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Cell Metabolism

Voluntary Running Suppresses Tumor Growth through Epinephrine- and IL-6-Dependent NK Cell Mobilization and Redistribution

Graphical Abstract

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In Brief

The beneficial effects of exercise are countless. Pedersen et al. now link exercise, cancer, and immunity and reveal that exercise decreases tumor incidence and growth by over 60% across several mouse tumor models through a direct regulation of NK cell mobilization and trafficking in an epinephrine- and IL-6-dependent manner.

Highlights

- Exercise reduces tumor incidence and growth in several mouse models

- Exercise increases NK cell infiltration, thereby controlling tumor growth

- Epinephrine mobilizes NK cells and β-blockade blunts the tumor suppression

- Exercise-induced muscle-derived IL-6 is involved in NK cell redistribution

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Voluntary Running Suppresses Tumor Growth through Epinephrine- and IL-6-Dependent NK Cell Mobilization and Redistribution

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SUMMARY

Regular exercise reduces the risk of cancer and disease recurrence. Yet the mechanisms behind this protection remain to be elucidated. In this study, tumor-bearing mice randomized to voluntary wheel running showed over 60% reduction in tumor incidence and growth across five different tumor models. Microarray analysis revealed training-induced upregulation of pathways associated with immune function. NK cell infiltration was significantly increased in tumors from running mice, whereas depletion of NK cells enhanced tumor growth and blunted the beneficial effects of exercise. Mechanistic analyses showed that NK cells were mobilized by epinephrine, and blockade of β-adrenergic signaling blunted training-dependent tumor inhibition. Moreover, epinephrine induced a selective mobilization of IL-6-sensitive NK cells, and IL-6-blocking antibodies blunted training-induced tumor suppression, intratumoral NK cell infiltration, and NK cell activation. Together, these results link exercise, epinephrine, and IL-6 to NK cell mobilization and redistribution, and ultimately to control of tumor growth.

INTRODUCTION

Epidemiological data document that regular exercise protects against the development of certain cancers and lowers the risk of disease recurrence (Brown et al., 2012; Christensen et al., 2014), prompting extensive research into exercise interventions in cancer patients (Jones and Alfano, 2013). Across a range of cancer diagnoses, exercise has been shown to improve functional capacity and patient-reported outcomes (Mishra et al., 2012). However, exercise may also directly suppress tumor growth, as suggested by decreased risk of disease recurrence in physically active cancer patients (Ballard-Barbash et al., 2012). Little is known about the mechanisms behind this protection, but exercise-mediated changes in body composition, sex hormone levels, systemic inflammation, and immune cell function have been suggested as possible mediators (McTiernan, 2008).

Exercise training comprises of acute bouts of physical exertion, followed by periods of recovery. During these acute bouts of exercise, plasma levels of stress hormones and muscle-derived myokines increase dramatically (Catoire and Kersten, 2015). Myokines may have direct anti-proliferative effects on cancer cells, as shown for oncostatin M on hormone-sensitive breast cancer cells (Hojman et al., 2011) and SPARC in colon cancer (Aoi et al., 2013). Yet during exercise, an acute mobilization of immune cells to the circulation is also seen (Pedersen and Hoffman-Goetz, 2000; Bigley et al., 2014). Cells of the immune system play dual roles in cancer. The immune system has a powerful capacity to combat cancer, but chronic inflammation has also been linked to tumorigenesis in several conditions (Vivier et al., 2012; Imai et al., 2000; Grivennikov et al., 2010). On the protective side, infiltrating cytotoxic immune cells have been demonstrated as positive prognostic factors for disease outcome and overall survival in several cancers (Fridman et al., 2012; Remark et al., 2013). Thus, mobilization of cytotoxic immune cells during exercise might represent an indirect defense mechanism against cancer growth.

