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Authors

Radom-Aizik, Shlomit
Zaldivar, Frank
Haddad, Fadia
et al.

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Impact of brief exercise on peripheral blood NK cell gene and microRNA expression in young adults

[Shlomit Radom-Aizik](#), [Frank Zaldivar](#), [Fadia Haddad](#), and [Dan M. Cooper](#)

Pediatric Exercise Research Center, Department of Pediatrics, University of California, Irvine, Irvine, California

Corresponding author.

Address for reprint requests and other correspondence: S. Radom-Aizik, Pediatrics Exercise Research Center, Dept. of Pediatrics, Univ. of California, Irvine, Irvine, CA 92697-4094 (e-mail: saizik@uci.edu).

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Abstract

Natural killers (NK) cells are unique innate immune cells that increase up to fivefold in the circulating blood with brief exercise and are known to play a key role in first-response defense against pathogens and cancer immunosurveillance. Whether exercise alters NK cell gene and microRNA (miRNA) expression is not known. Thirteen healthy men (20–29 yr old) performed ten 2-min bouts of cycle ergometer exercise at a constant work equivalent to an average of 77% of maximum O₂ consumption interspersed with 1-min rest. Blood was drawn before and immediately after the exercise challenge. NK cells were isolated from peripheral blood mononuclear cells using a negative magnetic cell separation method. We used Affymetrix U133+2.0 arrays for gene expression and Agilent Human miRNA V2 Microarray for miRNAs. A stringent statistical approach (false discovery rate < 0.05) was used to determine that exercise significantly altered the expression of 986 genes and 23 miRNAs. Using in silico analysis, we found exercise-related gene pathways where there was a high likelihood of gene-miRNA interactions. These pathways were predominantly associated with cancer and cell communication, including p53 signaling pathway, melanoma, glioma, prostate cancer, adherens junction, and focal adhesion. These data support the hypothesis that exercise affects the gene and miRNA expression pattern in the population of NK cells in the circulation and suggest mechanisms through which physical activity could alter health through the innate immune system.

Keywords: leukocytes, epigenetic, immune system, miRNA, cancer

WHETHER EXERCISE ALTERS NATURAL killer (NK) cell gene and microRNA (miRNA) expression was not known. The purpose of this study was to test hypotheses focused on genomics, specifically, gene expression and associated miRNA changes that might occur in the circulating population of NK cells following an intense bout of exercise. The study addresses key gaps in our understanding of how exercise influences the regulation of these dynamic cells (15% of the circulating lymphocyte population). Brief bouts of exercise can lead to large (4- to 5-fold) increases in the numbers of NK cells in the circulation. These

changes are larger than any other individual leukocyte cell type. NK cells are increasingly recognized to play key mechanistic roles in a wide variety of diseases, including innate immune recognition of pathogens (48), angiogenesis (22), and malignant cell surveillance (9). Whether the well-recognized positive health effects of exercise in many of these same diseases might be mediated through the NK cell population remains unknown.

miRNAs are short (~22 nt) noncoding RNAs that act on protein-encoding mRNAs, targeting them for translational repression or degradation (28). There is a small but growing literature showing that miRNAs do indeed play functional roles in NK cell gene expression and function, including NK cell development (5, 11), key receptor expression (15, 32), and cancer cell cytotoxicity (18). Moreover, Bezman et al. (4) outlined a critical role for miRNAs in controlling NK cell homeostasis and effector function, with implications for miRNAs regulating diverse aspects of NK cell biology. Consequently, we simultaneously determined both NK cell gene and miRNA expression following brief exercise in human participants and, using a previously published “intersecting” in silico analysis, identified gene pathways in circulating NK cells that were altered by exercise and possibly regulated through miRNAs.

There is mounting evidence that the alteration in population and composition of circulating NK cell subtypes is accompanied by change in NK cell function as well (7). Work from this and other laboratories supports the notion that exercise leads to activation of circulating immune cells that is similar in some ways, but unique in others, to immune cell activation that occurs in response to purely immunological exposures, such as lipopolysaccharide (44). We studied potential genomic and miRNA mechanisms in the circulating blood of young adults in response to a 20-min bout of heavy exercise. Emerging data demonstrate that NK cells are capable of genomic and functional modulation in response to a variety of stimuli and could play a key role in the cross talk among innate and adaptive immune cells (34); thus the NK cells are a potentially important vehicle through which exercise might modulate health.

MATERIALS AND METHODS

Participants. Thirteen healthy young men (20–29 yr old) participated in this study (Table 1). Due to RNA amount and quality issues, 12 volunteers were analyzed for gene expression and 11 for miRNA microarrays. The decision to include only men in this investigation was made primarily because we wanted, in these initial studies, to minimize possible confounding effects related to sex (e.g., menstrual cycle hormones, often difficult to account for precisely, that are known to influence stress and inflammatory responses). Elite athletes, individuals who participated vigorously in competitive sports, and potential volunteers with a history of any chronic medical conditions or medication use were excluded from participation. The Institutional Review Board at the University of California Irvine approved the study, and written, informed consent was obtained from all participants upon enrollment.

Research design. Subjects arrived at the Pediatric Exercise Research Center on two separate occasions. The first visit included anthropometric measurements, dual-energy X-ray absorptiometry scan, and a standardized aerobic fitness assessment. The second visit included an exercise challenge consisting of ten 2-min bouts of heavy (see below) cycle ergometer exercise at a constant work. Blood was drawn before and immediately after the exercise. Complete blood counts (CBC), plasma lactate levels, and circulating levels of plasma cytokines were measured. NK cells were isolated from peripheral blood mononuclear cells (PBMC) using a negative magnetic cell separation method and analyzed for gene and miRNA expression levels.

