Exercise training effects on natural killer cells: a preliminary proteomics and systems biology approach

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ABSTRACT

Background: Regular exercise, particularly moderate-intensity continuous training (MICT), can improve immune function. Natural killer (NK) cells, a subset of lymphocytes that react to infections, are the most responsive innate immune cells to exercise, but the mechanisms underlying this are poorly understood. A type of exercise training that is gaining popularity in recent years is high-intensity interval training (HIIT), but how it affects NK cells is largely unknown. In fact, intense exercise has been traditionally viewed as a potential stressor to immune homeostasis. The purpose of this study was to determine in healthy, previously untrained adults (N=8 [3 male; 40±6 years]) the effects of an intervention consisting of 4-week MICT followed by 4-week HIIT on NK cells as compared with a pre-training (baseline) state.

Methods: Participants were studied at three time points: baseline, mid-intervention (after MICT), and post-intervention (after HIIT). Main assessments included cytotoxicity assays, flow-cytometry analysis of NK cell surface markers, and interrogation of the cellular proteome using a systems biology approach.

Results: A significant time effect was found for NK cell cytotoxicity ($p<0.001$), which was increased ~10-fold at both mid- and post-intervention versus baseline. No significant intervention effect was found for NK surface receptor expression, except for CXCR3 determined as mean fluorescence intensity ($p=0.044$, although with no significant differences in post hoc pairwise comparisons).

The proteins showing a higher differential expression (Log2 fold-change>10 and false discovery rate [FDR] q-value<0.001) were COP9 signalosome subunit 3 (COPS3), DnaJ heat shock protein family member B11 (DNAJB11), histidyl-TRNA synthetase 1 (HARS), NIMA related kinase 9 (NEK9), nucleoporin 88 (NUP88), phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1), regulator of chromosome condensation 2 (RCC2), TAO kinase 3 (TAOK3), transducin beta like 2 (TBL2), and ring finger protein 40 (RNF40). All were upregulated at mid-intervention compared with baseline, with the exception of HARS, which was downregulated. Four enriched pathways (FDR <25%) were found: two related to transmembrane transport and cellular composition (downregulated at mid-intervention vs baseline), and two related to oxidation-reduction reactions (upregulated at post-intervention versus baseline).

Conclusions: A progressive exercise intervention of MICT followed by HIIT induces a remarkable improvement in NK function compared with the untrained state, although at the mechanistic level the pathways involved seem to differ over time during the intervention.

Keywords: immune system; high interval training; NK cell; cytotoxicity.

INTRODUCTION

Regular exercise, especially (but not only) if performed at low-moderate workloads (termed 'moderate-intensity continuous training' [MICT], typically ≥ three sessions/week of ≥ 30-minute duration at ~60 to 80% of peak oxygen uptake [VO_2peak] during several weeks), induces robust biological adaptations that positively impact human health, as reflected by an improvement in cardiorespiratory (or 'aerobic' fitness) indicators. The benefits of MICT may also extend to the immune system, particularly (but not only) the innate arm (73). Among the innate immune cell subtypes that can be potentially receptive to exercise, the evidence is especially strong for an exer-

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cise-induced mobilization of natural killer (NK) lymphocytes to the circulation, which might be accompanied by an improved cytotoxicity (or 'killing capacity') of these cells (28).

MICT has been shown to increase NK cytotoxic activity in preclinical models (13, 34) and human studies (for a review see Zimmer et al (80)); for instance, in women with mild obesity (45), in patients (52) or survivors of breast cancer (15), in patients with stomach cancer after curative surgery (38), or in children with pediatric cancer undergoing hematopoietic stem cell transplantation (9). Improvements have also been reported after resistance training in older women (35) or in women recovering from breast cancer (22). In addition, some cross-sectional studies have reported that NK cytotoxicity is greater in young/old athletes (36), trained marathon runners (40) and cyclists (40), female elite rowers (44), and endurance-trained older women (67 to 85 years) (42) than in their non-athletic/sedentary peers. And in general, NK cell cytotoxicity has been shown to be higher among older people who are active than in their less active age-matched controls (27, 61, 76). In other studies, however, no improvement was reported in patients with breast cancer after MICT alone (41) or combined with a healthy eating intervention (56), or in older women after 12-week MICT (42). Furthermore, 3 months of training with multicomponent exercises (strength, balance, walking, stretching) of gradually increasing intensities reduced NK cytotoxic activity in frail older adults (54) and one month of heavy exercise training during the pre-competition season had a similar effect in female volleyball players (65).

The underlying mechanisms by which MICT may increase NK number or function remain to be clearly elucidated. One pre-clinical study using tumor-bearing mice found that exercise training (wheel running) for six weeks induced the mobilization of NK cells and increased their infiltration into tumors (51). Mechanistic analyses revealed that NK cell mobilization to the circulation was dependent on epinephrine, the hormonal effector of the 'fight-or-flight' response that is activated during exertion, whereas tumor homing of NK cells was dependent on interleukin (IL)-6, a myokine released from contracting muscles. Although exercise did not enhance NK cell cytotoxicity per se, it 'prepared' the tumor environment for their infiltration by enhancing the expression of ligands for some NK cell-activating receptors (e.g., NKG2D and NKp46). This is consistent with human studies reporting acute transcriptomic (53) or epigenetic modifications (79) in NK cells after a single exercise session in humans, although a recent study found that a 12-week resistance training intervention failed to induce significant changes in the NK cell transcriptome of patients with breast cancer undergoing adjuvant therapy (48).

