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# Acute Exercise Increases NK Cell Mitochondrial Respiration and Cytotoxicity against Triple-Negative Breast Cancer Cells under Hypoxic Conditions

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**ABSTRACT**

CHO, E., J. STAMPLEY, R. WALL, R. MATTHEWS, E. ZUNICA, J. C. BROWN, N. M. JOHANNSEN, B. A. IRVING, and G. SPIELMANN. Acute Exercise Increases NK Cell Mitochondrial Respiration and Cytotoxicity against Triple-Negative Breast Cancer Cells under Hypoxic Conditions. *Med. Sci. Sports Exerc.*, Vol. 55, No. 12, pp. 2132–2142, 2023. **Purpose:** Triple-negative breast cancer (TNBC) is an aggressive, highly metastatic malignancy with high recurrence rates. Hypoxia is a hallmark of the TNBC tumor microenvironment, which promotes tumor growth while impairing natural killer (NK) cell cytotoxic functions. Although acute exercise improves NK cell function under normoxic conditions, the effect of exercise on NK cell cytotoxic functions under hypoxic conditions mimicking $O_2$ tensions observed in solid tumors is unknown. **Methods:** The cytotoxic functions of resting and postexercise NK cells isolated from thirteen young inactive healthy women were assessed against breast cancer cells expressing different levels of hormone receptors (MCF-7 and MDA-MB-231) under normoxic and hypoxic conditions. Mitochondrial respiration and $H_2O_2$ efflux rates of the TNBC-activated NK cells were assessed via high-resolution respirometry. **Results:** Under hypoxia, postexercise NK cells exhibited greater killing of TNBC than resting NK cells. Further, postexercise NK cells were more likely to kill TNBC under hypoxia than normoxic conditions. In addition, mitochondrial respiration associated with oxidative (OXPHOS) capacity of TNBC-activated NK cells was greater in postexercise cells than resting cells under normoxia, but not under hypoxia. Finally, acute exercise was associated with reduced mitochondrial $H_2O_2$ efflux by NK cells in both conditions. **Conclusions:** Together, we present crucial interrelationships between hypoxia and exercise-induced changes in NK cell functions against TNBC cells. By modulating their mitochondrial bioenergetic functions, we postulate that acute exercise improves NK cell function under hypoxic conditions. Specifically, NK cell $O_2$ and $H_2O_2$ flow ($pmol \cdot s^{-1} \cdot million\ NK\ cells^{-1}$) changes in response to 30-min cycling suggest that exercise primes NK cell tumor killing by reducing mitochondrial oxidative stress and, thus, rescuing their function when exposed to harsh hypoxic environments as observed in the microenvironment of breast solid tumors. **Key Words:** NK CELL CYTOTOXICITY, HYPOXIA ACUTE EXERCISE, TRIPLE-NEGATIVE BREAST CANCER, MITOCHONDRIAL RESPIRATION, MITOCHONDRIAL ROS

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Breast cancer is the most common cancer in the United States, with approximately one in eight women diagnosed with breast cancer during their lifetime (1), making breast cancer the second leading cause of cancer death in women. Among this heterogeneous disease, triple-negative breast cancer (TNBC) is the most common form of breast cancer diagnosed in premenopausal women (2). Clinically, TNBC is the most aggressive breast cancer because of its high rates of metastasis and recurrence (3). In addition, TNBC cells lack hormonal receptors, which prevent the use of traditional receptor blockade to prevent their proliferation and growth therapeutically.

Accumulating epidemiologic evidence suggests that regular physical activity reduces the risk of mortality and recurrence from various cancers while improving cancer treatment efficacy and health-related quality of life (4,5). Further, exercise training has been proposed as a cost-effective, noninvasive approach to enhance cancer therapy (6,7). Exercise-induced

alterations might mediate indirect effects on immune cell infiltration, vascularization, and the associated increase in oxygen concentrations in the tumor microenvironment (TME), which could ultimately lead to inhibition of tumor growth as seen in animal models (6,8,9). This is especially true for natural killer (NK) cells, which are among the most exercise-responsive immune cells (10). Per example, acute physical stress such as a single bout of moderate to vigorous-intensity exercise induces significant increases in NK cell numbers and effector functions characteristic of a highly differentiated phenotype (8,11). However, the effect of acute exercise on NK cell metabolic profiles is incompletely understood.

Emerging evidence suggests that increased effector functions are tightly linked to mitochondrial metabolism (12). Immune cell metabolic reprogramming requires biosynthesis, which is energy demanding (12). Preliminary data obtained in our laboratory showed that an acute bout of high-intensity exercise significantly increased routine (basal) mitochondrial respiration in isolated peripheral blood mononuclear cells (PBMC) (e.g., T cells, B cells, NK cells, and monocytes) in healthy collegiate swimmers (13). However, the effects of exercise on the metabolism of isolated NK cells under normoxic or hypoxic conditions are not well characterized.

One of the hallmarks of most malignant solid tumors is exposure to sustained hypoxia in the TME due to rapid tumor growth and uncontrolled angiogenesis. Hypoxia promotes tumor escape from immune surveillance by suppressing the function of immune cells, thus favoring the immunosuppressive pro-TME (14). Given that the TME is highly hypoxic, invasive cancer cells produce high levels of reactive oxygen species (ROS) due to abnormal cellular metabolism and impaired mitochondrial respiratory function, further limiting tumor clearance (15). Furthermore, the potential role of ROS and ROS-mediated signaling pathways on immune cell function, especially in NK cell, is poorly understood.

Because hypoxic stress impairs resting NK cell cytotoxic activity (NKCA) *in vitro* (16), hypoxia (as seen in TME), and hypoxia-driven ROS production likely also negatively affect the metabolic activity of NK cells. In light of the importance of cellular bioenergetics in supporting effector functions, low oxygen tension in the TME may reduce NK cell ability to clear tumors. This study aimed to characterize the effects of an acute bout of exercise on NK cell metabolic and effector functions against TNBC cells under physiologically relevant hypoxic conditions mimicking the TME. We hypothesized that acute exercise increases NK cells killing of tumor target cells by altering mitochondrial metabolism. Further, we hypothesized that the exercise-induced changes in NK cell mitochondrial metabolism are accentuated when exposed to hypoxic conditions.

## MATERIALS AND METHODS

### Participants

Thirteen young, healthy inactive women (mean ± SD; age = 26.9 ± 4.5 yr, weight = 63.7 ± 12.8 kg, height = 161.5 ± 7.6 cm) participated in this study. Their level of physical inactivity was confirmed to be lower than 7500 steps per day for the past 3 months via activity tracker (e.g., personal smartphone). All participants provided written informed consent before participating, which the Louisiana State University's Institutional Review Board approved (no. 4176). The lactate threshold was measured using an incremental exercise protocol.

### Experimental Design

The experimental protocol consisted of three study visits conducted within 2 wk. The first visit (V1) measured the participant's baseline characteristics, including their lactate threshold. The second and third visits (V2 and V3) were designed 1) to test cytotoxic ability of NK cells against target cells and 2) to assess the bioenergetics of target-activated NK cells. V2 and V3 were performed in a randomized order. A schematic experimental design can be found in Supplemental Figure 1, Supplemental Digital Content, http://links.lww.com/MSS/C886. Before V2 and V3, participants were asked to refrain from vigorous exercise, food, and drinks other than water for 12 h.

### Experimental Procedures

**Height, weight, body composition, Physical Activity Readiness Questionnaire, medical and health history form (V1).** During V1, participants completed the Physical Activity Readiness Questionnaire and a self-report medical and health history. In addition, height and weight were also measured using a stadiometer and a scale (Table 1). Finally, body composition was assessed using dual-energy x-ray absorptiometry (Horizon A, Hologic, Marlborough, MA).