Anthropometric measurements. Standard calibrated scales and stadiometers were used to determine

height and body mass.

Measurement of fitness. Each volunteer performed a ramp-type progressive cycle ergometer exercise test using the SensorMedics metabolic system (Ergoline 800S, Yorba Linda, CA). After sitting comfortably without pedaling ("resting") on the cycle ergometer for 3 min and 1 min of unloaded pedaling, the work rate (WR) was incremented at 20–30 W/min, every minute, to the limit of the subject's tolerance. Participants were vigorously encouraged during the high-intensity phases of the exercise protocol. Gas exchange was measured breath by breath, and the anaerobic (lactate) threshold and peak O₂ consumption were calculated using standard methods.

Exercise protocol. At least 48 h, but not exceeding 10 days following the completion of the ramp test, each volunteer performed an intermittent-exercise protocol. This exercise regimen was designed to more closely mimic naturally occurring patterns of physical activity and has been used extensively over the years in our laboratory. The protocol consists of ten 2-min bouts of constant WR cycle ergometry, with a 1-min rest interval between each bout. The participants were instructed not to perform any type of vigorous physical activity 48 h before the exercise challenge. The WR was individualized for each subject and was calculated to be equivalent to the WR, corresponding roughly to 50% of the WR between the ventilatory anaerobic threshold, measured by the V-slope analysis, and the peak oxygen uptake (as determined noninvasively from the ramp-type test). Gas exchange was measured breath by breath during the entire exercise challenge. On average, this WR was equivalent to 77% of the participants' peak O₂ consumption.

Blood sampling and analysis. Participants arrived at the laboratory between 7:30 and 8:30 AM after an overnight fast. An indwelling catheter was inserted into the antecubital vein. A baseline sample was taken 30 min after the placement of the catheter and before the onset of exercise. We waited 30 min to ensure that measurable physiological parameters of stress (e.g., heart rate and blood pressure) were at baseline levels. The volunteers then completed the intermittent exercise protocol, and additional blood samples were obtained immediately after exercise. CBC for white blood cell analysis were obtained by standard methods from the clinical hematology laboratory. Plasma lactate was measured before and after exercise using YSI 2300 STAT Plus Glucose and Lactate Analyzer. Circulating levels of plasma cytokines were measured using commercially available enzyme-linked immunosorbent assay kits manufactured by R&D Systems (Minneapolis, MN) and included interleukin-2 (IL-2) [intra-assay coefficient of variation (CV) 2.0–4.3, interassay CV 2.7–5.0, sensitivity < 7 pg/ml] and IL-15 (intra-assay CV 3.4–5.3, interassay CV 6.6–9.9, sensitivity < 2 pg/ml).

NK cell isolation. The duration from blood draw to stabilization of RNA never exceeded 150 min. PBMCs were isolated from EDTA-treated blood using OptiPrep Density Gradient Medium (Sigma). NK cells were isolated from PBMC using a negative magnetic cell separation methods (Miltenyi Biotec Kit no. 130-092-657 and the autoMACS Pro Separator). The NK Cell Isolation Kit was developed to isolate NK cells from human PBMCs with minimum perturbation. Our NK cell isolation procedure consistently yielded an average of $91.8 \pm 0.02\%$ purification using flow cytometry (cd3⁻/cd56⁺).

RNA extraction. Total RNA for gene and miRNA analysis was extracted using TRIzol (Gibco BRL Life Technologies, Rockville, MD). For gene expression study only, RNA was purified using Qiagen-RNeasy Mini Kit. RNA pellets were resuspended in diethyl pyrocarbonate-treated water. RNA integrity was assessed (before beginning target processing) using Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). We only analyzed samples with RNA integrity number ≥ 8.8 .

Gene expression microarrays. Microarray processing was performed as recommended by the manufacturer and is available in the Affymetrix GeneChip Expression Analysis Technical Manual

(Affymetrix, Santa Clara, CA). Briefly, first-strand cDNA is synthesized from 250 ng of isolated total RNA using an oligo(dT) primer with an attached T7 promoter sequence. After making the complementary second strand, the double-stranded cDNA is used to generate biotin-tagged cRNA from an in vitro transcription (IVT) using T7 RNA polymerase. Fifteen micrograms of the resulting biotin-tagged cRNA was fragmented to an average strand length of 100 bases (range 35–200 bases) following prescribed protocols (Affymetrix GeneChip IVT 3'IVT Express Kit User Manual). Subsequently, 10 µg of this fragmented target cRNA were hybridized at 45°C with rotation for 16 h (Affymetrix GeneChip Hybridization Oven 640) to probe sets present on an Affymetrix U133 plus 2 arrays. The GeneChip arrays were washed and then stained (SAPE, streptavidin-phycoerythrin) on an Affymetrix Fluidics Station 450. Arrays were scanned using GeneChip Scanner 3000 7G and Command Console Software version 3.2.3 to produce *.CEL intensity files.

miRNA microarrays. One hundred nanograms of total RNA were labeled with the fluorescent dye Cyanine 3-pCp (Cy3) using the miRNA Labeling Reagent and Hybridization Kit (Agilent Technologies, Palo Alto, CA) following the manufacturer's protocol. Cy3-labeled RNA from each sample was hybridized to Agilent human miRNA version 2 microarray. The hybridized array was then washed and scanned according to Agilent specifications, and data were extracted from the scanned image using Feature Extraction version 10.2 (Agilent Technologies).