RESULTS AND DISCUSSION

Voluntary Wheel Running Significantly Reduces Tumor Incidence and Growth

First, we evaluated the effect of wheel running before and/or during tumor challenge in a subcutaneous B16F10 melanoma model in female mice (Figure 1A). Four weeks of wheel running
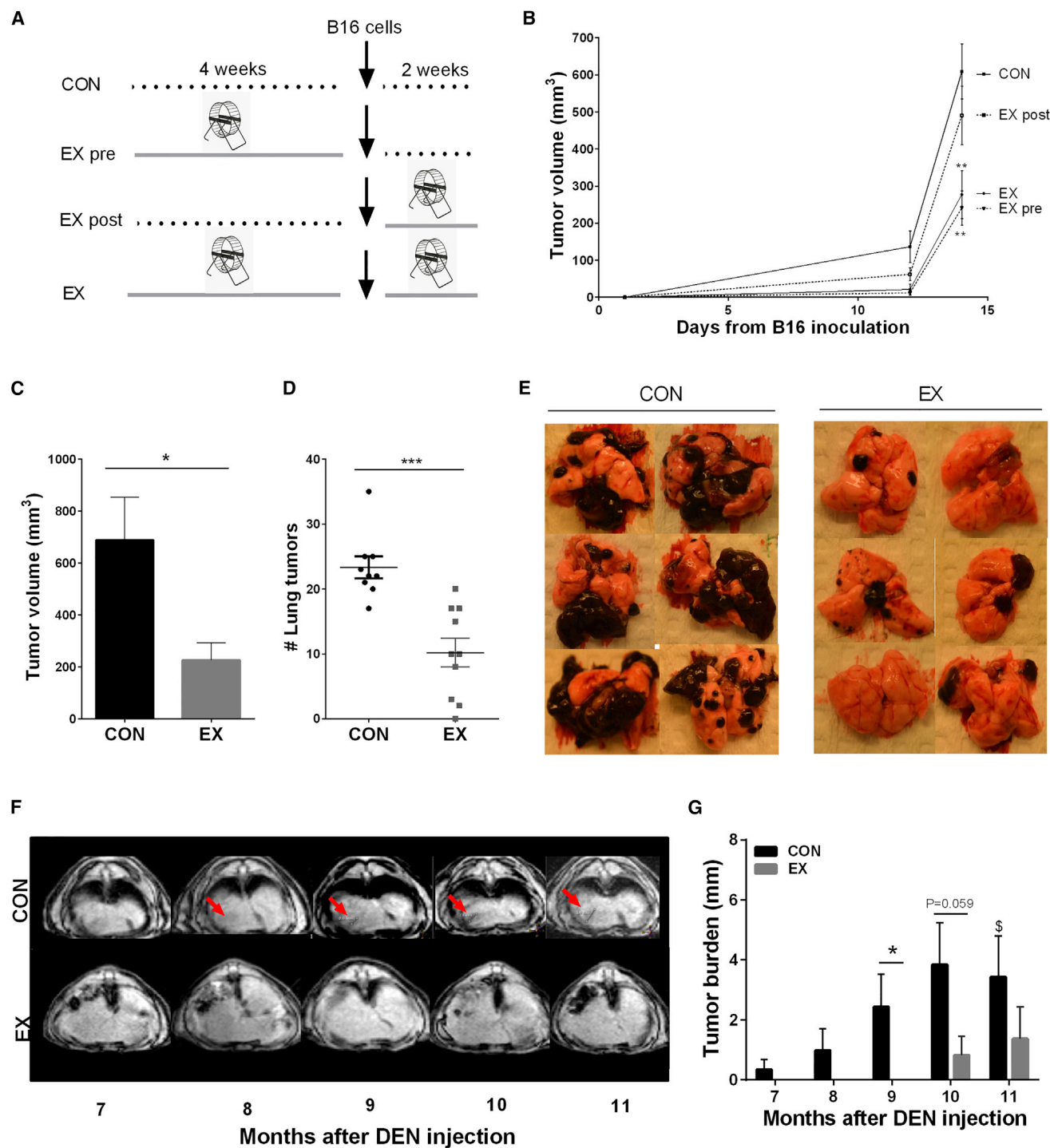
Figure 1. Wheel Running Reduces Tumor Incidence and Growth

(A) Experimental design.

(B) Tumor volume of subcutaneous B16 tumors in 3-month-old female C57BL/6 mice (n = 12, two-way ANOVA with Tukey's post hoc test).

(C) Tumor volume of subcutaneous B16F10 tumors in C57BL/6 mice (CON n = 11, EX n = 12, Students t test).

(D) Number of tumors per lung in mice injected i.v. with 1×10^5 B16F10 cells (CON = 9, EX = 10, Students t test).

(E) Representative pictures of B16 tumors in lungs of mice after i.v. injection.

(F) MRI of male NMRI mice injected with DEN (25 mg/kg body weight). The red arrows point to a liver tumor in a control mouse.

(G) Tumor burden assessed by MRI (n = 16 for both groups, two-way ANOVA with post hoc multiple t test). $: 11th month: EX = 16, CON = 14. See also Figure S1.

Means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

prior to tumor cell inoculation reduced tumor growth by 61% (p < 0.01, Figure 1B). Similar reductions in tumor volume of 67% and 53% with running were verified in female adult (3 months, p < 0.05, Figure 1C) and old mice (18 months, p < 0.001, Figure S1A). Wheel running also dramatically reduced lung metastases after intravenous (i.v.) injection of B16F10 melanoma cells (p < 0.001, Figures 1D and 1E). Next, the impact of wheel running on tumor growth was evaluated in three additional models. Male Naval Medical Research Institute (NMRI) mice were injected with diethylnitrosamine (DEN) at 4 weeks of age, which is known to cause liver tumors within 10 months. Here, wheel running reduced tumor incidence as only 31% of the running mice developed tumors, compared with 75% of the control mice (Figure 1F). Moreover, wheel running reduced tumor burden per mouse (p < 0.05, Figure 1G). In a Lewis Lung carcinoma model (LLC) in female mice, running decreased tumor volume by 58% (p < 0.01, Figure S1B) and tumor weight by 56% (p < 0.05, Figure S1C), and in Tg(Grm1)EPv transgenic male mice, which spontaneously develops melanoma, wheel running tended to delay formation of malignant lesions (p = 0.08, Figure S1D).

The average wheel running distance was 4.1 km/mouse/day for the B16-inoculated female mice and 6.8 km/mouse/day for the male DEN-injected mice. The presence of B16 tumors did not induce weight loss or cachexia (Figure S1E). In contrast, the presence of LLC tumors induced an average weight loss of -1.27 ± 1.74 g in the tumor-bearing control group. This weight loss was completely prevented in the running tumor-bearing mice (p < 0.05, Figures S1E and S1G).

Taken together, we find marked reductions in tumor incidence and growth with voluntary wheel running across 5 different tumor models. The delay in B16 melanoma progression required a 4-week pre-training period prior to tumor inoculation. During this period, the mice were habituated to wheel running, and the immune system was primed for the tumor challenge, suggesting that when using fast-growing transplantable tumor models, part of the exercise effect could potentially be killing of cancer cells at the inoculation site. In contrast, initiating running after tumor challenge in our slow-growing DEN-induced and Tg(Grm1)Epv models was sufficient to control tumor incidence and progression.