A type of exercise training that is gaining popularity in Western societies is high-intensity interval training (HIIT), because it is thought to stimulate aerobic fitness and muscle molecular adaptations that are comparable (if not superior) to those elicited by MICT despite a lower time commitment (i.e., lower total exercise volume) (19). The HIIT training model typically involves short, repeated bouts of intense effort (e.g., fast running or intensive bicycling), interspersed with short recovery periods (each lasting a few minutes or less). There is a paucity of data about the effects of HIIT on immune function, especially on NK cells, and the chronic effects of this training modality are unknown. Yet, this is an interesting question in light of the ongoing debate regarding the potential immunosuppressive effects of intense exercise training (of which HIIT is a good example), versus the documented beneficial effect of MICT for host immune defense (61). To the best of our knowledge, only one study found that an acute session of HIIT (performed at the end of a 6-week intervention of this training modality) increased NK cell count and activation (as assessed by the percentage of circulating CD56+CD3−NK cells) compared with pre-session levels in women with overweight/obesity (n=3) (4). It thus remains to be determined whether the effects of MICT on NK function are altered with the addition of subsequent vigorous training sessions (e.g., HIIT). An acute session of MICT (80% of VO_{2peak}) induced a rise in NK cell activity in well-trained young men that was not corroborated at lower intensities (50% of VO_{2peak}) (43). Yet, how this result translates to higher ('HIIT-like') intensities, especially with regard to chronic (i.e., training-induced) rather than acute effects, is unknown. One way to address this is to conduct a study where the same participants are assessed longitudinally along different training states. This might help to draw more conclusions about the effects of exercise training on NK function, particularly in light of the dearth of comparative studies currently available and the heterogeneity among them (particularly with regard to model [preclinical or human], participants' characteristics such as age and training status, or type of training programs). Mechanistically, proteomic analysis might provide important information on causal links as the proteome reflects the interaction of both inherent (genetics) and environmental factors in the responses and adaptations of tissues and cells to different stimuli (3). In addition, systems biology can help to understand the protein networks involved (55).

The purpose of the present study was to determine, in healthy adults, the effects of an exercise training intervention of progressive intensities (MICT followed by HIIT) on NK cells compared to a pre-training (baseline) state, using an integrative proteomics and systems biology approach.

METHODS

Participants and experimental design

The study was approved by the local ethics committee and was performed during 2018 in accordance with the Declaration of Helsinki. Inclusion criteria were as follows: (i) middle-aged (30–50 years) man/woman; (ii) not diagnosed with any major cardiorespiratory or systemic disease contraindicating exercise; (iii) not taking any medication; (iv) not having an infectious condition; and (v) not having performed structured exercise training (< 2 sessions/week of < 30 minutes) or practiced regularly any sport in the last 3 months. Of the 20 potentially eligible subjects originally contacted (University staff at two Faculties of the Universidad Europea de Madrid [UEM]), nine subjects meeting all the aforementioned criteria agreed to participate. One subject declined to continue in the study despite performing all the baseline assessments because he changed jobs and moved to a different city. Thus, eight apparently healthy individuals (3 male, 5 female; mean ± standard deviation [SD] age: 40 ± 6 years [range: 32, 50]; body mass index: 24.0 ± 2.1 $kg \cdot m^{-2}$) were finally enrolled in the study.

All the participants were assessed at three time points: (i) at baseline (untrained); (ii) after a 4-week MICT phase (mid-intervention); and (iii) after a subsequent 4-week HIIT phase (post-intervention). The study design is summarized in **Figure 1**.

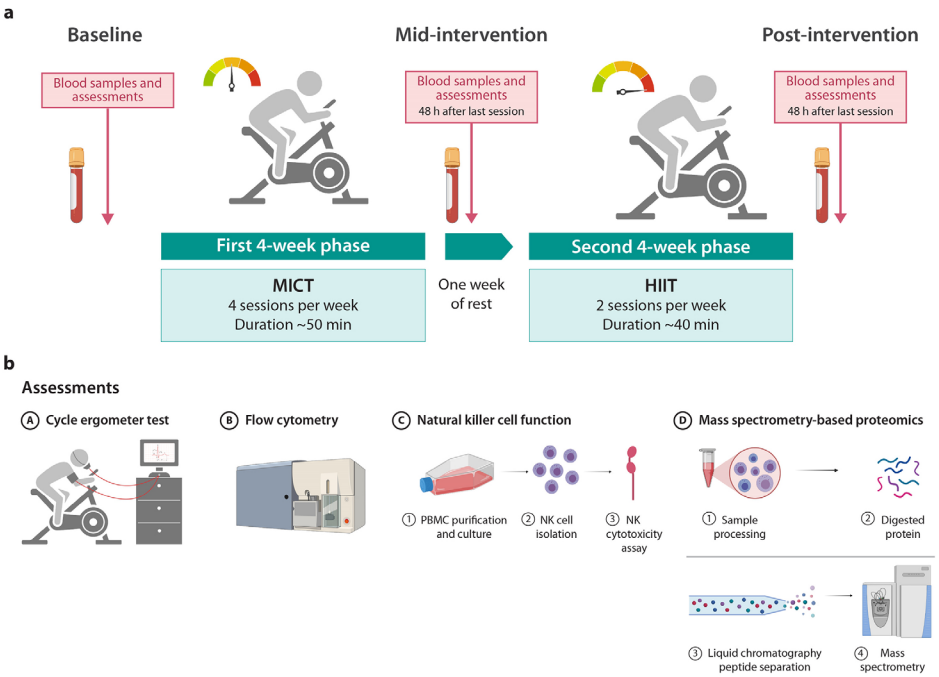

Figure 1. Study design. Protocol and intervention (a) and assessments (b).

Intervention

All exercise-training sessions were individually supervised (I.P.A. and L.B.A.), and were performed indoors with the same bicycle-ergometer (Ergometrics Ergoline 800; Jaeger, Bitz, Germany) that was used during the exercise tests for determination of VO_2peak, ventilatory threshold (VT) and respiratory compensation point (RCP), which are described later.

Moderate-intensity continuous training

This first phase started the week after baseline assessments and lasted four weeks in total, including four sessions per week (Monday, Wednesday, Friday and Sunday). The total duration of all the sessions was the same (~50 minutes), barring gradual build-up to this duration during the first week. The core part of the session lasted 40 minutes and the workload (power output [PO]) was gradually increased as follows: PO corresponding to the VT (first week), 5% above the VT (second week), midpoint between VT and RCP (third week), and 10% below the RCP (fourth week). All the sessions were preceded and followed by a 5–7-minute warmup below the VT and five minutes of cool-down (at the preferred PO), respectively.