**Lactate threshold test (V1).** An incremental exercise protocol was performed on a stationary cycle ergometer (Racermate, Inc., Seattle, WA) to determine the participant's lactate threshold. Respiratory gases ($\dot{V}E$, $\dot{V}O_2$, and $\dot{V}CO_2$) were collected and analyzed throughout the exercise test using a calibrated metabolic cart (ParvoMedics, Inc., Sandy UT).

TABLE 1. Physical characteristics and exercise performance of the participants ($n$ = 13).

| Measurements | All ($N$ = 13) | Range |
|---|---|---|
| Age (yr) | 26.85 ± 4.47 | 21–35 |
| Height (cm) | 161.52 ± 7.57 | 148.10–177.00 |
| Weight (kg) | 63.66 ± 12.81 | 48.70–89.60 |
| BMI ($kg \cdot m^{-2}$) | 24.26 ± 3.63 | 19.10–30.00 |
| BMC (kg) | 2.04 ± 0.37 | 1.48–2.75 |
| Fat mass (kg) | 21.22 ± 6.12 | 12.61–29.88 |
| Lean mass (kg) | 41.67 ± 7.66 | 31.92–59.34 |
| % Fat | 32.28 ± 4.79 | 24.00–43.80 |
| V1 | | |
| Mean $\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$) | 22.35 ± 4.04 | 17.30–30.60 |
| Mean max power (W) | 107.31 ± 31.73 | 13.60–28.80 |
| Mean max HR (bpm) | 173.23 ± 11.97 | 152–194 |
| Mean max RPE | 17.85 ± 2.23 | 13–20 |
| V2, V3 | | |
| LT + 10% (W) | 80.00 ± 22.64 | 45–125 |
| Mean $\dot{V}O_{2peak}$ (%$\dot{V}O_{2peak}$) | 89.90 ± 6.01 | 77.62–98.61 |
| Mean HR (bpm) | 155.89 ± 14.43 | 128.08–180.33 |
| Mean HR (% $HR_{max}$) | 80.61 ± 5.42 | 68.13–90.62 |

Data presented as mean ± SD.
Maximum HR estimated by the equation: 220 – age.
BMI, body mass index.

The test consisted of 3-min incremental stages preceded by 5 min of light warm-up. Initial workloads were determined between 15 and 30 W, with resistance increasing at each stage by 15–20 W. Participants were asked to maintain ~60 rpm. Earlobe blood samples were obtained at rest and at the end of each stage to measure lactate levels. The test was terminated when lactate concentration exponentially increased and passed 4 mmol/L or when participants were unable to maintain the pedaling rate. Heart rates (HR) were continuously monitored throughout the exercise protocol (Polar T31 coded transmitter; Polar Electro Oy, Kempele, Finland), and RPE values (Borg 6–20 [17]) were recorded during the last 30 s of each stage.

**Submaximal exercise protocol (visits 2 and 3).** During V2 and V3, participants cycled at power outputs corresponding to +10% above the lactate threshold for 30 min on the same cycle ergometer used in the lactate threshold test as previously described (8). Venous blood samples (EDTA; BD and Co., Franklin Lakes, NJ) were collected before and immediately after the exercise bout. Respiratory gases, HR, and RPE were monitored continuously to adjust the resistance based on biofeedback and ensure participants remained within +10% of the lactate threshold (Table 1).

## Outcome Assessments

**PBMC and NK cell isolation.** Complete blood counts were determined using an automated hematology analyzer in duplicate (Sysmex XN-330; Sysmex Co., Kobe, Japan). Immediately after complete blood counting, PBMC were isolated by density gradient centrifugation as previously described (Stem Cell Technologies, Vancouver, Canada) (18). After isolation, PBMC were counted using a flow cytometer (BD Accuri C6, Ann Arbor, MI) and an automated cell counter (Countess 3 FL; ThermoFisher Scientific Inc., Waltham, MA) Then, according to the manufacturer's instructions, NK cells were isolated by negative selection using magnetic-activated cell sorting separation beads (Miltenyi Biotec, Auburn, CA). Negatively sorted NK cells were washed at 400*g* for 10 min before resuspending with RPMI-1640. The purity of cells after separation was confirmed using anti-CD3 and anti-CD56 antibodies to ensure a minimum purity of 90% NK cells were achieved.

**Cell culture.** Human MDA-MB-231 (originally isolated from a 51-yr-old Caucasian woman), MCF-7 (originally isolated from a 69-yr-old woman), and K562 (originally isolated from a 53-yr-old chronic myelogenous leukemia patient) cell lines were used for this study. Considering the limited available literature on the effects of acute exercise on the NK cell killing of breast cancer tumor cell lines, K562 cells, an NK-sensitive target cell line, were included in the assay as a positive control for the NK cell cytotoxic assay. To compare the different breast cancer cell lines, two different breast cancer cell lines were included in the NKCA. MDA-MB-231 is a triple-negative (TNBC) cell line that has little to no expression of estrogen (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) (ER–, PR+–HER-2−), thus considered to be a hormone-insensitive breast cancer cell line. Meanwhile, MCF-7 is a luminal A subtype, expressing estrogen (ER), and progesterone receptor (PR) but no human epidermal growth factor receptor 2 (HER-2), thus considered to be a hormone-sensitive breast cancer cell line (ER+, PR+, HER-2−). MCF-7 and K562 were obtained from the American Type Culture Collection (Manassas, VA), and MDA-MB-231 was obtained from the Pennington Biomedical Research Center. All cell lines were cultured in RPMI 1640 medium with 10% fetal bovine serum and maintained in a 37°C humidified incubator with 5% $CO_2$. Low passages were used for the experiments to ensure adequate cellular viability (<10 cell passages).

**NK cell cytotoxic assay.** Target cells were stained with anti-CD71 APC for at least 20 min before washing with PBS. Then NK cells were cocultured with CD71-labeled target cells ($2.0 \times 10^5$ cells) at 10:1 NK cell–target cell ratios in a final volume of 2 mL into 96-well plates. In addition, NK cells and individual target cells alone were transferred to 96-well plates separately for both normoxia and hypoxia conditions. All plates were centrifuged at 400*g* for 10 min to ensure that maximum contact between NK cells and target cells was achieved. Both conditions were incubated for 4 h in 37°C humidified incubator. Hypoxic conditions were obtained by culturing cells in a hypoxia chamber (Stemcell Tech., Inc, Seattle, WA) with a mixture of 1% $O_2$, 5% $CO_2$, and 94% $N_2$, or normoxic conditions (21% $O_2$ mixture).

**Antibody labeling and flow cytometry.** NK cells were stained with monoclonal antibodies (mAbs) in a four-color direct immunofluorescence assay to phenotype them. The following mAbs were used in this study: an FITC-conjugated anti-CD3 (clone OKT3), anti-CD159C (NKG2C), anti–killer cell lectin-like receptor G1 (KLRG1 clone REA261), PE-conjugated anti–propidium iodide (PI), anti-CD158e (KIR3DL1), anti-CD56 (clone CMSSB), anti-CD57 (clone TB01), PE-Cyanine5.5-conjugated anti-CD56 (clone CMSSB), anti-CD3 (clone SK7), APC-conjugated anti-CD71, anti-CD314 (NKG2D), anti-CD159a (NKG2A), and anti-CD3 (clone UCHT1). FITC-conjugated anti-NKG2C, KLRG1, PE-conjugated anti-KIR3DL1, and anti-NKG2A were purchased from Miltenyi Biotec; PI and PE-cyanine5.5-conjugated anti-CD56 were purchased from eBiosciences; the anti-CD71 were purchased from BD Pharmingen; and the rest of antibodies were purchased from Invitrogen. Stained cells were incubated in the dark for 45 min at room temperature. A description of the function of receptors is presented in Supplemental Table 1, Supplemental Digital Content, NK cell surface marker combinations used to identify the level of differentiation, and expressing activatory, inhibitory receptors, http://links.lww.com/MSS/C886.