Data analysis. The results were analyzed using GeneSpring GX 10.0.2 software (Agilent Technologies).

Gene expression. Raw data were normalized using GC-RMA. Only probe sets that reached a signal value ≥ 50 in at least 100% of the values in any one out of the two conditions were included in the analysis. Overall, 17,313 of 54,675 probe sets represented on the array met these criteria. The microarray cell files and GC-RMA normalized data have been deposited in the GEO database, series accession number [GSE41915](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41915) (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41915>).

Traditional Student's paired *t*-test was first applied to each probe set, and fold change (FC; increase or decrease) > 1.5 and multiple testing correction (false discovery rate < 0.05) (Benjamini-Hochberg) procedure was carried out.

The final list of significantly changed probe sets was then additionally analyzed using the functional annotation tools provided by DAVID, the Database for Annotation, Visualization and Integrated Discovery (24, 25), to classify the genes into pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Only pathways with Expression Analysis Systematic Explorer (EASE) score ≤ 0.047 are presented in this analysis. EASE score is a modified Fisher exact *P* value in the DAVID system used for gene-enrichment analysis. EASE score *P* value = 0 represents perfect enrichment. *P* value ≤ 0.05 is considered as gene enrichment in a specific annotation category (http://david.abcc.ncifcrf.gov/helps/functional_annotation.html#summary).

miRNA microarrays. All raw signal values lower than 1 were adjusted to 1 and normalized using percentile shift (90th percentile). Only entities that had a present or marginal flag and passed the 20 percentile filtration in at least 100% of values in any one out of the two conditions were selected for further analysis. Overall, 240 out of 961 entities represented on the array met these criteria. The miRNA data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number [GSE41915](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41915) (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41915>). Traditional Student's paired *t*-test was first applied to each probe set, and FC (increase or decrease) ≥ 2 and false discovery rate (< 0.01) (Benjamini-Hochberg) procedure was carried out.

The final list of significantly changed miRNAs was then additionally analyzed using TargetScan

database, release 5.1 (provided by GeneSpring; <http://www.targetscan.org/>). We looked for the predicted target genes for each miRNA (context percentile = 50, conserved database). Briefly, TargetScan predicts gene targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region (positions 2–7 of a mature miRNA) of each miRNA.

An “intersecting” analysis of miRNA and gene expression level. See Table 4. Figure 1 presents the overall approach for the “intersecting” analysis. To identify genes and pathways that were potentially targeted specifically by the 23 miRNAs that had been significantly altered by exercise, we simultaneously analyzed the miRNA target genes and the 986 probe sets whose expression was shown to be affected. We then performed pathway analysis in this overlapping gene set that included miRNAs and their gene targets. We present only pathways with EASE score ≤ 0.05 . (N.B.: EASE score is a modified Fisher Exact P value in the DAVID system used for gene-enrichment analysis. An EASE score P value = 0 represents perfect enrichment; P value ≤ 0.05 was considered as significant gene-enrichment in a specific annotation category.)

(http://david.abcc.ncifcrf.gov/helps/functional_annotation.html#summary).

Gene and miRNA expression analysis by quantitative PCR (RT-PCR). For confirmation of gene expression and miRNA microarray expression findings, TaqMan assays were carried out on 11 subjects: 11 genes selected from cell communication pathways (e.g., leukocyte transendothelial migration, focal adhesion, antigen processing and presentation, and cytokine-cytokine receptor interaction) and five miRNAs that are known from the literature to play a role in the immune system function (e.g., miR-223, let-7e, miR-126, miR-363, and miR-29c).

The RT-PCR analysis was performed with the Applied Biosystems 7900HT PCR System by using TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression probes (Applied Biosystems) (ACTN1: assay ID, Hs00998100_m1; HLA-DPA1: assay ID, Hs01072899_m1; HLA-DRA: assay ID, Hs00219575_m1; FYN: assay ID, Hs00941600_m1; CXCR4: assay ID, Hs00976734_m1; IFNGR1 assay ID, Hs00988304_m1, CSF1R: assay ID, Hs00911250_m1, IL12RB2: assay ID, Hs01548202_m1, MYL9: assay ID, Hs00697086_m1, PTK2: assay ID, Hs01056457_m1, TNFSF4: assay ID, Hs00967195_m1, and VPS37B: assay ID, Hs01091832_m1). Actin beta was used as an endogenous control. For miRNA expression, we used Assays-on-Demand miRNA probes (Applied Biosystems) (miR-223: assay ID, 002295, let-7e: assay ID, 002406, miR-126: assay ID, 002228, miR-363: assay ID, 001271, and miR-29c: assay ID, 000587). RNU44 was used as an endogenous control.

NK cells subpopulations determined by flow cytometry. For NK cell enumeration, circulating levels of NK cells were identified using flow cytometry, as described by Timmons and coworkers earlier (54). Briefly, CD56^{bright} and CD56^{dim} cell populations were derived from lymphocyte event-gating based on forward- vs. side-scatter characteristics. Briefly, CD3 APC (total T cells) and CD56-PE-CY5 (NK cells) were used to identify NK cell population. Samples were acquired and analyzed using a C6 flow cytometer and C6-plus software (Becton Dickinson, San Jose, CA). A total of 100,000 events were collected in the lymphocyte gate based on forward- vs. side-scatter characteristics, and a dot plot of CD3 and CD56 fluorescence was created from events within the lymphocyte gate. Total NK cell counts of each NK cell subset were calculated by multiplying the percentage of cells with appropriate fluorescence with the adjusted absolute lymphocyte count.