Training-Dependent Reduction in Tumor Growth Is Associated with Induction of Immune-Related Pathways

To identify differentially regulated pathways in tumors from running mice, we performed microarray analysis on B16 tumors from Figure 1C (Table S1). Of the 92 upregulated gene ontology (GO) pathways, the majority (52%) was related to immunological and inflammatory pathways (Figure 2A). qPCR analysis confirmed the increased expression of both pro- and anti-inflammatory cytokines (Figure 2B), as well as markers of the cellular innate and adaptive immune systems in B16 tumors from running mice (Figure 2C). Also in LLC tumors, pro-inflammatory cytokines (IL-1α and iNOS) and markers for NK and T cells were upregulated with running (Figures 2D and 2E).

To exclude that these differences in immune pathways were merely related to tumor size, we repeated the evaluation now based on B16 tumors of identical volume (approx. 150 mm³, Figure 2F). Thus, the tumors were on average excised 2 days earlier

in the control group (Figure 2G). In these similar sized tumors, upregulation of the pro-inflammatory cytokines (IL-1α and iNOS) and immune cell markers with exercise was verified (Figures 2H and 2I).

Training Regulates Tumor Growth through Intratumoral NK Cell Infiltration

Next, we measured frequencies of immune cells in tumors by flow cytometry after 6 weeks of wheel running. In the subcutaneous B16 model, tumors from running mice showed markedly increased infiltration of NK cells (p < 0.001, Figures 3A, 3C, and 3D), as well as CD3 T cells and dendritic cells (p < 0.05, Figure 3A). Of note, the CD3 gate includes CD3$^+$ CD4$^-$ CD8$^-$ cells, and thus also includes cells such as gamma delta and NKT cells. In the i.v. B16 model, wheel running also increased NK cell infiltration in lung tumors (p < 0.05, Figure 3B), but without enhancement of other immune cell subtypes. The level of NK cell infiltration correlated inversely with tumor burden (p < 0.01, Figures S2A and S2B). Histological evaluation showed that exercise increased both NK1.1$^+$, CD8$^+$ and CD4$^+$cells with the absolute numbers of CD8$^+$ and CD4$^+$ cells being higher than that for NK cells (Figures 3E and S2C). In control mice, NK cells were rarely detectable. Infiltration of B cells did not change significantly with exercise (Figure S2D). In non-tumor-bearing mice, 6 weeks of running increased the frequencies of NK cells in bone marrow (p < 0.05), spleen (p < 0.01), and to a lesser extent peripheral blood mononuclear cells (PBMCs) (p = 0.17, Figure 3F), indicating an overall increase in the basal pool of NK cells with exercise. In tumor-bearing mice, running did not alter the frequency of NK cells in these organs (Figure 3G), yet these mice showed pronounced accumulation of NK cells in their tumors (Figure 3C). The numbers of T cells did not increase in blood, bone marrow, or spleen with 6 weeks of running in neither tumor-bearing nor non-tumor-bearing mice (Figures S3A and S3B).

We then evaluated the response to running in athymic mice, which lack functional T cells but retain NK cells. In these mice, a 66% reduction in B16 tumor volume persisted with 6 weeks of running (p < 0.05, Figure 3H), showing that T cells were not responsible for the suppressive effect of running on tumor growth. However, the athymic nude mice in general had larger tumors than wild-type immune-competent mice (WT) (Figures S3C and S3D), indicating that T cells aside from the exercise situation play a role in control of tumor growth. To further document the role of NK cells, circulating NK cells were depleted by administration of anti-asialo-GM1 antibodies (Figures S3E and S3F). Depletion of NK cells completely abolished the suppressive effect of running on tumor volume, and both anti-asialo-GM1-treated groups (control and exercise, respectively) showed enhanced tumor growth compared with isotype IgG-treated mice (p < 0.001, Figure 3I).