After 4 h of incubation, cells were collected, washed, and stained with PI and PE-cyanine5.5-conjugated anti-CD56 to quantify cell death and cytotoxicity. Specific lysis of NK cell cytotoxicity (NKCA) was quantified as percentage of specific lysis (% total lysis − % spontaneous cell death) and at the per cell level (the number of dead target cells per NK cell). After antibody labeling, cellular phenotypes were assessed on a

BD Accuri C6 flow cytometer (Accuri, Ann Arbor, MI), and data analyses were performed using FCS Express (FCS Express Version 7.0; De Novo Software, Pasadena, CA).

**High-resolution fluororespirometry.** After 4 h of incubation, target-activated NK cells (target-to-effector ratio of 10:1) were sorted by size using 10 μm cell strainers (Pluriselect, Leipzig, Germany) and washed with RPMI. After counting the recovered NK cells and checking for purity, we simultaneously measured the NK cell oxygen flow ($I_{O2}$, pmol·s$^{-1}$·million NK cells$^{-1}$) and $H_2O_2$ flow ($I_{H2O2}$, pmol·s$^{-1}$·million NK cells$^{-1}$) at 37°C as described by Komlódi and colleagues with modifications (19), while maintaining the oxygen concentration during the experimental protocol between ~50 and 200 μM using a high-resolution fluororespirometer (Oxygraph O2K, Oroboros, Austria). A detailed description of the high-resolution fluororespirometry methods can be found in the Supplemental Digital Content (see Supplemental Digital Content, High-resolution respirometry, http://links.lww.com/MSS/C886).

### Statistical Analysis

All statistical analyses were performed using JMP Pro 16.2 (SAS Inc., Cary, NC). Linear mixed models were used to test the different outcome measures (e.g., NKCA, OXPHOS, and ROS production) and the effects of time (preexercise vs postexercise) and oxygen tension (normoxia vs hypoxia) and their interaction. In addition, random effects representing the within- and between-subject error terms were also included in the model. Specific lysis of NK cell cytotoxicity at the different ratios in response to exercise was analyzed using the *post hoc* Student's *t*-test. To determine the differences in mobilized cell phenotypes, the numbers and the percentage change between pre- and postexercise independent sample *t*-tests were used to compare the number of the mobilized cells. Data are presented as mean ± SD. Statistical significance was declared at $P < 0.05$.

## RESULTS

### Participant Characteristics and Performance Output Measurements

All participants completed the 30-min cycling protocol at ~10% above their lactate threshold (80.0 ± 22.6 W). Table 1 presents the physical characteristics (age, weight, height, body composition, and $\dot{V}O_{2peak}$) and exercise outcomes. The mean HR and RPE values during were not different between visits ($P > 0.05$).

### Acute Exercise Redeploys NK Subsets with Highly Differentiated Phenotype and Activating Receptors in the Peripheral Blood Compartment

Because the expression of activatory (NKG2C and NKG2D) and inhibitory (NKG2A, KIR3DL1, and KLRG1) receptors tightly regulate NK cell function, we aimed to characterize the effect of a single bout of acute exercise on the mobilization of specific NK cell subsets further (see Supplemental Table 2, Supplemental Digital Content, Total number of peripheral blood lymphocytes, pan NK cells, CD56$^{dim}$, CD56$^{bright,}$ and their expressing receptors in response to acute exercise, http://links.lww.com/MSS/C886). The most exercise-responsive cells among the pool of pan NK cells were polarized toward an "activable" phenotype, expressing NKG2A-/NKG2C+ and increased by 559.4% (preexercise, 23 ± 24 cells per microliter, vs postexercise, 74 ± 92 cells per microliter; $P < 0.05$) in response to 30 min of cycling. Terminally differentiated KLRG1$^+$/CD57$^+$ (463.6%, preexercise, 24 ± 19 cells per microliter, vs postexercise, 82 ± 37 cells per microliter; $P < 0.0001$) were the second largest mobilized cell population in the blood compartment, followed by the highly mature NK phenotype expressing KLRG1$^-$/CD57$^+$ receptors (381.73%, preexercise, 30 ± 26 cells per microliter, vs postexercise, 100 ± 86 cells per microliter; $P < 0.01$). Like the exercise-induced mobilization observed among pan NK cells, CD56$^{dim}$ NK cells with an "inhibitable" phenotype, NKG2A+/NKG2C+, were mobilized by exercise. Specifically, their number in circulation increased by 1051.6% (preexercise, 3 ± 4 cells per microliter, vs postexercise, 12 ± 9 cells per microliter; $P < 0.01$) in response to exercise, followed by terminally differentiated subsets expressing KLRG1$^+$/CD57$^+$ (730.0%, preexercise, 16 ± 15 cells per microliter, vs postexercise, 61 ± 29 cells per microliter; $P < 0.0001$). Highly potent lytic IFN-γ-producing CD56$^{dim}$ NK cells, KLRG1$^-$/CD57$^+$ subsets (607.9%, preexercise, 19 ± 19 cells per microliter, vs postexercise, 76 ± 74 cells per microliter; $P < 0.01$), were the third largest mobilized subpopulation.

Among CD56$^{bright}$ NK cells, KLRG1$^+$/CD57$^-$ cells, with reduced proliferative capacity, were the most mobilized cells in response to acute exercise (416.0%, preexercise, 0.6 ± 1.4 cells per microliter, vs postexercise, 1.8 ± 4.3 cells per microliter; $P < 0.05$), followed by cells with an "activable" phenotype expressing NKG2D$^+$/KIR3DL1$^-$ (383.7%, preexercise, 3 ± 2 cells per microliter, vs postexercise, 7 ± 6 cells per microliter; $P < 0.01$), known for their high capacity at recognizing target cells. Finally, terminally differentiated mature but cytotoxic KLRG1$^-$CD57$^+$ NK cells (preexercise, 3 ± 3 cells per microliter, vs postexercise, 7 ± 9 cells per microliter; $P < 0.05$) increased by 278.6% in circulation after 30 min of moderate- to high-intensity cycling.

### NK Cell Cytotoxicity against HLA-Deficient Leukemia and Luminal A MCF-7 Cell Lines Increases in Response to Acute Exercise Cultured in Normoxia but Not against TNBC Cell Line

**HLA-deficient leukemia cell line K562.** The effects of an acute bout of exercise on NK cell–specific lysis (E:T 10:1) under normoxia are presented in Figure 1A. Under normoxic conditions, acute exercise increased NK cell killing of HLA-deficient K562 cell lines at 10:1 effector-to-target (E-T) ratio ($P < 0.01$). During hypoxic-cultured conditions, exercise also increased NK cell killing against HLA-deficient

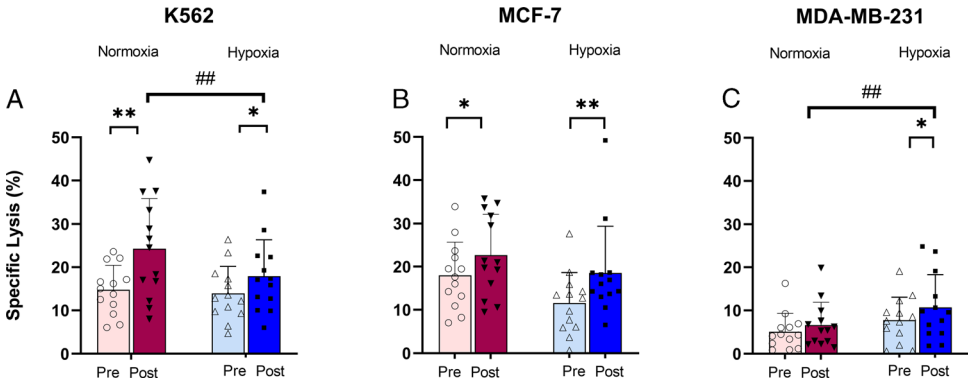


**FIGURE 1—Specific lysis (%) of NK cells against target cells in response to exercise at 10:1 ratio under either normoxic or hypoxia. A, Representative bar graph of specific lysis against HLA-deficient K562 cell, B, Lumina A subtype MCF-7 cell line. C, TNBC MDA-MB-231 cell line cocultured with NK cells under either normoxia or hypoxia at 10:1 ratio. *$P < 0.05$ and **$P < 0.001$. ##Different from normoxia postexercise NK cells. *Light pink*, preexercise NK cells under normoxia; *red*, postexercise NK cells under normoxia; *light blue*, preexercise NK cells under hypoxia; *blue*, postexercise NK cells under hypoxia.**