Physiological data analysis. The physiological data are presented as mean and SE. Two-sided paired t -test was applied for testing changes from before to after the exercise, and the significance level was set at 0.05.

RESULTS

Anthropometric and physiological characteristics. The anthropometric and physiological characteristics of the 13 subjects appear in [Table 1](#).

Plasma lactate. The exercise bout caused a mean increase of 10.2 ± 0.6 mmol/l in lactate levels in the plasma [before (1.3 ± 0.06 mmol/l) vs. after ($11.5.0 \pm 0.7$ mmol/l), $P = 1.9\text{E-}09$].

Plasma cytokine levels. Circulating levels of IL-15 were found to be slightly but significantly elevated after exercise (+8%) with no significant change on the expression level of IL-2 ([Fig. 2](#)).

Leukocyte response to exercise. As shown in [Table 1](#), the number of lymphocytes detected by CBC and NK cells detected by flow cytometry was significantly elevated at peak exercise ($P < 4.05\text{E-}04$). As expected, both dim and bright NK cell populations significantly increased by exercise (dim $442 \pm 75\%$ change, bright $244 \pm 79\%$ change), with a greater increase of the dim NK population (dim-to-bright ratio went up following exercise, 21.4 to 40.0).

Effects of exercise on NK gene expression. Our intermittent exercise protocol altered the expression level of 986 genes in the population of NK cells in the circulating blood. Twenty-three KEGG pathways ([Table 2](#)) were enriched with genes whose expression was altered by exercise (EASE < 0.05). Many of the pathways are related to cancer, cell communication, and inflammation ([Table 2](#)).

The effects of exercise on NK cell miRNA expression. The intermittent exercise protocol altered the expression level of 23 miRNAs [11 had higher expression ([Table 3](#))]. In silico analysis showed that 21 miRNAs had potential target genes (2 miRNAs did not have any known target genes).

Intersecting analysis of miRNA and gene expression level. See [Fig. 1](#). Intersecting analysis of gene and miRNA expression showed evidence of possible miRNAs regulation of seven pathways ([Table 4](#)) that are related to cancer and cell communication. The complete list of all 21 miRNAs and their potential interaction with the genes that were altered by exercise in this study can be found in Supplemental Table S1. (The online version of this article contains supplemental data.)

In [Table 4](#), we present five miRNAs and we show the following: 1) miRNAs that were affected by exercise; 2) the number of potentially targeted genes for each affected miRNA (in silico analysis); and 3) from the genes identified in no. 2, we show those genes that we found also to have actually been affected by exercise. KEGG pathway analysis of these sets of genes showed seven significant pathways that are related to cancer and cell communication: 1) p53 signaling pathway; 2) melanoma; 3) glioma; 4) focal adhesion; 5) prostate cancer; 6) pathways in cancer; and 7) adherens junction.

RT-PCR verification of specific genes and miRNAs. We used TaqMan assays to verify the expression level changes from before to after exercise of 11 genes ([Table 5](#)) and five miRNAs. The five miRNAs included the following: miR-29c (FC = 1.3, $P = 0.015$) and miRNA 363 (FC = 1.3, $P = 0.0005$), which were upregulated by exercise; and miR-126 (FC = 2.4, $P = 0.0006$), miR-223 (FC = 1.6, $P = 0.002$), and let 7e (FC = 1.5, $P = 0.008$), which were downregulated. TaqMan assays results (FC and significance) matched well with the microarray results for both gene expression ([Table 5](#)) and the miRNAs.

DISCUSSION

In this study, we showed for the first time that a relatively brief bout of moderate exercise affects both gene and miRNA expression pattern in circulating NK cells. Using a powerful tool, the microarray technology, which enabled us to analyze thousands of genes and hundreds of miRNAs simultaneously,

we found gene pathways in circulating NK cells that were altered by exercise and possibly regulated through miRNAs. Interestingly, the seven pathways that were identified are related mainly to cancer and cell communication: p53 signaling pathway, melanoma, glioma, prostate cancer, pathways in cancer, adherens junction, and focal adhesion. To the best of our knowledge, the pattern of changes in gene expression in the circulating population of NK in response to exercise cells is unique and does not simply reflect a more global stress response (36).

Study limitations. Although we found significant effects of exercise on gene and miRNA expression in the circulating pool of NK cells, we cannot determine from the present study whether such changes resulted directly from exercise on these cells, or, alternatively, from trafficking of NK cells located outside the circulation (3), where their gene and miRNA expression patterns were already different. While there is evidence that some specific exercise-associated factors (e.g., changes in pH, lactate, temperature, inflammatory mediators) can induce gene expression changes in NK cells in vitro (29, 37, 38), there have been, to the best of our knowledge, no studies in NK cell model systems in vitro or in vivo examining the collective impact of potential cell-stress exercise factors per se. An additional potential confounding factor is that, while the immediate effect of relative brief and intense exercise is to increase the number of NK cells, more prolonged and vigorous exercise may lead to a reduction in the number of NK cells (47, 56).