Next, we investigated if running affected NK cell cytotoxicity, but determined per cell basis, NK cells from control and running mice were equally effective in killing NK-sensitive YAC-1 and B16 cells (Figure 3J). To exclude that cancer cell killing was mediated by T cell contamination, the NK-depleted fraction (NK cell content of 2%) was used as a negative control and showed no YAC-1 or B16 cell killing. In contrast, within the tumors from the running mice, we found increased mRNA expression of NK-cell-activating receptor ligands (H60a, MULT1, Clr-b),
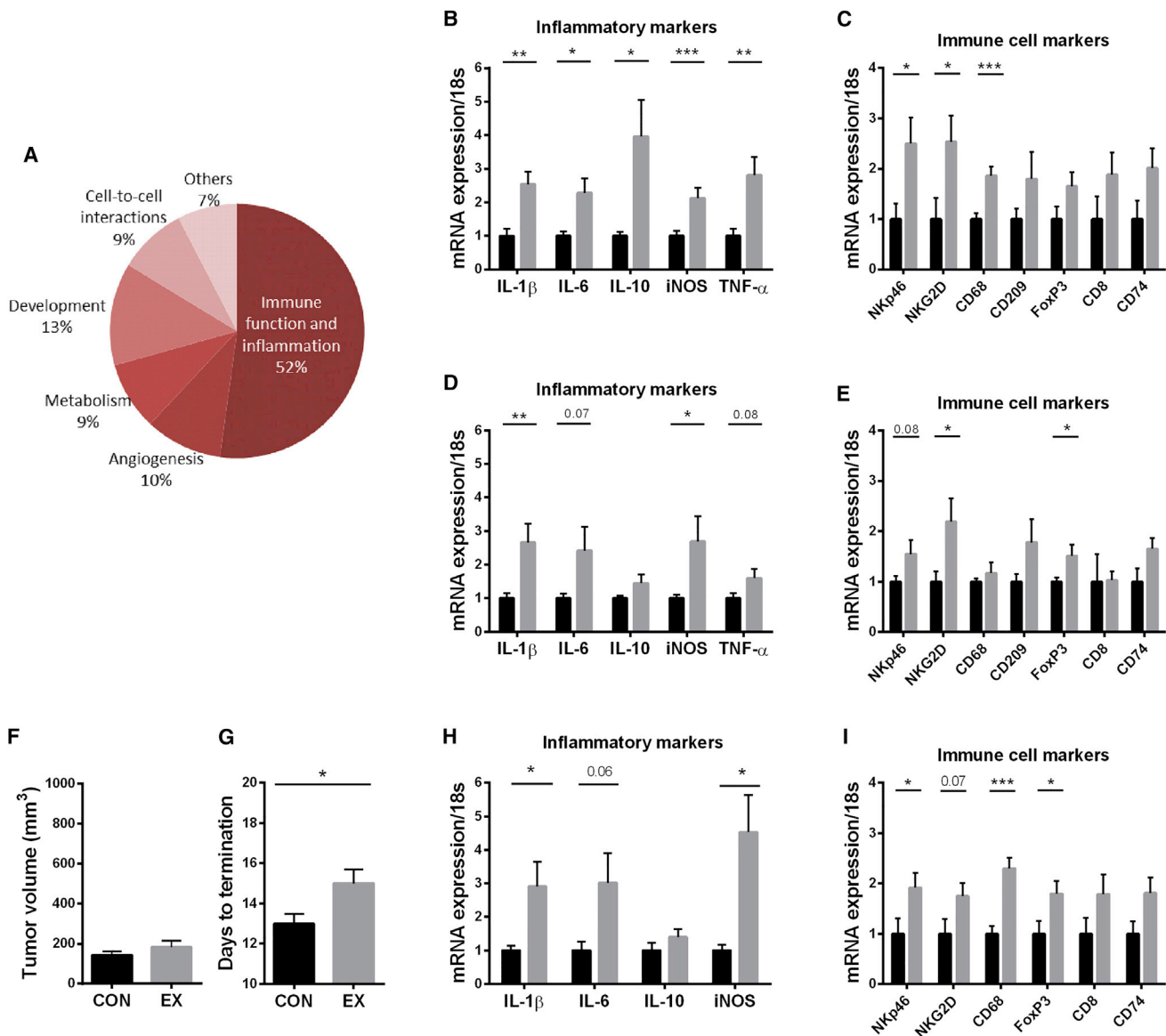
Figure 2. Running Is Associated with Induction of Immune Related and Inflammatory Pathways

(A–E) (A) Distribution of upregulated GO pathways (n = 5). See also Table S1. qPCR analysis of (B) inflammatory cytokines and (C) immune cell markers in B16 tumors from Figure 1C (CON = 12, EX = 11, multiple t testing), and (D) inflammatory cytokines and (E) immune cell markers in Lewis Lung tumors (CON = 9, EX = 10, multiple t testing).

(F–I) (F) Tumor volume of subcutaneous B16F10 tumors and (G) day of termination for tumors excised. qPCR analysis of (H) inflammatory cytokines and (I) immune cell markers from tumors in Figure 2F (n = 10, multiple t testing). Black bars = CON, gray bars = EX. Means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

as well as cytokines (IL-2, IL-15, IFNγ) and chemokines (CCL3, CXCL10, CX3CL1, Chemerin) related to NK cell activation and chemotaxis (Figure 3K). No changes in the expression of markers of angiogenesis (i.e., CD31 and VEGF-A) were observed (Figure 3K).

Taken together, these data point to a predominant role of NK cells in the training-dependent control of tumor growth. NK cells represent a critical component of the innate immune defense, recognizing transformed cells independently of antibodies or major histocompatibility complex (MHC) restriction (Vivier et al., 2012; Brodin et al., 2012), while T cells are cyto-