leukemia K562 cell line at the same E-T ratio. For example, specific lysis of NK killing in the postexercise NK cells was higher compared with resting NK cells cultured in hypoxia (hypoxic-cultured preexercise, 13.92 ± 6.29, vs hypoxic-cultured postexercise, 17.88 ± 8.46; $P < 0.05$), showing that exercise rescues killing capability initially impaired by hypoxia (Fig. 1A). Although resting NK cell–specific lysis between $O_2$ tension was not different ($P = 0.66$), specific lysis from postexercise NK cells in hypoxic-cultured conditions had a lower killing than postexercise NK cells in normoxic-cultured conditions ($P = 0.004$), showing that delta (Δ) killing was larger in normoxic- than in hypoxic-cultured conditions (Δ in normoxia, 9.5% ± 6.9%, vs Δ in hypoxia, 4.0% ± 5.6%; $P < 0.01$).

**Breast cancer cell lines: MCF-7.** In response to 30 min of moderate- to high-intensity cycling, normoxic-cultured postexercise NK cells showed an increased killing against estrogen and progesterone receptors (ER and PR) expressing MCF-7 cells at 10:1 E-T ratio when compared with resting NK cells at the same E-T ratio (preexercise, 18.0% ± 7.7%, vs postexercise, 22.7% ± 9.5%; $P = 0.011$) (Fig. 1B). Furthermore, under hypoxic conditions, postexercise NK cell–specific lysis against MCF-7 cell line at an E-T ratio of 10:1 showed increased killing ability compared with resting NK cells cultured in hypoxia ($P < 0.0001$). Interestingly, in specific lysis at 10:1 E-T ratio in hypoxic-cultured conditions, postexercise NK cell killing of MCF-7 was not different from postexercise NK cell killing under normoxic-cultured conditions (normoxia postexercise, 22.68% ± 9.47%, vs hypoxia postexercise, 18.55% ± 10.83%; $P = 0.214$), showing a single bout of exercise rescues the NK cell killing ability against ER+, PR+ expressing luminal A subtype, and MCF-7 cell line (Fig. 1B).

**Breast cancer cell lines: MDA-MB-231.** When cultured under normoxic conditions, the acute exercise showed little effect on MDA-MB-231 cells, as there was no difference in NK cell–specific lysis between resting and postexercise cells (preexercise, 5.1% ± 4.2%, vs postexercise, 6.7% ± 5.2%; $P = 0.228$) (Fig. 1C). However, under hypoxic conditions, specific lysis at a 10:1 E-T ratio of postexercise NK cells was increased against a TNBC-type MDA-MB-231 cell line in response to the acute bout of exercise (hypoxia preexercise, 7.80 ± 5.27, vs hypoxia postexercise, 10.67 ± 7.63; $P < 0.05$). In addition, postexercise NK cells cultured in hypoxia at a 10:1 E-T ratio showed a higher killing ability compared with those cultured in normoxia ($P < 0.01$) (Fig. 1C).

## Oxygen Flow ($I_{O2}$) in Pre- and Postexercise NK Cells Cocultured with MD-MB-231 Cells under Normoxic and Hypoxic Conditions

No differences in $I_{O2}$ were detected in preexercise NK cells after 4 h of coculture with MDA-MB-231 cells under normoxia or hypoxia (Figs. 2 and 3 and Supplemental Table 3, Supplemental Digital Content, Flow of O2 ($I_{O2}$) of preexercise NK cells under normoxic and hypoxic conditions at cellular and tissue level, http://links.lww.com/MSS/C886). The $I_{O2}$ in preexercise NK cells after 4 h of coculture with MDA-MB-231 cells under normoxia compared with hypoxia was measured to assess the effect of hypoxia on target-activated NK cell mitochondrial respiratory functions. Across the different respiratory states, no differences in $I_{O2}$ were found between the preexercise NK cells cocultured under normoxia compared with hypoxia, whether expressed at the cellular level or the tissue level.

Cellular-level oxygen flow ($I_{O2}$, pmol·s$^{-1}$·million NK cells$^{-1}$) in response to a single bout of exercise increased OXPHOS and ET capacity in NK cells cocultured with MDA-MB-231 under normoxic conditions (Fig. 2 and Supplemental Tables 3 and 4, Supplemental Digital Content, $I_{O2}$ in response to acute exercise culture under normoxic and hypoxic conditions at cellular and tissue level, http://links.lww.com/MSS/C886). The addition of saturating ADP concentrations revealed that under the OXPHOS state ($NS_P$), there was a higher $I_{O2}$ expressed at the cellular level in the post- compared with the preexercise NK cells cocultured with MDA-MB-231 ($NS_P$: $P < 0.05$). Furthermore, higher $I_{O2}$ was also measured during all three ET capacity states in the post- compared with preexercise NK cells cocultured with MDA-MB-231 under normoxic conditions

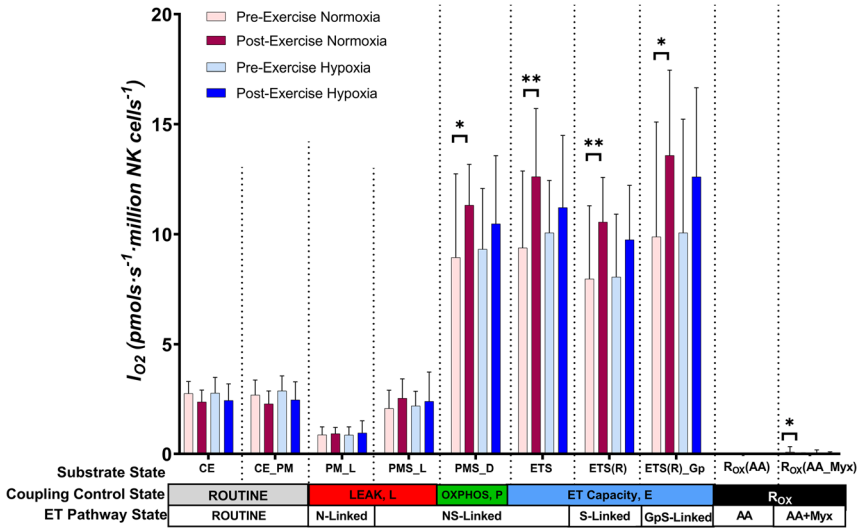


**FIGURE 2—Cellular-level oxygen flow ($I_{O2}$, pmol·s$^{-1}$·million NK cells$^{-1}$) was measured in pre- and postexercise NK cells activated by coculturing with MDA-MB-231 at a 10:1 ratio under normoxia (~21% $O_2$) or hypoxia (1% $O_2$) for 4 h. The $I_{O2}$ was measured in the intact cells (CE, routine) and chaconine-permeabilized NK cells in the LEAK, OXPHOS, and ET capacity coupling control states, using endogenous (routine), N-Linked (PM, pyruvate + malate), NS-Linked (PMS, addition of succinate), S-Linked (Rot, addition of rotenone), and GpS-Linked (GpS, addition of glycerol-3-phosphate) substrates that were titrated serially. LEAK was measured in the absence of ADP, OXPHOS capacity was measured in the presence of 2.5 mM ADP, and ET capacity was measured after titrating in an uncoupler, carbonyl cyanide m-chlorophenyl hydrazone (CCCP). All data were background corrected for residual oxygen consumption measured in the presence of antimycin A ($R_{OX}$(AA)). In addition, $R_{OX}$ was also measured after the addition of myxothiazol ($R_{OX}$(AA_Myx). *$P < 0.05$ and **$P < 0.01$ for pre- vs postexercise comparison within oxygen tension. Data presented as mean ± SD ($n$ = 11).**

($NS_E$, $P < 0.01$; $S_E$, $P < 0.01$; $GpS_E$, $P < 0.05$). Similarly, a greater $I_{O2}$ was measured in the residual oxygen consumption state in the presence of antimycin A plus myxothiazol ($R_{OX\ +\ Myx}$) in the post- compared with preexercise NK cells cocultured with MDA-MB-231 under normoxic conditions ($R_{OX\ +\ Myx}$, $P < 0.05$). However, the exercise-induced changes in $I_{O2}$ noted above were not statistically significant when the NK cells were cocultured with MDA-MB-231 under hypoxic conditions.