The subjects refrained from doing any exercise sessions for one week after the MICT intervention and then started the HIIT program.

High-intensity interval training

This second phase also lasted four weeks in total, and included two sessions per week (Monday and Wednesday). The total duration of all the sessions was ~40 minutes. Each session started with a ~15-minute warmup and ended with a 5–10-minute cool-down period (at the preferred PO). For the Monday sessions, after a ~15-minute warmup slightly below the VT, participants performed three (in the first week) to five (in the remainder of weeks) 3-minute intervals at a PO above the RCP (starting from just above the RCP in the first week and increasing the PO by 2-3% per week thereafter) interspersed with 3-minute rest periods. For the Wednesday sessions, participants performed 10 (in the first week) to 15 (in the last two weeks) 30-second sprints (at maximal or near-maximal intensity) interspersed with 80-second rest periods. Rating of perceived exertion (RPE, on a 0 to 10 scale) were recorded in each session, with subjects instructed to aim at an RPE value ≥ 9 during the intervals versus 4 and 2 in the recovery periods between the 3-minute intervals and 30-second sprints, respectively.

Measurements

Exercise tests

All the participants performed a graded cycle ergometer test until volitional exhaustion, at baseline and at mid- and post-intervention, respectively (after a 48-hour rest period from the last training session). All tests were performed between 9:00 am and 12:00 pm at the exercise physiology laboratory of the UEM. Before the tests (~08:00 am), venous blood samples were collected from the antecubital vein into VacutainerR (sodium-heparin) tubes (BD Biosciences, San José, CA) for performing all the studies on NK cells that are described in the following sections.

Gas exchange data were recorded 'breath-by-breath' with a metabolic cart (Vmax 29C; SensorMedics Corp., Yorba Linda, CA). The test started at 20 watts and the load was increased in a ramp-like fashion (5 watts/12 sec [20 watts/minute on average]) while cadence was kept constant at 60 to 70 rpm. Participants were verbally encouraged to continue pedalling until volitional exhaustion, and were continuously monitored electrocardiographically. The VO_2peak was defined as the highest value (mean of 30 seconds) reached during the test. We also determined the VT and RCP (also termed 'second ventilatory threshold') using the following criteria: VT, the point at which the ventilatory equivalent for oxygen (ventilation [VE]·VO_2^{-1}) starts to increase with no concomitant increase in the ventilatory equivalent for carbon dioxide (VE·VCO_2^{-1}) and with departure from linearity of VE; RCP, the point at which both VE·VO_2^{-1} and VE·VCO_2^{-1} increase together with a decrease in end-tidal pressure of carbon dioxide (31).

Flow cytometry

Flow cytometry analyses (FACSCantoTM II, BD Biosciences) were conducted to assess the presence of various cell surface markers related to cellular activation processes (**Table 1**). The gating strategy was based on dead/live cells and doublets discrimination. When possible, a minimum of 10,000 events of the population of interest was analyzed. FACSDiva™ software (BD Biosciences) was used for analysis.

Natural killer cell function

Peripheral blood mononuclear cell purification and cultures. Heparinized blood was diluted two-fold with phosphate buffered saline (PBS) and layered on top of Ficoll-Paque in a 50 mL conical polypropylene tube (BD Biosciences). After centrifugation, peripheral blood mononuclear cells (PBMC) were recovered and washed twice in calcium- and magnesium-free PBS. Cells were seeded in culture flasks containing Rosewell Park Memorial Institute 1640 medium and 10% fetal bovine serum (FBS) (both from Gibco/Thermo Scientific, Waltham, MA). After 48 hours, cell suspensions were stimulated with 10 µg/mL phytohemagglutinin (PHA) (Sigma Aldrich, St Louis, MO) (18, 49) and functional assays were performed as indicated below.

Natural killer cell isolation. NK cells were purified from PBMC using the NK Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Briefly, non-NK cells (T, B, stem, dendritic and erythroid cells, monocytes, and granulocytes) were magnetically labeled using a cocktail of biotin-conjugated antibodies and the NK Cell MicroBead Cocktail (Miltenyi Biotech). Isolation of highly pure NK cells was achieved by depletion of magnetically-labeled cells.

Natural killer cell cytotoxicity assay. K562 cells were used as NK target cells, and were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% FBS. To assess NK cytotoxic capacity, K562 cells were loaded with bis[acetoxymethyl] 2,2′:6′,2′′-terpyridine-6,6′′-dicarboxylate (BATDA), a hydrophobic ligand that penetrates the plasma membrane quickly, according to the DELFIA® EuTDA Cytotoxicity Kit (Perkin Elmer, Waltham, MA). The assay was performed with 5×10^3 BATDA-loaded K562 cells and 100 µL of primary NK cells at various concentrations (2×10^4, 1×10^4, 5×10^3 and 2.5×10^3 cells). After cytolysis, the released ligand (TDA) reacts with a europium solution to constitute a fluorescent chelate. The fluorescent signal correlates directly with the amount of lysed cells:

$$\% \text{ Specific release} = \frac{\text{Experimental release (counts)} - \text{Spontaneous release (counts)}}{\text{Maximum release (counts)} - \text{Spontaneuos release (counts)}} \times 100$$