It would be difficult, if not impossible, in human subjects to access marginal NK cell pools (e.g., NK cells residing in the pulmonary circulation) and then directly test the impact of exercise on the redistribution of the cells and their gene and miRNA expression profiles. What is clear is that relatively brief exercise, patterned after naturally occurring physical activity, can substantially change the gene and miRNA expression patterns of NK cells in the circulation. How these changes influence the subsequent gene and miRNA expression patterns and redistribution of circulating NK cells during the recovery from acute exercise is not known. It is intriguing that Shakhar et al. (46) found in murine models that NK cells residing in the pulmonary circulation [likely to be released to the central circulation with exercise (1)] responded differently than NK cells residing in the peripheral circulation to immunostimulation and to modulation by stress hormones. In these studies, the pulmonary NK cells were more effective than the circulating NK cells at killing malignant cells in in vivo experimental models, an observation recently corroborated by Takeda et al. (51). Thus an exercise-associated shift in NK cells (or other leukocytes) from marginal pools, in which the NK cell gene expression and functional profile differ from the profiles in the circulation, might prove to be as effective a biological mechanism as a direct effect of exercise on the gene and functional profile of the circulating cells themselves.

NK cells are sensitive to behavioral stress factors (2). Exercise leads to increases in mediators, such as epinephrine, growth hormone, and cortisol, which do accompany behavioral stress, but exercise also is accompanied by distinct and unique physiological changes not seen in behavioral stress, like prolactin, and other factors that have immunomodulatory effects (20, 56). Indeed, we found a small increase in circulating IL-15, but not IL-2, inflammatory-related cytokines known to stimulate NK cells (6, 40). The changes in IL-15 concentration were remarkably consistent, but, nonetheless, could have been influenced by hemoconcentration. Most research in this area has focused on prolonged exercise (13), and more recent work suggests that estimating plasma volume changes solely on erythrocyte concentration variables may not be fully accurate (50). The impact of brief exercise on plasma volume remains unclear.

The immediate systemic, cell-stress related, physiological effects of brief exercise include the following: reduction in pH, increases in inflammatory cytokines, increase in temperature, release of catecholamines and stimulation of the sympathetic nervous systems, and increased turbulent flow

within the circulation (19). How exercise "stress" alters NK cell gene expression and function differently than behavioral "stress" is still unknown.

NK cell subtype (phenotype) and changes in NK cell gene expression. How might the exercise-associated change in the distribution of the NK cell phenotypes within the circulation impact overall NK cell gene expression? As noted above, distinct NK cell subsets have diverse functions. CD3-CD56^{dim} cells are more cytotoxic than CD3-CD56^{bright} cells. In contrast, the "bright" NK cells (~10% of the circulating NK cells) produce abundant cytokines (12). Moreover, there is growing evidence that NK cell subsets differ in both gene and protein expression (21, 57). CD56^{dim} cells have higher gene expression of cytolytic molecules than CD56^{bright} subsets (with the exception of Granzyme K), whereas expression of molecules involved in adhesion, migration, and cell-to-cell cross talk is generally higher in the CD56^{bright} subset. Different gene and protein expression in the NK cell subpopulation in the unstimulated state is particularly relevant from a physical activity perspective, considering that both subsets are mobilized into the peripheral circulation with acute exercise.

As noted in previous reports (53), both dim and bright NK cell populations significantly increased by exercise in our study (dim $442 \pm 75\%$ change, bright $244 \pm 79\%$ change), with a greater increase of the dim NK population (dim-to-bright ratio went up following exercise, 21.4 to 40.0). Consistent with this change in the relative proportion of dim and bright NK cells in the central circulation, we found that the pattern of change in gene expression of known NK genes was consistent with the change in the ratio of these NK cell phenotypes. For example, expression of killer cell immunoglobulin-like receptors (KIRs), KIR2DL4, KIR2DS3, and KIR2DS4, increased after exercise, consistent with a greater ratio of dim to bright NK cells. In addition, we found that genes with higher expression at rest in the NK bright population were downregulated following exercise (e.g., *CXCR3*, *IL7R*, *CCR7*, *GZMK*), again, in line with the change in the ratio of these cells in the circulation following exercise. Based on these findings, we speculate that some of the gene expression changes we observed might be mediated by distributional shifts in NK cell subsets.

NK cell and exercise. Previous studies from this and other laboratories have shown that gene expression changes in response to exercise in the circulating population of PBMCs and neutrophils (43, 44). The gene expression changes are accompanied by alterations in the expression of miRNAs as well (45). As noted, circulating NK cells show the greatest proportional increases in response to exercise. This sensitivity to physiological stress, combined with their role in innate immune defense, suggests that these cells may mediate a link between regular physical activity and overall health status. This is the first study to look at the NK cell genomic response to exercise.

KIR receptors are considered to be the key receptors that control the development and function of human NK cells (35). Only a few studies have examined the effect of exercise on KIR gene expression patterns in general, and the data are restricted to PBMCs only (8). In this study, we show for the first time specific upregulation of three KIR activation receptors, KIR2DL4, KIR2DS3 and KIR2DS4, in the circulating NK population in response to exercise, suggesting potential subsequent impact of exercise on NK cell function.

miRNA and NK cells. We have discovered that, in the larger population of PBMCs, brief heavy exercise was sufficient to change the expression pattern of miRNAs (42). Using the same in silico intersecting analysis that we employed in the present study of NK cells, we identified PBMC gene pathways (e.g., MAPK signaling pathway) in which regulation by miRNA was possible. When exercise-induced miRNA expression in PBMCs was compared with neutrophils (45), we found that, of the 34 miRNAs affected in PBMCs and of the 38 in neutrophils, only 6 miRNAs changed similarly. Thus it is essential to examine

the miRNA and gene expression response to exercise in the individual leukocyte cell types.