Tissue-level oxygen flow ($I_{O2\text{-}Tissue}$, pmol·s$^{-1}$·mL blood$^{-1}$) in response to a single bout of exercise increased in all respiratory states in NK cell cocultured with MDA-MB-231 cells under both normoxic and hypoxic conditions (Fig. 3 and Supplemental Table 4, Supplemental Digital Content, Flow of $O_2$ ($I_{O2}$) in response to acute exercise culture under normoxic and hypoxic conditions at cellular and tissue level, http://links.lww.com/MSS/C886). After normalizing the $I_{O2}$ to the tissue level to account for the exercise-induced mobilization of NK cells, the $I_{O2\text{-}Tissue}$ measured under all respiratory states was higher in the post- compared with preexercise NK cells cocultured with MDA-MB-231 (all $P < 0.05$), independent of oxygen tension. However, the $I_{O2\text{-}Tissue}$ under the $NS_E$ state in the postexercise

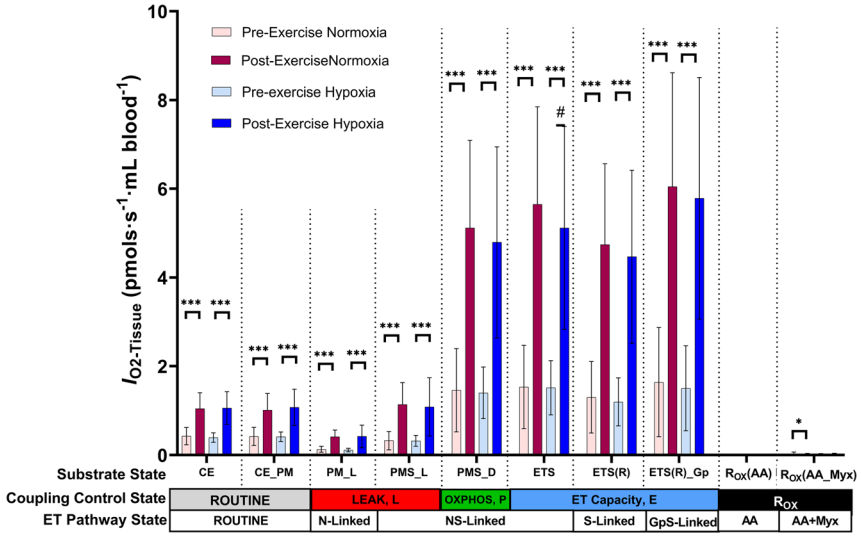


**FIGURE 3—Tissue-level oxygen flow ($I_{O2\text{-}Tissue}$, pmol·s$^{-1}$·mL blood$^{-1}$) measured in pre- and postexercise NK cells activated by coculturing with MDA-MB-231 at a 10:1 ratio under normoxia (~21% $O_2$) or hypoxia (1% $O_2$) for 4 h. The $I_{O2\text{-}Tissue}$ was measured as described in Figure 2. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ for pre- vs postexercise comparison within oxygen tension. #$P < 0.05$ for hypoxia vs normoxia comparison within the postexercise cells. Data are presented as mean ± SD ($n$ = 11).**

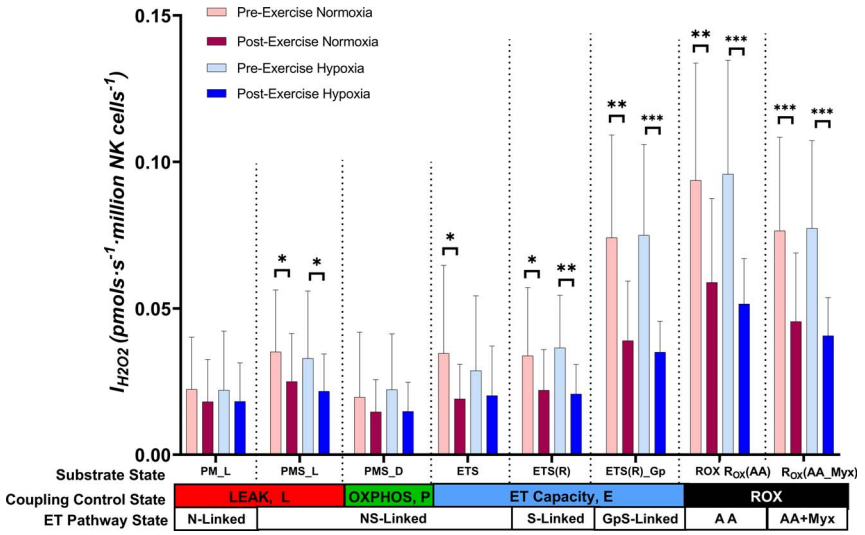


**FIGURE 4—Cellular-level hydrogen peroxide flow ($I_{H2O2}$, pmol·s$^{-1}$·million NK cells$^{-1}$) was measured in pre- and postexercise NK cells activated by coculturing with MDA-MB-231 at a 10:1 ratio under normoxia (~21% $O_2$) or hypoxia (1% $O_2$) for 4 h. These measurements were made simultaneously with the measurements of $I_{O2}$ in the cells discussed in Figure 2 using Amplex Ultrared (AMR), horseradish peroxidase, and superoxide dismutase after permeabilizing the cell membrane with chaconine. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Data presented as mean ± SD ($n = 11$).**

NK cells was lower in the NK cells cocultured with MDA-MB-231 under hypoxia than those cultured under normoxia ($NS_E$, $P < 0.05$). This effect was lost when analyzing $I_{O2}$ at the cellular level ($NS_{E\_cellular}$, $P = 0.07$).

## Hydrogen Peroxide Flow ($I_{H2O2}$) in Pre- and Postexercise NK Cells Cocultured with MDA-MBA-231 under Normoxic and Hypoxic Conditions

No differences in $I_{H2O2}$ were detected in preexercise NK cells after 4 h of coculture with MDA-MB-231 cells under normoxic and hypoxic conditions (Figs. 4 and 5 and Supplemental Table 5, Supplemental Digital Content, $H_2O_2$ flow of preexercise NK cells under normoxic and hypoxic conditions at cellular and tissue level, http://links.lww.com/MSS/C886). We aimed to determine whether there were differences in resting NK cell $H_2O_2$ flow rates ($I_{H2O2}$) between the NK cells cocultured with MDA-MB-231 cells under normoxic and hypoxic conditions. No differences in $I_{H2O2}$ were found across the different respiratory states between the normoxic and the hypoxic conditions, whether expressed at the cellular level or the tissue level.

Cellular-level hydrogen peroxide flow ($I_{H2O2}$, pmol·s$^{-1}$·million NK cells$^{-1}$) in response to acute exercise decreased under both normoxic and hypoxic conditions (Fig. 4 and Supplemental Tables 5 and 6, Supplemental Digital Content, Flow of $H_2O_2$ in response to acute exercise culture under normoxic and hypoxic conditions at cellular and tissue level, http://links.