Mass spectrometry-based proteomics

Sample processing and protein digestion. Frozen sample aliquots of 1–3 × 10^6 NK cells were lysed in 150 µL SDT-lysis buffer (4% SDS and 0.1 M DTT in 0.1 M Tris/HCl, pH 7.6) using 1:10 sample to buffer ratio, at 95°C for 5 minutes. Samples were briefly sonicated to reduce the viscosity of the lysates and then centrifuged at 16,000 × g for 10 minutes. Sample processing was based on the procedure of Wisniewski et al. (75) with minor modifications. Aliquots (100 µL) of lysates were loaded onto spin ultrafiltration units (Amicon Ultra-0.5 Ultracel-30 membrane; Merck, Kenilworth, NJ) and washed with 355 µL of buffer UA (8 M urea in 0.1 M Tris/HCl, pH 8.5). Spin unit were then centrifuged at 14,000 × g for 15 minutes and washed again with 375 µL of buffer UA. Alkylation of cysteine residues in the samples was carried out by adding 100 µL of freshly prepared iodoacetamide solution (50 mM in UA buffer) into the spin filters and incubating the samples in the dark for 20 minutes. Filters were washed three times with 200 µL of UA followed by three washes with 100 µL of 50 mM ammonium bicarbonate. On-filter digestion was performed overnight with trypsin at 37°C. The released peptides were collected by centrifugation at 14,000 × g for 10 minutes followed by an additional wash with 50 µL of 50 mM ammonium bicarbonate. The peptide-containing eluates were finally desalted using C-18 spin columns (C-18 Micro Spin Column; Harvard Apparatus, Holliston, MA) and dried in a speed vacuum (Thermo Scientific). Samples were resuspended in 0.1% formic acid and peptide concentration was measured with the Qubit Protein Assay Kit (Thermo Scientific). Before the liquid chromatography-mass spectrometry (LC-MS/MS) analysis, 200 ng of each biological replicate (baseline, MICT, HIIT) and 0.66 µg sample was analyzed.

Liquid chromatography-tandem mass spectrometry. LC-MS/MS analysis was performed using a Q Exactive mass spectrometer interfaced with an Easy-nLC 1000 nanoUPLC System (both from Thermo Scientific). Digested peptides were loaded onto an Acclaim PepMap100 precolumn (75 µm × 2 cm) connected to an Acclaim PepMap RSLC (50 µm × 15 cm) analytical column (Thermo Scientific). Peptides were eluted with a 180 minute linear gradient of 3–30% acetonitrile in 0.1% formic acid at a flow rate of 300 nL/minute directly onto the nanoES Emitter (Thermo Scientific). The mass spectrometer was operated in a top 10 data-dependent mode. Survey scans were acquired at a resolution of 70,000 (m/z 200) and fragmentation spectra at 17,500 (m/z 200). Peptide selection was done with an isolation window of 2.0 Th and normalized collision energy of 28 was applied for peptide fragmentation. The maximum injection time was 120 milliseconds for survey, and MS/MS scans and automatic gain control target values of 3e6 for survey scans and 5e5 for MS/MS scans were used. Acquired raw data files were processed with MaxQuant (10) software (v1.6.0.16) using the internal search engine Andromeda, and searched against the UniProt database restricted to Homo sapiens entries (release 2018_11). Carbamidomethylation was set as fixed modification, whereas methionine oxidation and protein N-terminal acetylation were defined as variable modifications. Mass tolerance was set to 8 and 20 ppm at the MS and MS/MS level, respectively. Enzyme specificity was set to trypsin, allowing for a maximum of two missed cleavages. The "match between runs" option was enabled with a 1.5-minute time window and a 20-minute alignment window to match identification across samples. The minimum peptide length was set to seven amino acids. The false discovery rate (FDR) for peptides and proteins was set to 1%. Normalized spectral protein label-free quantification intensities were calculated using the MaxLFQ algorithm.

MaxQuant output data was analyzed with the Perseus module (v1.6.5.0) (67). Proteins only identified by site, contaminants and reverse hits were removed. Changes in protein

abundance higher than 4-fold in proteins identified with more than two unique peptides were considered as relevant protein differences.

Statistical analyses

Exercise capacity and natural killer cell numbers/cytotoxicity

Data are presented as mean ± SD. Normal distribution (Shapiro-Wilk test) and homoscedasticity (Levene's test) of the data were checked before any statistical treatment. A one-way repeated-measures ANOVA was used to examine differences across the three conditions (i.e., baseline, mid-intervention, and post-intervention). Bonferroni's test was performed post hoc when a significant condition (or 'time') effect was present. The Greenhouse-Geisser correction was applied when Mauchly's test of sphericity was violated. Statistical analyses were conducted using a statistical software package (SPSS v23; IBM, Amonk, NY) setting the significance level at α=0.05.

Proteomics

Candidate proteins. To identify proteins in plasma that were differentially expressed across the three conditions (baseline, mid-intervention and post-intervention) the magnitude of the changes was calculated by taking the base 2 logarithm of the mean fold change (log2FC). For proteins with no expression in either of the three samples, log2FC values were adjusted by adding one to each mean and then calculating the ratio. Negative values indicate down-regulation whereas positive values indicate up-regulation. Multi-test correction was performed according to the Benjamini-Hochberg method, that is, p-values were adjusted with FDR correction (5).

Processing of protein expression data. Proteins identified in the plasma profiles were filtered to unique human-reviewed protein entries according to the UniProt Knowledgebase (UniProtKB) (67). To reduce potential interference for high signals, keratin protein entries were excluded from the analysis. In addition, the protein entries that were traced to an unreviewed UniProtKB entry were manually curated to find a valid reviewed entry. When more than one protein entry was traced to the same reviewed UniProtKB entry, the following criteria were applied to prevent duplications: (i) to prioritize protein entries with an identical protein ID to the one noted in UniProtKB; (ii) to prioritize protein entries that were automatically traced to the reviewed UniProtKB entry over those that were first traced to an unreviewed or deleted one and then manually curated; (iii) to prioritize protein entries with valid signals; (iv) to prioritize whole protein entries over those referring to fragments; and (v) to apply an alphabetical criterion when all the criteria above were not applicable.

Contextualization of the differentially expressed proteins within "natural killer cell" protein network. Effector proteins were used to focus the analysis on the biological condition of interest in the human biological network. The relationships between the differentially expressed proteins and the immune system were assessed. Different publicly available databases were consulted for the human protein network generation (Reactome, Molecular INTeraction database (MINT) and Biological General Repository for Interaction Datasets [BioGrid]) (25, 26).