In the present study, the “intersecting” in silico analysis identified seven unique gene pathways that were possibly regulated through miRNAs. The specific pathways, as noted, are related mainly to cancer and cell communication. Only the adherens junction pathway was in common with the PBMC analysis, and no pathways were in common with observations we made earlier in neutrophils. Thus the impact of exercise on miRNA expression in NK cells is unique to that cell type.

NK cells and cancer. The mechanisms through which physical activity modulates cancer risk [now generally accepted in breast and colon cancer ([30](#), [41](#))] is not known, but we speculated that finding exercise alterations in NK cell gene expression might lead to a series of useful and testable hypotheses. The ability of NK cells to kill tumor cells without the need to recognize tumor-specific antigens and the relative ease of NK cell collection and isolation have rendered these cells appealing as potential effectors for cancer immunotherapy ([39](#)). NK cell recognition of targets is regulated through a balance of activating and inhibitory signals. They can induce tumor apoptosis via the perforin/granzyme pathway or through death receptor ligands. Such ligands include tumor necrosis factor-related, apoptosis-inducing ligand (TRAIL) or Fas ligand ([31](#), [49](#)). Years ago, MacNeil and Hoffman-Goetz ([33](#)) showed that splenic NK cell activity was elevated in a group of wheel-running mice compared with sedentary controls 3 wk after cessation of exercise. The exercised mice displayed lower tumor cell retention in the lungs relative to nonexercised mice with no difference in tumor incidence.

In the present study, we detected a 1.9-fold decrease in the expression of GZMK, which is a cell-death inducing serine protease that has trypsin-like protease activity ([14](#)). While we did not detect a significant change in the expression level of the TRAIL or Fas ligands, we did find gene expression alteration in other, functionally related tumor necrosis factor (TNF) family members, e.g., a 2.1-fold increase in TNFAIP3, an inhibitor of programmed cell death playing a role in the function of the lymphoid system, a 2-fold decrease in TNFSF13B, and a 3.2-fold decrease in TNFSF4, which can stimulate cytokine production. Thus our data do point toward possible NK cell-related mechanisms that might explain how physical fitness acts to prevent certain malignancies.

As noted, our intersecting analysis identified significant exercise-associated changes in gene pathways (see [Table 4](#)) that have predominantly been found within tumor cells themselves and not in innate immunity, cytotoxic cells like the NK cell. It is intriguing that each of the cancer-related pathways that we identified in our intersecting analysis plays a role in the interaction between tumor cells and potential cytotoxic cells, such as the NK cells. For example, p53 signaling pathway and pathways in cancer involve mechanisms that alter apoptosis or cellular senescence within tumor cells ([16](#)). Moreover, immune genes are epigenetically silenced in tumor cells, and Khan et al. ([27](#)) have suggested that reversing these effects could potentially be used to develop therapeutic vaccines in melanoma. The conversion of cancer cells to antigen presenting cells, or, alternatively, rendering innate immune cells, such as NK cells, more effective in the tumor recognition process might also benefit therapy ([17](#)). Indeed, NK cells are showing promise as adoptive cell transfer therapies for malignant glioma ([26](#)), prostate cancer ([52](#)), and melanoma ([10](#)). How the exercise alteration of the expression of these pathways within NK cells might influence the role of NK cells in tumor suppression remains unknown.

Implications of this study for “exercise is medicine”. There is a growing understanding of the profound role that exercise plays in disease therapy and prevention ([23](#)). Our approach provides new insights into the complex molecular mechanisms of the exercise-induced stress response and modulation of immune function. Moreover, NK cells in particular are seen to play substantial roles in cancer surveillance ([55](#)). The genomic and epigenetic mechanisms through which exercise alters the

function of key immune mediators like NK cells may ultimately prove beneficial in optimizing the clinical exercise prescription or in uncovering pathways that can contribute directly to the development of new therapies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.R.-A. and D.M.C. conception and design of research; S.R.-A. and F.P.Z. performed experiments; S.R.-A., F.P.Z., and F.H. analyzed data; S.R.-A., F.P.Z., F.H., and D.M.C. interpreted results of experiments; S.R.-A., F.H., and D.M.C. prepared figures; S.R.-A. and D.M.C. drafted manuscript; S.R.-A., F.H., and D.M.C. edited and revised manuscript; S.R.-A., F.P.Z., F.H., and D.M.C. approved final version of manuscript.

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Figures and Tables

Table 1.

Anthropometric characteristics and exercise responses of the 13 subjects

	Mean ± SE
Age, yr	24 ± 0.9
Height, cm	174.2 ± 2.8
Body mass, kg	73.2 ± 2.8
BMI, kg/m ²	24.1 ± 0.6
Peak $\dot{V}_{O_2}$, ml·kg ⁻¹ ·min ⁻¹	48.2 ± 2.7
Average $\dot{V}_{O_2}$ during the constant work rate as percentage of peak $\dot{V}_{O_2}$	76.6 ± 2.7
Lymphocytes, %change from before to after exercise, (<i>P</i> = 1.4E-06)	171 ± 30
NK cells, %change from before to after exercise (<i>P</i> = 4.05E-04)	432.5 ± 74

BMI (body mass index); peak $\dot{V}_{O_2}$, peak oxygen uptake; NK, natural killer.