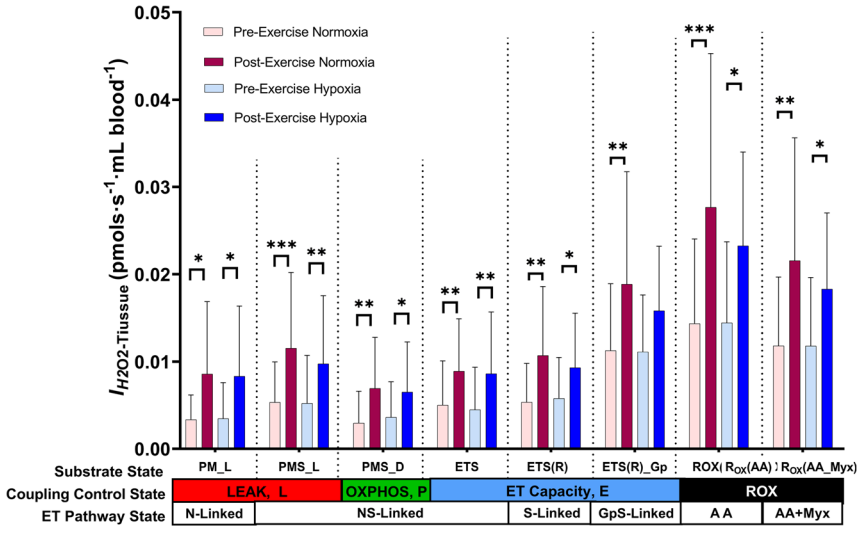


**FIGURE 5—Tissue-level hydrogen peroxide flow ($I_{H2O2-Tissue}$, pmol·s$^{-1}$·mL blood$^{-1}$) was measured in pre- and postexercise NK cells coculturing with MDA-MB-231 at a 10:1 ratio under normoxia (~21% $O_2$) or hypoxia (1% $O_2$) for 4 h. These measurements were made as described in Figure 4. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Data presented as mean ± SD ($n = 11$).**

lww.com/MSS/C886). A lower $I_{H2O2}$ in the post- compared with the preexercise NK cells was found under LEAK ($NS_L$), ET capacity ($NS_E$, $S_E$, and $G_PS_E$), and $R_{OX}$ ($R_{OX(AA)}$, $R_{OX(AA\_Myx)}$) states when cocultured with MDA-MB-231 cells under normoxic conditions at cellular level (all $P < 0.05$). Similar results were also observed in the NK cells cocultured with MDA-MB-231 cells under hypoxic conditions with one exception. The lower $I_{H2O2}$ under the $NS_E$ state in the post- compared with preexercise NK cells cocultured with MDA-MB-231 cells under hypoxic conditions did not reach statistical significance ($NS_E$, $P = 0.17$).

Tissue-level hydrogen peroxide flow ($I_{H2O2\text{-}Tissue}$, $pmol \cdot s^{-1} \cdot mL\ blood^{-1}$) in response to a single bout of exercise increased in all respiratory states in NK cell cocultured with MDA-MB-231 under both normoxic and hypoxic conditions (Fig. 5 and Supplemental Tables 5 and 6, Supplemental Digital Content, Flow of $H_2O_2$ in response to acute exercise culture under normoxic and hypoxic conditions at cellular and tissue level, http://links.lww.com/MSS/C886). After normalizing the $I_{H2O2}$ to the tissue level to account for the exercise-induced mobilization of NK cells, the $I_{H2O2\text{-}Tissue}$ measured under LEAK, OXPHOS, ET capacity, and $R_{OX}$ states was higher in the post- compared with preexercise NK cells cocultured with MDA-MB-231 under hypoxic conditions (all $P < 0.05$), independent of oxygen tension with one exception. The $I_{H2O2\text{-}Tissue}$ under the $GpS_E$ state in the post- compared with preexercise NK cells cocultured with MDA-MB-231 cells under hypoxic conditions was not statistically different ($P = 0.06$).

## DISCUSSION

This study examined the effects of a single bout of exercise on the cytotoxic capabilities of NK cells against different breast cancer cell lines under normoxia and hypoxia when isolated from sedentary healthy women. The present data suggest that the cytotoxic activity of NK cells varies when cultured under physiologically low oxygen tension, such as 1% $O_2$ mixture, compared with a 21% $O_2$ mixture. Furthermore, when cocultured under normoxic conditions, the NK cells' ability to specifically lyse various breast cancer cell lines differed from the NK cells' ability to kill the same tumor cells under the physiologically relevant hypoxic conditions found in the TME. This novel finding suggests that actual oxygen tension directly affects the interactions between NK and cancer cells. Here we also found that a single bout of acute exercise rescues the cytotoxic function of NK cells when cocultured under hypoxic conditions, in a cell line–specific manner, with the greatest effect seen in cells known for promoting hypoxia in the TME (MDA-MB-231) and little to no effect against cells less prone to creating hypoxic TME (MCF-7). Moreover, we investigated two likely mechanistic underpinnings of the exercise-induced improvements in NK cell killing of TNBC using flow cytometry and high-resolution respirometry. Overall, we showed that while the preferential mobilization of inhibitory $KIR^+/KAR^-$ cells led to decreases in NK cell killing, exercise was associated with an influx of "activable" $KAR^+/KIR^-$, which correlated with tumor cell death. We also showed that acute exercise leads to an increase in NK cell oxygen utilization under normoxic conditions. Indeed, postexercise NK cells had a greater $I_{O2}$ than resting cells under OXPHOS and ET capacity. This was true both at the cellular and tissue levels, suggesting that the increase in $I_{O2}$ is independent of exercise-induced mobilization. Furthermore, in pre- and postexercise NK cells cocultured with MDA-MB-231 cells under hypoxia, we showed that acute exercise increases mitochondrial oxygen flow under all respiratory states when expressed at the tissue-level $I_{O2\text{-}Tissue}$ to account for the exercise-induced NK cell mobilization. Likewise, the tissue-level hydrogen peroxide flow ($I_{H2O2\text{-}Tissue}$) in the MDA-MB-231 cells activated by NK cells was generally elevated across all respiratory states, independent of oxygen tension. Interestingly, the cellular-level hydrogen peroxide flow ($I_{H2O2}$) in the MDA-MB-231 cells activated by NK cells was generally reduced across all respiratory states, independent of oxygen tension.

To our knowledge, this is the first study to identify the interrelationship between hypoxia and exercise-induced changes in NK cell functions against breast cancer cell lines and its association with the metabolism of the tumor (MDA-MB-231)-activated NK cells. Indeed, the majority of studies that investigated the effects of exercise on NK cell activity against tumor cells *in vitro* mainly were conducted using whole blood samples or pan lymphocytes (8,20–23), under normoxic conditions alone (21% $O_2$) (8,22–24), or conducted against HLA-deficient K562 cell line, regarded as NK-sensitive cancer cells (20,21). However, because 5%–15% of circulating lymphocytes consist of NK cells, using freshly isolated pan PBMC limits the ability to exclude the roles of cytotoxic T cells when measuring cytotoxic capacity against target cells. In addition, physiological $O_2$ tension never reaches 21% *in vivo*, especially within solid tumors. The physiological oxygen level of healthy breast tissue reaches 42 mm Hg (median, 5.5% $O_2$), whereas hypoxia-induced solid breast tumor tissues arise at 10 mm Hg (median, 1.3% $O_2$) and vary by tissue origin of the tumors (25). This is particularly relevant because hypoxic conditions are known to reduce expression of activatory receptors and impair cytotoxic capabilities through activating inhibitory phosphatases (16,26). Similarly, exposure to hypoxic conditions affects not only the function of NK cells but also the cancer cells. MDA-MB-231 is a TNBC that lacks hormone receptors regarded as more aggressive and with a poorer prognosis than MCF-7, whereas MCF-7 is a luminal A submolecular type expressing estrogen status (3). This has been suggested to be partly due to breast cancer cells' increased migration and viability properties in response to low oxygen tension, suggesting that hypoxia increases metastatic ability (27), while facilitating their immune evasion (28).