toxic effector cells of the adaptive immune response. Both immune cell types are known to be regulated by exercise. During exercise, circulating lymphocytes increase in number and frequency and then fall below pre-exercise levels (Pedersen and Hoffman-Goetz, 2000). Of these lymphocytes, NK cells are the most responsive cells to the exercise-dependent mobilization, followed by CD8 T cells, CD4 T cells, and lastly B cells, which respond poorly to exercise (Walsh et al., 2011). Thus, the importance of NK cells in the training-dependent control of tumor growth follow their superior responsiveness to exercise.
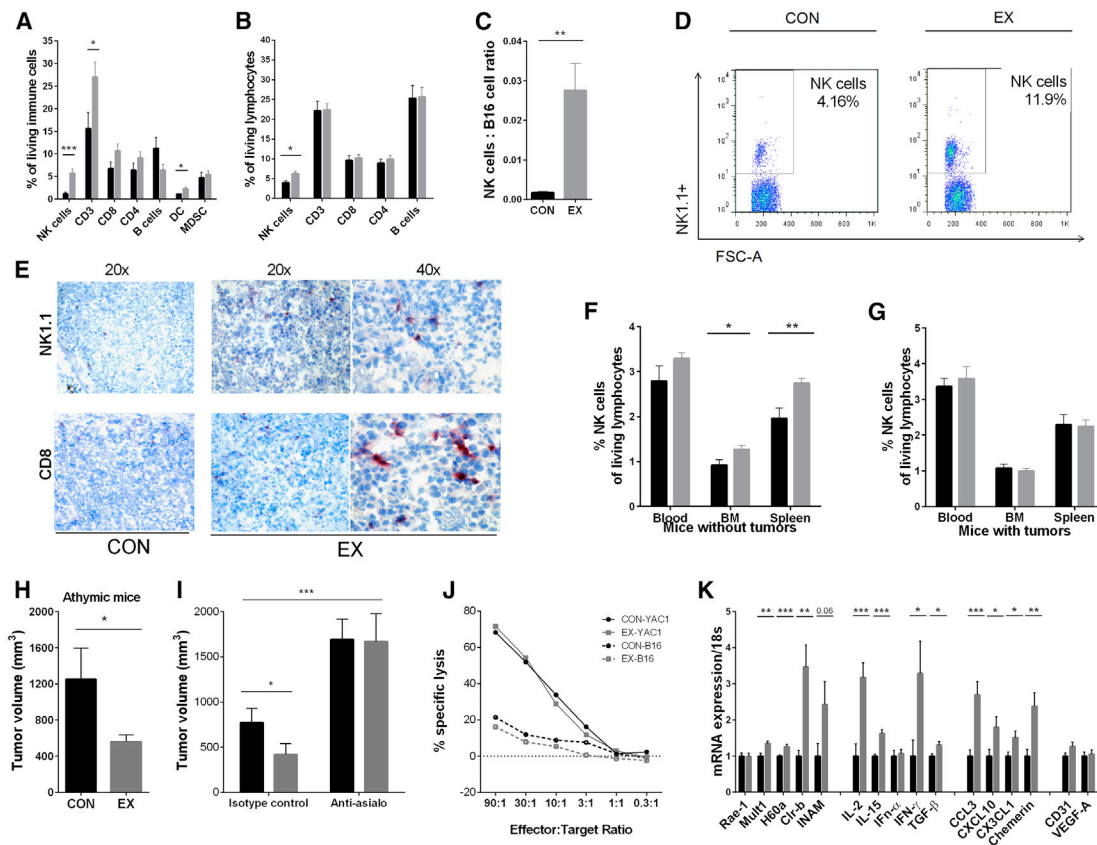
Figure 3. Training Controls Tumor Growth through Intratumoral NK Cell Infiltration

(A) Frequency of immune cells in subcutaneous B16 tumors from CON and EX mice (Gating criteria: total T cells [CD3$^+$], CD8 T cells [CD3$^+$ CD8$^+$], CD4 T cells [CD3$^+$ CD4$^+$], B cells [CD3$^-$ CD19$^+$], NK cells [CD3$^-$ NK1.1$^+$], DC [CD11b$^+$ GR-1$^-$ CD11c$^+$], MDSC [CD11b$^+$ GR-1b$^+$]) (CON = 8, EX = 9, multiple t testing).

(B) Frequency of immune cells in lungs from Figure 1D (n = 8, multiple t testing).

(C) NK cells per total B16 cells in subcutaneous B16 tumors (CON = 8, EX = 9, Students t test).

(D) Flow cytometry panels of tumor infiltrating NK1.1$^+$ cells.

(E) Histological analysis of tumor sections stained for NK1.1 or CD8 in red.

(F and G) NK cell frequency in bone marrow, blood, and spleen from non-tumor-bearing (F) and tumor-bearing mice (G).

(H) Tumor volume of subcutaneous B16 tumors in athymic mice (CON = 9, EX = 11, Students t test).

(I) Tumor volume of subcutaneous B16 tumors in C57BL/C mice after NK cell depletion (n = 12, two-way ANOVA with post hoc Bonferroni test). See also Figure S3.

(J) NK-cell-mediated killing of NK-cell-sensitive YAC-1 cells (full line) and B16F10 tumor cells (dotted line).

(K) qPCR analyses of mRNA expression of NK-cell-activating receptor ligands, cytokines, chemokines, and angiogenic factors (CON = 10, EX = 11, multiple t testing). Black bars = control, gray bars = EX. Means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

In addition to the mobilization of the NK cells during exercise, we found increased expression levels of NK cell-related activating receptor ligands, stimulatory cytokines, and chemoattractant chemokines in the tumors of running mice, suggesting that exercise works both on the mobilization of NK cells, and on the tumor microenvironment to generate a NK cell activating milieu. NK cells are regulated by a multitude of activating and inhibitory receptor-ligand interactions. Here, we show that ligands for the activating receptor NKG2D, MULT1 and H60a, as well as Clr-b, a ligand for NKR-P1B, which has proven important in the education of NK cells, were upregulated in the tumors from running mice (Chen et al., 2015; Rahim et al., 2015). Previously, it has been shown that B16F10 cells do not express Clr-b (Carlyle et al., 2004). The methodology employed in this study did not allow for precise identification of whether Clr-b expression was attributable to tumor cells, infiltrating immune cells, or other cells in the tumor microenvironment. Furthermore, we found increased expression of the activating receptor NKp46 with wheel running. NKp46 has been shown to mediate control of B16 metastasis, which correlates well with our results (Glasner et al., 2012).

