changes in tumor growth and metastasis. Thus, further investigation of these immune-mediated changes and their role in mediating the beneficial effect of the dual intervention on tumor outcomes and survival is warranted. In contrast, a protective effect of PA was not observed on primary tumor growth, metastatic burden, or survival in mice that gained weight over the course of the study. These data suggest that weight gain–induced disturbances in metabolic, inflammatory, or immunologic function can override the activity-induced benefits observed in weight-stable mice.

The 4T1 (28) and 4T1.2 mammary tumor cells produce cytokines (e.g., G-CSF, IL6) that dysregulate hematopoiesis, resulting in the expansion and accumulation of immature, myeloid-lineage cells into the blood, spleen, TME, and metastatic niches. As 4T1.2 tumors advance, we have demonstrated a progressive decrease in lymphoid-lineage cells and an increase in myeloid-lineage cells in the spleen. This dysregulated immune phenotype can generate an immunosuppressive environment that dampens antitumor effector functions, which promote tumor escape and survival. The combination of PA+ER reduced splenomegaly and MDSC accumulation at day 35 posttumor implantation, and when tumor volumes were equal, suggesting that the combination of PA+ER may be influencing myelopoiesis and/or the recruitment and trafficking of MDSCs to secondary sites. MDSCs suppress antitumor immunity and contribute to tumor progression and metastases (16); thus, their reduction by PA+ER may be contributing to the delayed tumor progression in this group. In addition, the beneficial effect of PA+ER on tumor and immune outcomes varied by volume of daily activity with more running wheel activity providing greater benefit. In contrast, no difference in tumor and immune outcomes was detected between mice in the low and high activity groups that were fed ad libitum. These data provide further evidence that the combination of PA and energy restriction to prevent weight gain is critical to achieve beneficial effect on tumor and immune outcomes. In addition, these findings support data in clinical and epidemiologic studies demonstrating a dose-dependent effect of PA on breast cancer outcomes (7).

In a previous study using non–tumor-bearing mice, we demonstrated that moderate, voluntary wheel activity in weight-stable mice enhances antigen-specific CD4⁺ T-cell responses (18). In this study, we report that these findings are consistent in the 4T1.2 mammary tumor model. The enhancement of T-cell function in active mice could play a role in maintaining immunosurveillance mechanisms that are critical in preventing the metastatic process via the recognition of disseminating tumor cells and promotion of immune-mediated dormancy (29). Thus, the combination of PA+ER may be impacting primary tumor growth and metastases via both the augmentation of antitumor immunity and the reduction in immunosuppressive cells.

Moderate activity, energy restriction, and the combination alter the TME, and these effects change over time as tumor volume increases. In tumors that were harvested from both ER groups, there was a downregulation of the gene expression level of chemokines important for chemotaxis and the recruitment of MDSCs and Tregs into the TME (e.g., Ccl5, Ccl20, Ccl22; refs. 30–33) compared with SED+AL tumors. The findings from the gene array are consistent with the observed reduction in splenic MDSC subsets in the SED+ER and PA+ER groups at both time points. When tumors were equal in size, the combination of PA+ER resulted in a significant downregulation of genes important in metastasis (Aica), immune checkpoint molecules (Pdcd1), and molecules associated with...
immunosuppressive populations (Ido1, Ifng, Foxp3). The treatment-induced downregulation of Ido1, Ifng, and Foxp3, was also detected in tumors collected at day 35. In addition, the combination of PA+ER increased the percent of CD8+ T cells, and reduced the percent of total MDSC and MDSC subsets in tumors, thus altering the ratio of effector to immunosuppressive cells both early in tumor growth, when tumor volumes were equal, and at day 35 posttumor implantation. These findings suggest that PA, energy restriction, and the combined intervention induce changes in the TME that result in a reduction in immunosuppressive factors, which may allow better immunosurveillance and effector cell infiltration of the tumor. With the emergence of immunotherapy as the fourth pillar of cancer therapy, interventions that shift the TME to favor an antitumor response may be useful adjuvant therapies to improve response rates to immune-based interventions.

In conclusion, the combination of PA and the prevention of weight gain via mild dietary restriction is necessary to delay tumor growth, metastatic progression, and improve survival in the 4T1.2 metastatic breast cancer model. The beneficial effects of the combined intervention on tumor outcomes and survival were associated with a reduction in MDSC accumulation, enhancement of CD4+ T-cell proliferation, a reduction in the expression level of metastatic and immunosuppressive genes in the TME, and favorable changes in immune cell infiltrates into the tumor. These findings provide a biological rationale for future studies using both PA and the prevention of weight gain to prevent metastatic progression in patients with breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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