Moreover, we identified differences in specific lysis between the different cell lines studied, both at rest and postexercise. Three cell lines were included in this study based on their differential expression of major histocompatibility complex (MHC) molecules, ranging from high expression (MDA-MB-231) to

low expression (MCF-7) or even complete lack of MHC-I and II (K562) (29). Because MHC molecules play a crucial role in inhibiting NK cell killing, the amount of MHC-I expression expressed on a tumor cell is directly associated with its likelihood of being lysed by an NK cell (29,30). Thus, the lower expression of MHC-I on MCF-7 cell lines compared with MDA-MB-231 induces fewer inhibitory signals in NK cells, increasing their sensitivity to NK cell–mediated killing. Further, the complete lack of MHC-I molecules on K562 also explains their great sensitivity to either resting or postexercise NK cells.

Here we found that a single bout of exercise redeploys a highly differentiated phenotype of NK cells with activating receptors and potent lytic activity markers. As for pan NK cells, $NKG2A^{-}/NKG2C^{+}$ cells were the most mobilized, followed by $KLRG1^{+}/CD57^{+}$ cells. This suggests that exercise preferentially mobilizes/enhances the numbers of circulating NK cells armed with activatory receptors that confers them potent antitumor functions and thus enhances immune surveillance against malignant cells. Moreover, MHC class I-interactive NKG2D expressing $CD56^{bright}$ cells were found to significantly increase in circulation in response to the acute bout of exercise, whereas KIR3DL1+ $CD56^{bright}$ remained unchanged, which suggests that exercise preferentially mobilizes subsets armed with activatory NK cells to the detriment of inhibitory NK cells. This further advocates for the role of acute exercise in enhancing cytokine production and degranulation, which ultimately increases target cell lysis (31), and enhances immune surveillance rather than suppressing immune function as previously believed (32,33). This is of particular importance for the use of exercise as a therapeutic adjuvant against aggressive and hormone-insensitive breast cancer types such as MDA-MB-231.

We found that a single bout of exercise restored postexercise NK cell cytotoxicity against HLA-deficient K562 cell lines under hypoxic conditions when compared with NK cells cocultured with target cells under normoxic conditions. This result replicates previous findings from other groups, who also observed a dampened NK cell activity against HLA-deficient K562 cell lines under hypoxia (34). However, the ecological relevance of such findings is debatable because K562 does not naturally form solid tumors and thus does not promote hypoxia *in vivo*. Although this cell line was used as an internal validity control for our experiment, our study also showed for the first time that MDA-MB-231 and MCF-7 cell lines have different sensitivities to NK cells in response to oxygen tension. Under hypoxia, increased killing of MDA-MB-231 cells by NK cells was observed, whereas the dampened activity of NK cells against luminal A MCF-7 cell lines was seen when compared with normoxic conditions.

Although the exercise-induced preferential mobilization of NK cells expressing activatory receptors is of great importance, emerging evidence suggests that immune metabolic functions are another driver of pathogenic and tumor clearance (35). Hypoxia is a known detrimental characteristic of the TME and is associated with increased ROS production levels and dampened immune cell function in solid tumors (15,16). This is of specific relevance because high levels of ROS production have been shown to induce oxidative stress (36) and immunosuppression in immune cells. By contrast, adequately low levels of ROS promote cellular metabolism and signaling activation (37). In particular, NK cells have high sensitivity to ROS-induced oxidative stress, which dramatically impairs their cytotoxic functions (38). In our study, we saw decreased levels of $H_2O_2$ flow ($I_{H2O2}$, $pmol \cdot s^{-1} \cdot million\ NK\ cells^{-1}$) in NK cells cocultured with MDA-MB-231 after acute bout of exercise, suggesting that exercise primes NK cells to elevate their function by decreasing their level of net mitochondrial ROS efflux. Reductions in $I_{H2O2}$ could be due to reductions in ROS production and/or increases in ROS scavenging. Recent work has shown that the activity of thioredoxin, a potent ROS scavenger, enhances the ability of NK cells to infiltrate tumors and increases their cytotoxicity (38). One potential explanation for the reduced $I_{H2O2}$ in the postexercise NK cells compared with the preexercise cells is an increase in thioredoxin activity within the postexercise NK cells. The exercise-induced increase in thioredoxin activity could be driven by elevations in thioredoxin concentrations or through concomitant reductions in the binding of the thioredoxin-interacting protein to thioredoxin (38). Although we did not measure thioredoxin in our study, a recent study suggests that an acute bout of exercise increased thioredoxin activity by as much as 200% in PBMC (39). We should note that Auronafin, a thioredoxin inhibitor, was used in the fluororespirometry experiments to facilitate the detection of $H_2O_2$ in NK cells that are rich in thioredoxin. The oxidative ROS burst during killing also likely comes from the NADPH pathway, and the reduction in mitochondrial ROS may provide some compensatory protection. These results further advocate for the role of exercise as a way to compensate for the hypoxic-mediated ROS milieu. Furthermore, reduced ROS level after exercise is in accordance with our cytotoxic assay, where postexercise NK cells had increased killing compared with preexercise NK cells, likely via nonmitochondrial ROS-mediated pathways, such as perforin or granzyme B secretion (40).

This study is not without limitations. One limitation is using sedentary, otherwise healthy participants instead of breast cancer patients or survivors. Although our results cannot directly be applied to clinical practice, the study is clinically relevant because our participants classified as having "poor" fitness level by the American College of Sports Medicine match the expected fitness level of breast cancer patients undergoing therapy. Thus, we hypothesize that the exercise benefits on NK cell effector function observed in this study would translate to a clinical population. Furthermore, technical limitations prevented us from assessing changes in KIRs and KARs expression on the surface of NK cells in response to 4 h of incubation under normoxic or hypoxic conditions. Although we did not observe significant changes in the number or proportions of NK cells subsets after incubation, we cannot rule out that the hypoxic environment could have altered NK cell proliferative capacity or expression of KIRs and KARs, thus affecting their cytotoxic activity. Finally, future studies should

attempt to identify further the mechanistic underpinnings of exercise-induced changes in NK cell mitochondrial functions, including changes in mitochondrial respiratory function, ROS production, antioxidant defense, and redox balance. For example, future studies should assess whether changes in circulating catecholamine concentration could also affect NK cell bioenergetic functions, regardless of cellular shifts in the peripheral blood compartment.

In conclusion, this study showed that a single acute bout of exercise recues the NK cell function lost due to hypoxic conditions, and that the level by which NK cell killing is rescued depends on the tumor cell type. Considering the aggressiveness and poor prognosis associated with TNBC, the finding that acute exercise exerts the most beneficial effects on this specific cell line is of great clinical relevance. Furthermore, target cell (MDA-MB-231)–activated NK cells after a single bout of exercise had increased mitochondrial respiration under OXPHOS and ET capacity and decreased $H_2O_2$ flow independently of oxygen tension, suggesting that aerobic exercise primes NK cells by reducing oxidative stress and, thus, protecting the ROS-sensitive NK cells TME.

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The authors have no conflicts of interest to declare. All coauthors have seen and agree with the contents of the manuscript. The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation. The results of the present study do not constitute endorsement by the American College of Sports Medicine.