IL-6-Sensitive NK Cells Are Recruited by Exercise through β-Adrenergic Signaling

Exercise has been shown to mobilize NK cells through epinephrine (EPI) (Bigley et al., 2014; Dimitrov et al., 2010). Five hours into the dark period, when wheel running was at its highest, serum EPI levels were 561.2 ± 157.4 pg/ml in the CON group and 1,169.3 ± 340.2 pg/ml in the EX group (p < 0.001, Figure 4A). To mimic this acute running response, we injected low and high doses of EPI (0.5 mg/kg and 2 mg/kg, respectively) and found a reduction in both spleen volume (Figure 4B) and the
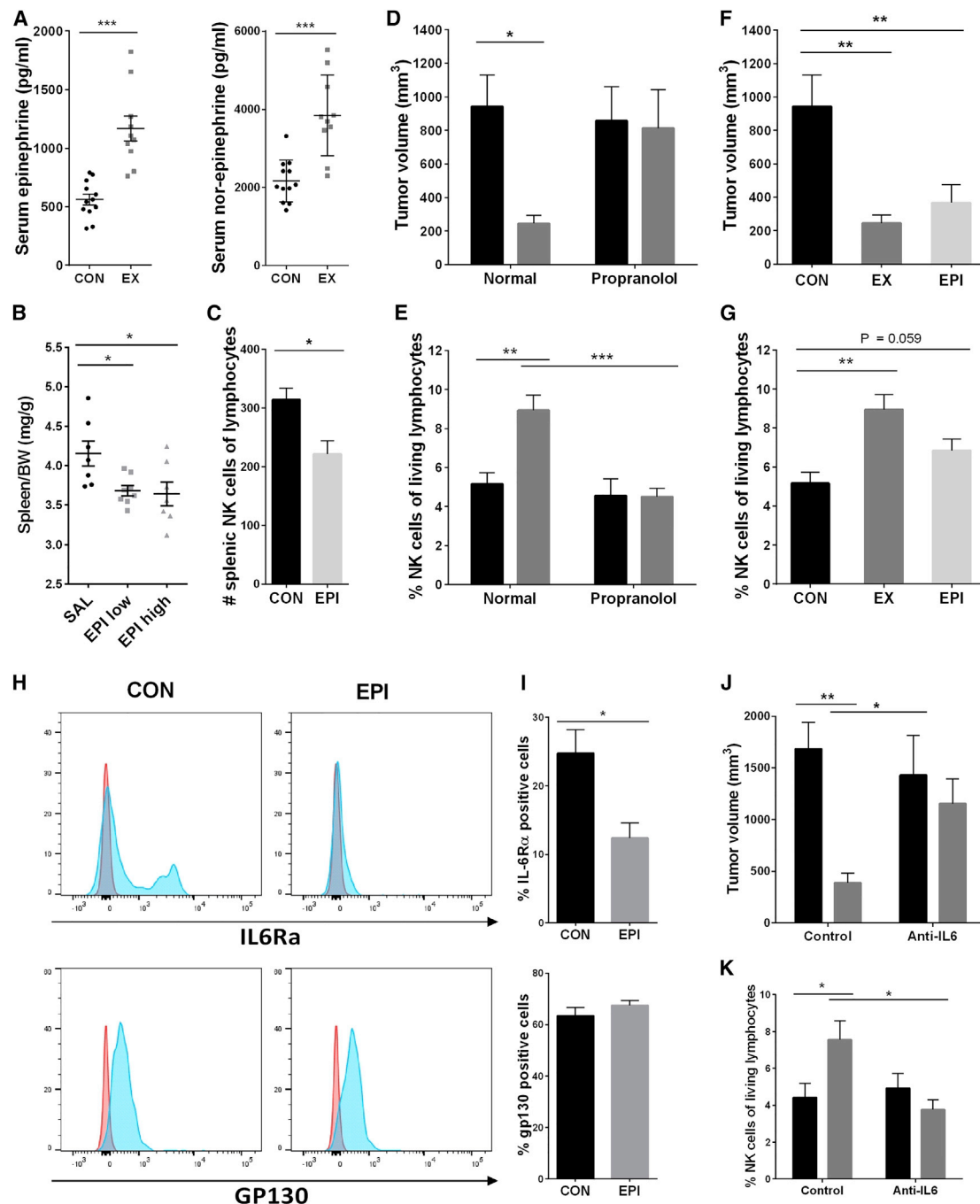
Figure 4. IL-6-Sensitive NK Cells Are Mobilized through β-Adrenergic Signaling

(A) Serum epinephrine and nor-epinephrine (CON = 12, EX = 10, Students t test).

(B) Weight of the spleen 30 min after epinephrine injection at 0.5 mg/kg (EPI low, n = 7) and 2 mg/kg (EPI high, n = 8, one-way ANOVA with Tukey's post hoc test).

(C) Number of NK cells in the spleen (n = 6, Students t test).

(D and E) (D) Tumor volume of subcutaneous B16 tumors after propranolol treatment (n = 11) and (E) NK cell infiltration in the tumors from (D) (n = 8, both two-way ANOVA with Bonferroni post hoc test). See also Figure S4.

(F and G) (F) Tumor volume of subcutaneous B16 tumors from mice injected daily with low-dose 0.5 mg/kg epinephrine (n = 11) and (G) NK cell infiltration in the tumors from (F) (n = 8, both one-way ANOVA with Tukey's post hoc test).

(H and I) (H) Flow cytometry panels of IL-6Rα (top) and gp130-positive (bottom) splenic NK cells in control (CON) or epinephrine (EPI, 0.5 mg/kg 30 min prior to sampling) and (I) average IL-6Rα and gp130 expression (n = 6, Students t test).