Gene set enrichment analysis. Proteomic data were analyzed using the Gene Set Enrichment Analysis (GSEA) tool (63) to compare the differential pathways and molecular processes between conditions. This approach categorizes genes according to a specific metric, ranking the most statistically significant genes (focusing on log2FC values) at the top end of the list. The FDR cutoff was set at 25% to maximize hypothesis generation. Specifically, the enrichment was run over the following databases: Gene Ontology (GO) terms (Biological Process, Cellular Component, Molecular Function) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Visualization as a network of the relationship between enriched pathways. Cytoscape 3.5.1. software was used to represent the relationship between the enriched pathways identified by GSEA (based on the number of common annotated genes between datasets).

RESULTS

No adverse effect was noted during the exercise sessions and no participant reported any medical condition (e.g., infection/s) during the study that could have altered the results.

Exercise capacity

No significant time effect ($p = 0.455$) was found for participants' body mass (baseline: 69.5 ± 9.5 kg; mid-intervention: 69.1 ± 9.0 kg; and post-intervention: 69.4 ± 9.1 kg). A significant time effect ($p = 0.016$) was found for VO_2peak, which showed an increasing trend during the study (baseline: 34.9 ± 4.8 $mL \cdot kg^{-1} \cdot min^{-1}$; mid-intervention: 38.7 ± 7.6 $mL \cdot kg^{-1} \cdot min^{-1}$; and post-intervention: 39.5 ± 7.5 $mL \cdot kg^{-1} \cdot min^{-1}$). In post hoc pairwise comparisons, a quasi-significant difference was found for post-intervention versus baseline ($p = 0.071$). The peak PO (PPO) attained during the tests ($p = 0.004$ for time effect; baseline: 2.4 ± 0.2 $watts \cdot kg^{-1}$; mid-intervention: 2.8 ± 0.4 $watts \cdot kg^{-1}$; and post-intervention: 2.9 ± 0.4 $watts \cdot kg^{-1}$), as well as the PO eliciting the RCP ($p = 0.006$ for time effect; baseline: 2.1 ± 0.2 $watts \cdot kg^{-1}$; mid-intervention: 2.4 ± 0.4 $watts \cdot kg^{-1}$; and post-intervention: 2.5 ± 0.3 $watts \cdot kg^{-1}$) also showed an increasing trend during the study, with significant post hoc differences for post-intervention versus baseline ($p = 0.028$ for PPO and 0.044 for RCP). No significant time effect ($p = 0.154$) was, however, found for the PO eliciting the VT (baseline: 1.1 ± 0.1 $watts \cdot kg^{-1}$; mid-intervention: 1.1 ± 024 $watts \cdot kg^{-1}$; and post-intervention: 1.2 ± 1.2 $watts \cdot kg^{-1}$).

NK number and cytotoxicity

A significant time effect was found for PBMC ($p < 0.001$, **Figure 2A**) and NK ($p < 0.001$, Figure 2B) cell counts, with both cell counts considerably increasing at both mid- and post-intervention compared with baseline, and with a clear training time-dependent effect (i.e., a further, significant increase was found at post-intervention as compared with mid-intervention).

A significant time effect was also found for NK cell cytotoxicity ($p < 0.001$, **Figure 2C**); which significantly increased at both mid- and post-intervention compared with baseline; however, no training time-dependent effect was found (i.e., no significant differences were found for mid- versus post-intervention).

Flow cytometry

No significant condition effect was found except for the surface expression of the chemokine receptor CXCR3 expressed as mean fluorescence intensity ($p = 0.044$, with a trend towards a decrease at mid- and post-intervention compared with baseline), although no significant differences were found in post hoc pair-