Fig. 1.

An intersecting analysis of the specific natural killer (NK) microRNAs and genes whose expression was significantly altered by exercise and genes whose expression was also significantly altered by exercise. This approach identified six significant pathways and provided direction for further investigation of how microRNAs altered by exercise regulate NK cells function.

Fig. 2.

Effect of brief exercise on circulating interleukin (IL)-2 and IL-15 in the sample population. The figure shows individual responses before (Pre) and after (Post) the exercise bout. There was a significant increase in IL-15 but not in IL-2 ($P = 0.0001$ using paired t -test).

Table 2.

Twenty-three gene expression KEGG pathways altered by exercise in NK cells

KEGG Pathway	No. of Genes	EASE Score
Systemic lupus erythematosus	18	4.60E-06
Antigen processing and presentation	14	1.70E-04
Intestinal immune network for IgA production	9	2.30E-03
Asthma	7	2.40E-03
Gap junction	12	3.80E-03
Focal adhesion	20	4.10E-03
Viral myocarditis	10	7.30E-03
Allograft rejection	7	7.30E-03

Hematopoietic cell lineage	11	8.80E-03
Graft-versus-host disease	7	1.10E-02
Prostate cancer	11	1.10E-02
Type I diabetes mellitus	7	1.50E-02
Regulation of actin cytoskeleton	19	1.70E-02
Pathogenic <i>Escherichia coli</i> infection	8	2.00E-02
Vascular smooth muscle contraction	12	2.00E-02
Melanoma	9	2.20E-02
Leukocyte transendothelial migration	12	2.90E-02
Cytokine-cytokine receptor interaction	21	3.10E-02
Glioma	8	3.30E-02
Adherens junction	9	3.40E-02
Autoimmune thyroid disease	7	3.70E-02
Chemokine signaling pathway	16	4.00E-02
p53 signaling pathway	8	4.70E-02

KEGG, Kyoto Encyclopedia of Genes and Genomes; EASE, Expression Analysis Systematic Explorer.

Table 3.

Twenty-three microRNAs altered by exercise in NK cells

microRNA	Fold Change	Direction	P Value	FDR
hsa-let-7e	2.6	Down	0.00853	
hsa-miR-126	3.2	Down	0.00884	
hsa-miR-126*	2.9	Down	0.00524	
hsa-miR-130a	2.9	Down	0.01147	
hsa-miR-151-5p	2.8	Down	0.00435	
hsa-miR-199a-3p	3.1	Down	0.00903	
hsa-miR-199a-5p	2.9	Down	0.01292	
hsa-miR-221	2.1	Down	0.00324	
hsa-miR-223	2.9	Down	0.00506	
hsa-miR-326	2.3	Down	0.00524	
hsa-miR-328	2.3	Down	0.00103	
hsa-miR-652	2.2	Down	0.00466	
hsa-miR-142-3p	3.0	Up	0.00126	
hsa-miR-142-5p	2.5	Up	0.00161	
hsa-miR-192	2.2	Up	0.00299	
hsa-miR-29a	2.0	Up	0.00253	
hsa-miR-29b	3.5	Up	0.00096	
hsa-miR-29c	2.3	Up	0.00147	
hsa-miR-30e	2.1	Up	0.00076	
hsa-miR-338-3p	2.2	Up	0.00103	

hsa-miR-363	2.1	Up	0.00191
hsa-miR-590-5p	2.6	Up	0.00094
hsa-miR-7	2.2	Up	0.00383

FDR, false discovery rate.

Table 4.

Summary of key findings: association between microRNA and gene expression in response to brief exercise in human NK cells and gene pathways that were significantly affected