(J and K) (J) Tumor volume of subcutaneous B16 tumors (CON = 12, anti-IL6 = 10,) and (K) NK cell infiltration (n = 8, both two-way ANOVA with Bonferroni post hoc test) in subcutaneous B16 tumors of mice receiving anti-IL-6 antibodies or saline treatment. Black bars = control, gray bars = EX, light gray = daily epinephrine injections (0.5 mg/kg). Means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

number of NK cells in the spleen (Figure 4C). Blocking of the EPI-dependent mobilization of NK cells by propranolol during wheel running completely blunted the effect of running on tumor volume (Figure 4D) and abolished the increased NK cell infiltration in tumors from running mice (Figure 4E). Propranolol administration did not alter training-dependent changes in body weight and muscular expression of the exercise-responsive factor, PGC-1α (Figures S4A and S4B). To confirm the role of EPI, we mimicked the exercise-related EPI surge by daily injections of low-dose (0.5 mg/kg) EPI in non-running mice. This resulted in a 61% reduction in tumor volume ($p < 0.01$, Figure 4F) and tended to increase tumor infiltration of NK cells compared with non-treated controls ($p = 0.059$, Figure 4G). This increased infiltration did, however, not match the effect seen by running alone. In addition to the blockade of NK cell infiltration, propranolol administration tended to block the infiltration of CD3$^+$, CD4$^+$, and CD8$^+$ cells, while EPI injection tended to mimic the exercise-induced mobilization of these cells (Figures S4C and S4D).

Plasma IL-6 increases dramatically during exercise due to release from contracting muscles and might be the additional exercise factor, involved in tumor homing (Pedersen and Febbraio, 2012). In our model, serum IL-6 increased from 4.3 ± 3.5 pg/ml (range: 1.6–12.4 pg/ml, n = 12) in the CON group to 29.3 ± 32.6 pg/ml (range: 5.7–106.5 pg/ml, n = 10, $p < 0.05$) during wheel running. In the spleen, $24.8\% \pm 8.4\%$ of the NK cells expressed IL-6Rα and $63.4\% \pm 7.9\%$ gp130 (Figures 4H and 4I). After EPI injection, the fraction of IL-6Rα-positive splenic NK cells decreased to $12.4\% \pm 5.3\%$, suggesting an EPI-dependent mobilization of IL-6-sensitive NK cells. Blocking of training-induced IL-6 by anti-IL-6 antibodies diminished the exercise-induced inhibition of tumor growth (Figure 4J) and inhibited the infiltration of NK cells into tumors (Figure 4K). In contrast, daily injections of 100 ng IL-6, which increased serum IL-6 from 2.7 ± 0.9 pg/ml to 301.1 ± 130.3 pg/ml and 263.8 ± 950 pg/ml at 30 and 60 min, respectively, did not mimic the training-induced reduction in tumor growth (Figure S4E) or enhanced NK cell infiltration (Figure S4E). Thus, with this IL-6 concentration, the redistribution of NK cells seen during running could not be mimicked, suggesting that the increment in NK cell infiltration is dependent on concurrent exercise-induced mobilization of immune cells. Both running and IL-6 injections decreased the frequency of immature (CD11b$^-$, CD27$^-$) NK cells ($p < 0.05$, Figure S4E) and tended to increase the frequency of cytotoxic (CD11b$^-$, CD27$^+$) NK cells ($p = 0.11$). In contrast, anti-IL-6 antibody treatment blocked the training-induced increase in cytotoxic (CD11b$^-$, CD27$^+$, $p < 0.05$) NK cells but increased the frequency of cytokine producing (CD11b$^+$, CD27$^-$, $p < 0.05$) NK cells (Figure S4H).

The epinephrine surge during exercise can mobilize NK cells to the blood stream through activation of their β-adrenergic receptors, increasing the NK cell frequency but not their cytotoxicity (Dimitrov et al., 2010). Early studies suggest that a relatively small increase in epinephrine level is sufficient to mobilize NK cells. Thus, to obtain the physiological changes, which we observe within the tumors, additional stimuli must be present. To this end, we observed a selective mobilization of IL-6Rα-positive NK cells after epinephrine injection. In further support of the role of IL-6 in NK cell redistribution and activation, recombinant human IL-6 infusion has been

shown to mimic the acute and transient lymphopenia seen during the recovery from exercise (Steensberg et al., 2003), and stimulation of human NK cells with IL-6 has been shown to increase their expression of adhesion molecules (Rabinowich et al., 1993).

Yet, the role of IL-6 in cancer is complex. Chronic elevated plasma levels of IL-6 have been associated with poor disease outcome across a number of cancer diagnoses, whereas increased IL-6 expression within tumors is a positive prognostic marker for overall and disease-free survival (Dethlefsen et al., 2013; Mauer et al., 2015). In particular for the DEN-induced liver model, used in this study, Naugler and colleagues showed that IL-6 KO mice were resistant to DEN-induced tumor formation (Naugler et al., 2007). In contrast to these models, plasma IL-6 displays a dynamic response during exercise. The exercise-induced surge in plasma IL-6 may signal directly to IL-6Rα-expressing cells or through its alternative pathway, IL-6 trans-signaling (Kraakman et al., 2015; Scheller et al., 2014). We show that about 25% of the splenic NK cells express IL-6Rα and are thus directly sensitive to classical IL-6 signaling. Regarding IL-6 trans-signaling, IL-6 binds the soluble IL-6 receptor and then this complex binds to membrane-bound gp130. The concentration of the soluble IL-6 receptor is about 40 ng/ml, thus there is a large buffer capacity for IL-6 binding, when IL-6 increases as seen during wheel running. Thus, the systemic IL-6 increase during wheel running can signal either directly through the classical IL-6 signaling or through trans-signaling to NK cells. In Figure 2, we showed a 2.3- to 3.0-fold increase in IL-6 expression in the tumors of running mice, yet following on the importance of classical IL-6 signaling and IL-6 trans-signaling, this suggests that the exercise-induced increase in systemic IL-6 levels play a greater role than the intratumoral concentration of IL-6 in NK cell redistribution and activation.