## REFERENCES

1. Brown JC, Damjanov N, Courneya KS, et al. A randomized dose-response trial of aerobic exercise and health-related quality of life in colon cancer survivors. *Psychooncology*. 2018;27(4):1221–8.
2. Kumar P, Aggarwal R. An overview of triple-negative breast cancer. *Arch Gynecol Obstet*. 2016;293(2):247–69.
3. Dai X, Cheng H, Bai Z, Li J. Breast cancer cell line classification and its relevance with breast tumor subtyping. *J Cancer*. 2017;8(16): 3131–41.
4. Brown JC, Huedo-Medina TB, Pescatello LS, Pescatello SM, Ferrer RA, Johnson BT. Efficacy of exercise interventions in modulating cancer-related fatigue among adult cancer survivors: a meta-analysis. *Cancer Epidemiol Biomarkers Prev*. 2011;20(1):123–33.
5. West-Wright CN, Henderson KD, Sullivan-Halley J, et al. Long-term and recent recreational physical activity and survival after breast cancer: the California Teachers Study. *Cancer Epidemiol Biomarkers Prev*. 2009;18(11):2851–9.
6. Dethlefsen C, Hansen LS, Lillelund C, et al. Exercise-induced catecholamines activate the hippo tumor suppressor pathway to reduce risks of breast cancer development. *Cancer Res*. 2017;77(18):4894–904.
7. Pedersen L, Idorn M, Olofsson GH, et al. Voluntary running suppresses tumor growth through epinephrine- and IL-6-dependent NK cell mobilization and redistribution. *Cell Metab*. 2016;23(3):554–62.
8. Bigley AB, Rezvani K, Chew C, et al. Acute exercise preferentially redeploys NK-cells with a highly-differentiated phenotype and augments cytotoxicity against lymphoma and multiple myeloma target cells. *Brain Behav Immun*. 2014;39:160–71.
9. Yang G, Shi R, Zhang Q. Hypoxia and oxygen-sensing signaling in gene regulation and cancer progression. *Int J Mol Sci*. 2020;21(21):8162.
10. Simpson RJ, Kunz H, Agha N, Graff R. Exercise and the regulation of immune functions. *Prog Mol Biol Transl Sci*. 2015;135:355–80.
11. Graff RM, Kunz HE, Agha NH, et al. β2-adrenergic receptor signaling mediates the preferential mobilization of differentiated subsets of CD8+ T-cells, NK-cells and non-classical monocytes in response to acute exercise in humans. *Brain Behav Immun*. 2018;74:143–53.
12. Choi C, Finlay DK. Optimising NK cell metabolism to increase the efficacy of cancer immunotherapy. *Stem Cell Res Ther*. 2021;12(1):320.
13. Irving BA, Johannsen N, Wang H, et al, editors. *Impact of Acute Exercise on PBMC Respiratory Function and Methodological Considerations*. Hradec Kralove, Czech Republic: MITOEagle Consortium Conference; 2017.
14. Vitale M, Cantoni C, Pietra G, Mingari MC, Moretta L. Effect of tumor cells and tumor microenvironment on NK-cell function. *Eur J Immunol*. 2014;44(6):1582–92.
15. Al Tameemi W, Dale TP, Al-Jumaily RMK, Forsyth NR. Hypoxia-modified cancer cell metabolism. *Front Cell Dev Biol*. 2019;7:4.
16. Balsamo M, Manzini C, Pietra G, et al. Hypoxia downregulates the expression of activating receptors involved in NK-cell-mediated target cell killing without affecting ADCC. *Eur J Immunol*. 2013;43(10):2756–64.
17. Borg G. *Borg's Perceived Exertion and Pain Scales*. Human Kinetics; 1998.
18. Cho E, Theall B, Stampley J, et al. Cytomegalovirus infection impairs the mobilization of tissue-resident innate lymphoid cells into the peripheral blood compartment in response to acute exercise. *Viruses*. 2021;13(8):1535.
19. Komlódi T, Sobotka O, Krumschnabel G, et al. Comparison of mitochondrial incubation media for measurement of respiration and hydrogen peroxide production. *Methods Mol Biol*. 2018;1782:137–55.
20. Woods JA, Ceddia MA, Wolters BW, Evans JK, Lu Q, McAuley E. Effects of 6 months of moderate aerobic exercise training on immune function in the elderly. *Mech Ageing Dev*. 1999;109(1):1–19.
21. McFarlin BK, Flynn MG, Phillips MD, Stewart LK, Timmerman KL. Chronic resistance exercise training improves natural killer cell activity in older women. *J Gerontol A Biol Sci Med Sci*. 2005;60(10): 1315–8.
22. Nieman DC, Miller AR, Henson DA, et al. Effects of high- vs moderate-intensity exercise on natural killer cell activity. *Med Sci Sports Exerc*. 1993;25(10):1126–34.
23. Gupta P, Bigley AB, Markofski M, Laughlin M, LaVoy EC. Autologous serum collected 1 h post-exercise enhances natural killer cell cytotoxicity. *Brain Behav Immun*. 2018;71:81–92.
24. Millard AL, Valli PV, Stussi G, Mueller NJ, Yung GP, Seebach JD. Brief exercise increases peripheral blood NK cell counts without immediate functional changes, but impairs their responses to ex vivo stimulation. *Front Immunol*. 2013;4:125.
25. Muz B, de la Puente P, Azab F, Azab AK. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia (Auckl)*. 2015;3:83–92.
26. Teng R, Wang Y, Lv N, et al. Hypoxia impairs NK cell cytotoxicity through SHP-1-mediated attenuation of STAT3 and ERK signaling pathways. *J Immunol Res*. 2020;2020:4598476.
27. Cronin PA, Wang JH, Redmond HP. Hypoxia increases the metastatic ability of breast cancer cells via upregulation of CXCR4. *BMC Cancer*. 2010;10:225.
28. You L, Wu W, Wang X, et al. The role of hypoxia-inducible factor 1 in tumor immune evasion. *Med Res Rev*. 2021;41(3):1622–43.
29. Rozanov DV, Rozanov ND, Chiotti KE, et al. MHC class I loaded ligands from breast cancer cell lines: a potential HLA-I-typed antigen collection. *J Proteomics*. 2018;176:13–23.
30. Jørgensen N, Sayed A, Jeppesen HB, et al. Characterization of HLA-G regulation and HLA expression in breast cancer and malignant melanoma

cell lines upon IFN-γ stimulation and inhibition of DNA methylation. *Int J Mol Sci*. 2020;21(12):4307.
31. Idorn M, Hojman P. Exercise-dependent regulation of NK cells in cancer protection. *Trends Mol Med*. 2016;22(7):565–77.
32. Nieman DC, Pedersen BK. Exercise and immune function. *Sports Med*. 1999;27(2):73–80.
33. Nieman DC. Exercise effects on systemic immunity. *Immunol Cell Biol*. 2000;78(5):496–501.
34. Sarkar S, Germeraad WT, Rouschop KM, et al. Hypoxia induced impairment of NK cell cytotoxicity against multiple myeloma can be overcome by IL-2 activation of the NK cells. *PLoS One*. 2013;8(5):e64835.
35. Terrén I, Orrantia A, Vitallé J, Zenarruzabeitia O, Borrego F. NK cell metabolism and tumor microenvironment. *Front Immunol*. 2019;10:2278.
36. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. *Curr Biol*. 2014;24(10):R453–62.
37. Kennel KB, Greten FR. Immune cell-produced ROS and their impact on tumor growth and metastasis. *Redox Biol*. 2021;42:101891.
38. Yang Y, Neo SY, Chen Z, et al. Thioredoxin activity confers resistance against oxidative stress in tumor-infiltrating NK cells. *J Clin Invest*. 2020;130(10):5508–22.
39. Wadley AJ, Chen YW, Bennett SJ, et al. Monitoring changes in thioredoxin and over-oxidised peroxiredoxin in response to exercise in humans. *Free Radic Res*. 2015;49(3):290–8.
40. Abel AM, Yang C, Thakar MS, Malarkannan S. Natural killer cells: development, maturation, and clinical utilization. *Front Immunol*. 2018;9:1869.