microRNA (Direction/Fold Change)	No. of Target Genes	Genes Affected by Exercise 1) Known To Be Targeted by the Specific miRNA; and 2) Whose Expression Level Was Altered by Exercise	Common Genes Enriched in KEGG Pathways EASE ≤ 0.05
hsa-let-7e ↓2.6	566	26 Genes: WASL(↓),TAF9B(↓), PMAIP1 (↓), CREM(↓), MGAT4A(↓), THBS1 (↑), PGRMC1(↑), CDKN1A (↑), IGF2BP3(↑), NAP1L1(↑), ITGB3, XK(↑), NAP1L1(↑), SNAP23(↑), PTPRO(↑), FRMD4B(↑), NAP1L1(↑), ITGB3(↑), SNN(↑), PLEKHO1(↑), NLK(↑), MST150(↑), MGC13057(↑), ANKRD28(↑), KLHL6(↑), MGC13057(↑), ANKRD28(↑)	p53 signaling pathway
hsa-miR-363 ↑2.1	555	54 Genes: ATRX(↓), BCL11B(↓), BTG2(↓), C10orf118(↓), CD69(↓), CDK5R1(↓), CHST7(↓), CUGBP2(↓), DDIT4(↓), DDX3X(↓), DNAJB9(↓), DUSP10(↓),FNDC3B(↓), FOSL2(↓), JMY(↓), JOSD1(↓), KIAA1128(↓), MEF2D(↓), NUFIP2(↓), PAPD5(↓), PHF3(↓), PTGER4(↓), RAB8B(↓), SERTAD3(↓), SMAD7(↓), TAGAP(↓), TBL1XR1(↓), TGIF1(↓), TMCC3(↓), TOB1(↓), TOB2(↓), WASL (↓), CDKN1C(↑), CXCL5(↑), FSTL1(↑), LMO2(↑), MMD(↑), MPP1(↑), MSRB3(↑), NLK (↑), PRKAR2B(↑), PTPRO(↑), RNF11(↑), SNN(↑), VCL (↑), ZFYVE21(↑)	Adherens junction
hsa-miR-29a ↑2.0	614	39 Genes: MCL1(↓), CCND2 (↓), BTG2(↓), JOSD1(↓), PDGFRB (↓), TRIB2(↓), SLC2A3(↓), TNFAIP3 (↓), LARP4(↓), AKAP13(↓), BAT2D1(↓), PIK3R1 (↓), DDX3X(↓), AGPAT4(↓), BCL11B(↓), CDK6 (↓), HDAC4(↓), AKAP5(↓), SPTY2D1(↓), HELZ(↓), NASP(↓), KIAA1128(↓), TACC1(↓), C16orf72(↓), MGAT4A(↓), CSDA(↑), C5orf13(↑), IFI30(↑), TPM1(↑), FSTL1(↑), NCOA4 (↑), RHOBTB1(↑), SPARC(↑), MAFB(↑), PDGFC (↑), C19orf22(↑), GNB4(↑), SESTD1(↑), C4orf32(↑)	Melanoma, glioma, focal adhesion, prostate cancer, pathways in cancer. OMIM: common variants at CD40 and other loci confer risk of rheumatoid arthritis
hsa-miR-29b ↑3.5	615	39 Genes: MCL1(↓), CCND2 (↓), BTG2(↓), JOSD1(↓), PDGFRB (↓), TRIB2(↓), SLC2A3(↓), TNFAIP3 (↓), LARP4(↓), AKAP13(↓), BAT2D1(↓), PIK3R1 (↓), DDX3X(↓), AGPAT4(↓), BCL11B(↓), CDK6 (↓), HDAC4(↓), AKAP5(↓), SPTY2D1(↓), HELZ(↓), NASP(↓), KIAA1128(↓), TACC1(↓), C16orf72(↓), MGAT4A(↓), CSDA(↑), C5orf13(↑), IFI30(↑), TPM1(↑), FSTL1(↑), NCOA4 (↑), RHOBTB1(↑), SPARC(↑), MAFB(↑), PDGFC (↑), C19orf22(↑), GNB4(↑), SESTD1(↑), C4orf32(↑)	Melanoma, glioma, focal adhesion, prostate cancer, pathways in cancer. OMIM: common variants at CD40 and other loci confer risk of rheumatoid arthritis
hsa-miR-29c ↑2.3	613	40 Genes: MCL1(↓), CCND2 (↓), BTG2(↓), JOSD1(↓), PDGFRB (↓),	Melanoma, glioma,

TRIB2(↓), SLC2A3(↓), TNFAIP3 (↓), LARP4(↓), AKAP13(↓), BAT2D1(↓), PIK3R1 (↓), DDX3X(↓), AGPAT4(↓), BCL11B(↓), CDK6 (↓), CSNK1G1(↓), HDAC4(↓), AKAP5(↓), SPTY2D1(↓), HELZ(↓), NASP(↓), KIAA1128(↓), TACC1(↓), C16orf72(↓), MGAT4A(↓), CSDA(↑), C5orf13(↑), IFI30(↑), TPM1(↑), FSTL1(↑), NCOA4 (↑), RHOBTB1(↑), SPARC(↑), MAFB(↑), PDGFC (↑), C19orf22(↑), GNB4(↑), SESTD1(↑), C4orf32(↑)	focal adhesion, prostate cancer, pathways in cancer. OMIM: common variants at CD40 and other loci confer risk of rheumatoid arthritis
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Bolded genes are those associated with specific pathways.

Table 5.
Comparison of microarray and RT-PCR gene expression data

Gene Symbol	Gene Name	P Value	RT-PCR FC	Microarrays FC
<i>PTK2</i>	PTK2 protein tyrosine kinase 2	3.53E-05	↓2.5	↓2.4
<i>ACTN1</i>	Actinin, α1	1.17E-05	↓2.9	↓3.0
<i>MYL9</i>	Myosin, light chain 9, regulatory	1.57E-05	↓3.9	↓3.8
<i>CSF1R</i>	Colony stimulating factor 1 receptor	1.60E-02	↓1.5	↓1.7
<i>HLA-DRA</i>	Major histocompatibility complex, class II, DR α	3.90E-03	↓1.8	↓2.3
<i>HLA-DPA1</i>	Major histocompatibility complex, class II, DP α1	5.80E-03	↓1.4	↓1.6
<i>TNFSF4</i>	Tumor necrosis factor (ligand) superfamily, member 4	2.84E-05	↓2.8	↓2.4
<i>CXCR4</i>	Chemokine (C-X-C motif) receptor 4	3.01E-07	↑2.5	↑1.7
<i>IFNGR1</i>	Interferon-γ receptor 1	2.00E-04	↑2.0	↑1.5
<i>IL12RB2</i>	Interleukin 12 receptor, β2	5.03E-02	↑1.4	↑1.5
<i>VPS37B</i>	Vacuolar protein sorting 37 homolog B (<i>S. cerevisiae</i>)	1.31E-03	↑2.3	↑1.8

FC, fold change.