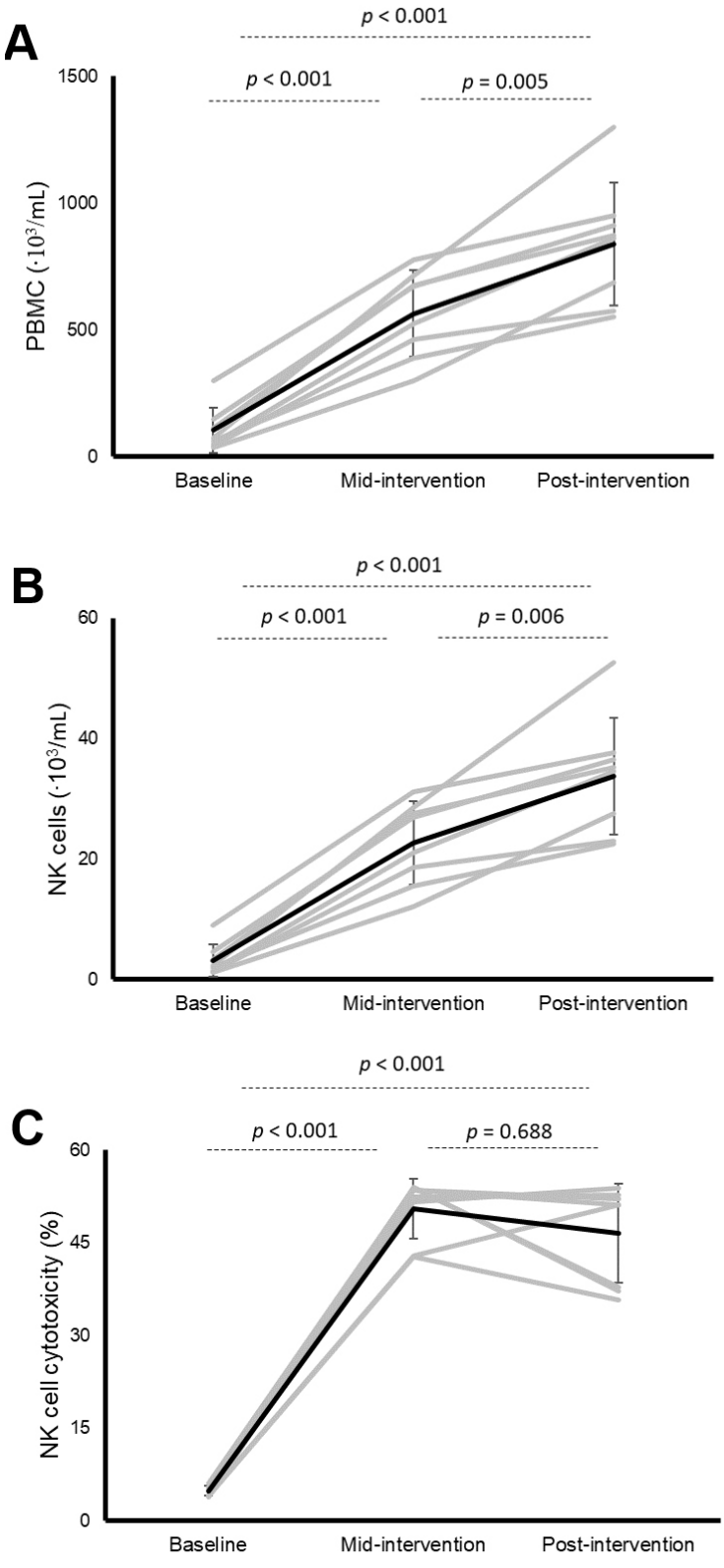

Figure 2. Within-subject comparison of the number of peripheral blood mononuclear cells ([PBMC], **panel A**), and of the number (**panel B**) and cytotoxicity (**panel C**, N=8) of natural killer (NK) cells. Cytotoxicity was expressed as % specific fluorescence release (see text for more details) at a ratio of effector (NK) to target (K562) cells of 1:1, with the results replicated (i.e., same p-values) at different E:T ratios (1:2, 2:1 and 4:1).

wise comparisons (p = 0.244 and 0.093 for baseline versus mid- and post-intervention, respectively; and p = 1.000 for mid- versus post-intervention) (**Table 2**).

Proteomics

A total of 2538 human proteins were identified by LC-MS/MS, of which 2448 valid protein entries were used in the next steps of the study and 90 were filtered out (i.e., potential contaminants, reverse and only identified by site).

Protein candidates

Mid-intervention versus baseline. The expression of 74 plasma proteins was significantly different between mid-intervention and baseline (FDR q-value < 0.01), of which 49 and 25 were upregulated and downregulated, respectively, at mid-intervention (**Table 3**).

Moreover, one and nine unique proteins were identified (FDR q-value < 0.01) at baseline and at mid-intervention, respectively. Among the candidate proteins, 17 are known to be potentially related to immune function (**Figure 3**):

Proteins upregulated at mid-intervention (versus baseline): N-acylsphingosine amidohydrolase 1 (ASAH1); V-type proton

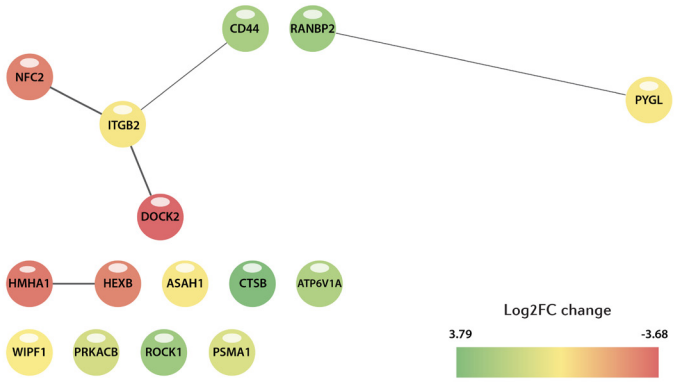

Figure 3. Visual protein interaction network of the proteins related to the immune system that were differentially expressed (upregulated or downregulated) at mid-intervention compared to baseline.

ATPase catalytic subunit A (ATP6V1A); CD44; cathepsin B (CTSB); integrin beta chain-2 (ITGB2, also known as 'CD18'); nucleoporin 88 (NUP88); phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1); cAMP-dependent protein kinase catalytic subunit beta (PRKACB); proteasome (prosome, macropain) subunit, alpha 1 (PSMA1); glycogen phosphorylase, liver isoform (PYGL); RAN binding protein 2 (RANBP2, also known as 'nucleoporin 358'); Rho-associated coiled-coil containing protein kinase 1 (ROCK1); and WAS/WASL interacting protein family member 1 (WIPF1).

Proteins downregulated at mid-intervention (versus baseline): dedicator of cytokinesis 2 (DOCK2); hexosaminidase subunit beta (HEXB); minor histocompatibility protein HA-1 (HMA1, also known as 'Rho GTPase activating protein 45'); and neutrophil cytosolic factor 2 (NCF2).

Post-intervention versus baseline. The expression of 76 plasma proteins was significantly different between post-intervention and baseline, of which 22 and 54 were upregulated and downregulated, respectively, at post-intervention (**Table 4**). No unique proteins were identified (FDR q-value < 0.01) at post-intervention compared with baseline.

Table 1. Antibodies per tube and per peripheral blood sample used for flow cytometry analysis.

	AC	APC	APC-Cy7	FITC	PB	PE	PE-Cy7	PerCP/Cy5.5
Tube 1	CD45	IgG	IgG	IgG	CD3	IgG	CD56	IgG
Tube 2	CD45	NKp46	NKG2D	CD94	CD3	NKp30	CD56	NKp44
Tube 3	CD45	CXCR3	CD69	CD25	CD3	CD57	CD56	CXCR4

Antibody	Reference	Brand
AC CD45 V500-C	655873	BD Horizon
APC IgG Fc	409306	BioLegend
APC CD335 (NKp46)	331918	BioLegend
APC CD183 (CXCR3)	353708	BioLegend
APC/Cy7 CD314 (NKG2D)	320824	BioLegend
APC/Cy7 CD69	310914	BioLegend
APC/Cy7 IgG Fc	409314	BioLegend
FITC IgG Fc	409310	BioLegend
FITC CD25	555431	BD Pharmingen
FITC CD94	305504	BioLegend
PB V405 CD3	560365	BD Horizon
PE IgG Fc	409304	BioLegend
PE CD337 (NKp30)	325208	BioLegend
PE CD57	322312	BioLegend
PE-Cy7 CD56	335826	BD Biosciences
PerCP/Cy5.5 IgG Fc	409312	BioLegend
PerCP/Cy5.5 CD184 (CXCR4)	306516	BioLegend
PerCP/Cy5.5 CD336 (NKp44)	325114	BioLegend

Abbreviations: AC, AmCyan; APC, Allophycocyanin; APC-Cy7, Allophycocyanin cyanin-7; Ig, Immunoglobulin; FITC, Fluorescein isothiocyanate; PB, Pacific blue; PE, Phycoerythrin; PE-Cy7, Phycoerythrin cyanin-7; PerCP-Cy7, Peridinin chlorophyll protein.