In further support of the beneficial role of IL-6 exclusively in the exercise setting, we found no protective beneficial effect of IL-6 injection alone on tumor growth or intratumoral NK cell infiltration, stressing the dependence on prior training-dependent mobilization of NK cells. IL-6 and exercise have previously been shown to increase tumor vascularization (Taniguchi and Karin, 2014), yet we did not find any effect of IL-6 or running on CD31 and VEGF-A mRNA expression levels, nor did we detect any marked increases in capillarization in our histological analyses (data not shown).

In conclusion, voluntary wheel running inhibits tumor onset and progression across a range of tumor models and anatomical locations. This occurs through a direct regulation of NK cell trafficking, involving an epinephrine-dependent mobilization of NK cells to the circulation and an IL-6-dependent redistribution to tumors. The clinical relevance of infiltrating T cells is evident (Fridman et al., 2012), while the potential of tumor-infiltrating NK cells is still being unraveled (Remark et al., 2013). NK cells are part of the early innate immune response and can activate other immune cells through secretion of IFNγ (Gross et al., 2013). Thus, a key action of NK cells is to deliver the initial "spark" that activates other cell types of the immune system. Currently, clinical attention is on generating an inflammatory intratumoral environment through among others immune checkpoint blockade therapy (Topalian et al., 2015). The present study indicates that exercise might deliver such therapeutic

intratumoral adaptations with increased immune cell infiltration and generation of an inflammatory intratumoral environment.

EXPERIMENTAL PROCEDURES

All methods and interventions are described in details in the Supplemental Experimental Procedures.

Animal Studies and Interventions

Studies were compliant with the ARRIVE guidelines and approved by the Danish Inspectorate for Research Animals. Exercise were provided by running wheels (diameter 12 cm). The mice were inoculated subcutaneously with 2×10^5 B16F10 melanoma or Lewis Lung cells (cells obtained from ATCC) or i.v. through the tail vein with 1×10^5 B16F10 cells. For DEN-induced liver tumors, 23-day-old NMRI male mice obtained from Harlan, were injected intraperitoneally (i.p.) with DEN (25 mg/kg body weight). The Tg(Grm1)EPv mice were obtained from the University of Graz, after 2 weeks of acclimatization, running wheels were placed in the home cages.

Anti-Asialo Treatment

To deplete NK cells, mice received anti-Asialo GM_1 antibodies (1 mg/ml, EBio-Science) or isotype antibodies as control (Rabbit IgG, 0.5 mg/ml, Southern Biotech) every second day from 1 week before B16 inoculation. Five days after tumor cell inoculation, NK cells content was evaluated.

Propranolol/Epinephrine Study

Eight-week-old C57BL/6 mice were randomized to drinking water containing 0.5 g/l propranolol or nothing. Propranolol administration started 1 week before B16 inoculation. An additional group received daily (Monday to Friday) injections of epinephrine (0.5 mg/kg, 200 μl i.p.) from 1 week before B16 inoculation. For the acute effect of epinephrine, mice were injected with 0.5 mg/kg or 2 mg/kg epinephrine, and sacrified after 30 min, where blood, spleen, and muscle tissue were collected.

Anti-IL-6/IL-6 Study

Eight-week-old C57BL/6 mice were randomized to groups, receiving i.p. injections of anti-IL-6 antibodies (100 μg/mouse, R&D systems, #AB-406-NA) or vehicle injections twice a week from 1 week before B16 inoculation. An additional group received daily (Monday to Friday) injections of IL-6 (100 ng/mouse, R&D systems, #406-ML-025/CF) from 1 week before B16 inoculation.

Statistics

For comparisons of exercise and other interventions, two-way ANOVA followed by post hoc tests with Bonferroni corrections were performed. Two-way ANOVA with repeated-measures was used when the effects of experimental factors was analyzed at different timepoints. In the injection studies, one-way ANOVA with Tukey's post hoc test was performed. The statistical significance of mRNA and protein expression levels in CON and EX groups was obtained from two-tailed multiple t tests. Results are depicted as means ± SEM. The criterion for significance was $p < 0.05$.

ACCESSION NUMBERS

The microarray data generated in this publication have been deposited in NCBI's GEO and are accessible through GEO: GSE62628 (reviewer's link at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cbgzksoktzerxuz&acc=GSE62628).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.01.011.

AUTHOR CONTRIBUTIONS

L.P., B.K.P., and P.H. conceived the study. L.P., M.I., B.L., J.G., B.K.P., P.t.S., and P.H. designed the experiments. I.N. and J.N. performed microarray analysis. R.H.H. and H.H.J. performed the MR scans and image analyses. J.C.B. performed the histological analysis. M.I. and B.L. performed FACS analyses and cytotox assays. L.P., M.I., G.H.O., B.L., K.S.P., and C.D. carried out experiments. L.P., M.I., and P.H. analyzed the data. L.P. and P.H. wrote the paper with contributions from all authors. All authors approved the final version of the paper.

CONFLICTS OF INTEREST

None of the authors declare any conflicts of interest.

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