Table 2. Flow cytometry results in NK cells.

A

Variable	Baseline	Mid-intervention	Post-intervention	p-value
CD94	5.5 ± 1.6	6.2 ± 2.0	5.4 ± 2.2	0.305
NKp30	190 ± 149	53 ± 52	25 ± 21	0.367
NKp44	0.10 ± 0.06	0.09 ± 0.1	0.13 ± 0.08	0.595
NKp46	619 ± 449	214 ± 324	246 ± 379	0.057
NKG2D	123 ± 149	146 ± 172	172 ± 127	0.676
CD25	0.79 ± 0.09	0.75 ± 0.09	0.74 ± 0.11	0.527
CD57	11.2 ± 24.8	3.1 ± 4.1	2.1 ± 2.8	0.318
CXCR4	0.16 ± 0.13	0.15 ± 0.20	0.18 ± 0.12	0.708
CXCR3	436 ± 302	171 ± 274	138 ± 166	**0.044**
CD69	13.9 ± 17.1	11.2 ± 11.7	13.4 ± 18.1	0.946

B

Variable	Baseline	Mid-intervention	Post-intervention	p-value
CD94	61.3 ± 13.9	58.6 ± 14.9	59.6 ± 16.2	0.574
NKp30	66.6 ± 20.3	62.4 ± 16.3	65.3 ± 21.2	0.697
NKp44	0.06 ± 0.09	0.08 ± 0.12	0.06 ± 0.05	0.950
NKp46	51.0 ± 17.0	50.8 ± 13.2	54.8 ± 18.2	0.660
NKG2D	31.0 ± 13.9	17.3 ± 10.6	17.7 ± 9.8	0.078
CD25	0.21 ± 0.20	0.24 ± 0.24	0.18 ± 0.13	0.818
CD57	5.9 ± 5.0	6.0 ± 4.9	6.9 ± 6.1	0.246
CXCR4	0.19 ± 0.25	0.16 ± 0.09	0.08 ± 0.07	0.393
CXCR3	30.0 ± 11.5	26.6 ± 7.1	30.6 ± 9.2	0.513
CD69	1.8 ± 0.9	1.4 ± 0.7	1.5 ± 1.4	0.611

Table footnote. Values are mean ± SD of fluorescence intensity (**A**) or of percent positive (**B**) for each NK cell receptor. The only significant p-value for the time (condition) effect is in bold. Abbreviations: CXCR3, C-X-C chemokine receptor type 3; CXCR4, C-X-C chemokine receptor type 4 (also known as fusin or CD184 [cluster of differentiation 184]).

Table 3. List of proteins differentially expressed at mid-intervention versus baseline.

UniProt	Gene	Number of unique peptides	Log2FC change	FDR (q-value)	Related to immune system	Expression at mid-intervention compared to baseline
Q9UBS4	DNAJB11	3	27.921	<.001	No	↑
P27986	PIK3R1	3	27.050	<.001	Yes	↑
Q9UNS2	COPS3	3	27.023	<.001	No	↑
Q9P258	RCC2	3	26.768	<.001	No	↑
Q99567	NUP88	3	26.728	<.001	Yes	↑
Q9H2K8	TAOK3	3	26.724	<.001	No	↑
O75150	RNF40	3	26.628	<.001	No	↑
Q8TD19	NEK9	3	26.468	<.001	No	↑
Q9Y4P3	TBL2	3	26.246	<.001	No	↑
Q9UHD8	SEPT9	8	3.957	<.001	No	↑
P07858	CTSB	3	3.788	<.001	Yes	↑
O75439	PMPCB	5	3.500	<.001	No	↑
Q13464	ROCK1	8	3.386	<.001	Yes	↑
P49792	RANBP2	5	3.379	<.001	Yes	↑
P46060	RANGAP1	4	3.319	<.001	No	↑
P10412	HIST1H1E	8	3.319	<.001	No	↑
P16070	CD44	4	3.250	<.001	Yes	↑
Q14683	SMC1A	8	3.157	<.001	No	↑
O15372	EIF3H	5	3.146	<.001	No	↑
P38606	ATP6V1A	5	3.136	<.001	Yes	↑
Q9H4G4	GLIPR2	3	3.006	<.001	No	↑
P34897	SHMT2	3	2.963	<.001	No	↑
O94776	MTA2	3	2.849	<.001	No	↑
Q9UQ35	SRRM2	7	2.698	<.001	No	↑
Q15424	SAFB	4	2.678	<.001	No	↑
P22694	PRKACB	4	2.671	<.001	Yes	↑
Q92878	RAD50	5	2.670	<.001	No	↑
Q9H583	HEATR1	3	2.668	<.001	No	↑
P55769	NHP2L1	3	2.609	<.001	No	↑
P25786	PSMA1	3	2.568	<.001	Yes	↑
Q92888	ARHGEF1	6	2.554	<.001	No	↑
P49588	AARS	6	2.498	<.001	No	↑
O00410	IPO5	3	2.467	<.001	No	↑
Q13045	FLII	11	2.424	<.001	No	↑
P05198	EIF2S1	5	2.375	<.001	No	↑
Q12907	LMAN2	4	2.306	<.001	No	↑
Q14980	NUMA1	19	2.237	<.001	No	↑
O43516	WIPF1	6	2.203	<.001	Yes	↑
P00390	GSR	3	2.202	<.001	No	↑
Q9NTI5	PDS5B	9	2.195	<.001	No	↑
Q3KQU3	MAP7D1	3	2.162	<.001	No	↑
Q13510	ASAH1	5	2.103	<.001	Yes	↑
Q96HE7	ERO1L	3	2.098	<.001	No	↑
Q5JSL3	DOCK11	4	2.079	<.001	No	↑
Q99497	PARK7	5	2.054	<.001	No	↑
P05107	ITGB2	11	2.052	<.001	Yes	↑
P06737	PYGL	4	2.050	<.001	Yes	↑
P23634	ATP2B4	3	2.045	<.001	No	↑
Q15942	ZYX	9	2.040	<.001	No	↑
Q9UBQ7	GRHPR	4	-2.188	<.001	No	↓
O75964	ATP5L	3	-2.214	<.001	No	↓
P84243	H3F3A	3	-2.231	<.001	No	↓
P07686	HEXB	4	-2.272	<.001	Yes	↓
Q7Z2W4	ZC3HAV1	6	-2.366	<.001	No	↓
P19878	NCF2	5	-2.386	<.001	Yes	↓
Q6P2Q9	PRPF8	11	-2.478	<.001	No	↓
P16403	HIST1H1D	5	-2.487	<.001	No	↓
P17844	DDX5	4	-2.547	<.001	No	↓
Q92896	GLG1	8	-2.552	<.001	No	↓
P45954	ACADSB	4	-2.656	<.001	No	↓
Q92619	HMHA1	7	-2.817	<.001	Yes	↓
P62081	RPS7	3	-2.874	<.001	No	↓
Q9H0U4	RAB1B	3	-2.945	<.001	No	↓
P57737	CORO7	5	-3.053	<.001	No	↓
O94919	ENDOD1	3	-3.131	<.001	No	↓
Q00325	SLC25A3	5	-3.370	<.001	No	↓
O94906	PRPF6	5	-3.448	<.001	No	↓
P52209	PGD	5	-3.634	<.001	No	↓
Q92608	DOCK2	10	-3.683	<.001	Yes	↓
P00505	GOT2	7	-4.115	<.001	No	↓
P46781	RPS9	4	-4.234	<.001	No	↓
P47985	UQCRFS1	4	-4.541	<.001	No	↓
Q9H299	SH3BGRL3	3	-4.923	<.001	No	↓
P12081	HARS	3	-26.319	<.001	No	↓

Table footnote. Symbols: ↑ upregulation at mid-intervention compared to baseline; ↓ downregulation at mid-intervention compared to baseline. Abbreviations (other than protein names): FDR, false discovery rate; Log2FC, log 2 fold change. Abbreviations (proteins): AARS, alanyl-tRNA synthetase 1; ACADSB, acyl-coA dehydrogenase short/branched chain; ARHGEF1, Rho guanine nucleotide exchange factor 1; ASAH1, N-acylsphingosine amidohydrolase 1; ATP2B4, ATPase plasma membrane Ca^{2+} transporting 4; ATP5L, ATP synthase membrane subunit G; ATP6V1A, ATPase H^{+} transporting V1 subunit A (also known as V-type proton ATPase catalytic subunit A); CD44, CD44 molecule; COPS3, COP9 signalosome subunit 3; CORO7, CORO7-PAM16 readthrough; CTSB, cathepsin B; DDX5, DEAD-box helicase 5; DNAJB11, DnaJ heat shock protein family (Hsp40) member B11; DOCK11, dedicator of cytokinesis 11; DOCK2, dedicator of cytokinesis 2; EIF2S1, eukaryotic translation initiation factor 2 subunit alpha; EIF3H, eukaryotic translation initiation factor 3 subunit H; ENDOD1, endonuclease domain containing 1; ERO1L, endoplasmic reticulum oxidoreductase 1 alpha; FLII, FLII actin remodeling protein; GLG1, Golgi glycoprotein 1; GLIPR2, GLI pathogenesis related 2; GOT2, glutamic-oxaloacetic transaminase 2; GRHPR, glyoxylate and hydroxypyruvate reductase; GSR, glutathione-disulfide reductase; H3F3A, H3.3 histone A; HARS, histidyl-TRNA synthetase 1; HEATR1, hEAT repeat containing 1; HEXB, hexosaminidase subunit beta; HIST1H1D, H1.3 linker histone, cluster member; HIST1H1E, H1.4 linker histone, cluster member; HMHA1, minor histocompatibility protein HA-1 (also known as Rho GTPase activating protein 45); IPO5, importin 5; ITGB2, integrin subunit beta 2 (also known as 'CD18'); LMAN2, lectin, mannose binding 2; MAP7D1, MAP7 domain containing 1; MTA2, metastasis associated 1 family member 2; NCF2, neutrophil cytosolic factor 2; NEK9, NIMA related kinase 9; NHP2L1, NHP2-like protein 1 (also known as 'small nuclear ribonucleoprotein 13) [SNU13]); NUMA1, nuclear mitotic apparatus protein 1; NUP88, nucleoporin 88; PARK7, parkinsonism associated deglycase; PDS5B, PDS5 cohesin associated factor B; PGD, Phosphogluconate dehydrogenase; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PMPCB, mitochondrial-processing peptidase subunit beta; PRKACB, protein kinase CAMP-activated catalytic subunit beta; PRPF6, pre-MRNA processing factor 6; PRPF8, pre-MRNA processing factor 8; PSMA1, proteasome (prosome, macropain) subunit alpha 1; PYGL, glycogen phosphorylase L; RAB1B, RAB1B, member RAS oncogene family; RAD50, RAD50 double strand break repair protein; RANBP2, RAN binding protein 2; RANGAP1, Ran GTPase activating protein 1; RCC2, regulator of chromosome condensation 2; RNF40, ring finger protein 40; ROCK1, Rho associated coiled-coil containing protein kinase 1; RPS7, ribosomal protein S7; RPS9, ribosomal protein S9; SAFB, scaffold attachment factor B; SEPT9, septin 9; SH3BGRL3, SH3 domain binding glutamate rich protein like 3; SHMT2, serine hydroxymethyltransferase 2; SLC25A3, solute carrier family 25 member 3; SMC1A, structural maintenance of chromosomes 1A; SRRM2, serine/arginine repetitive matrix 2; TAOK3, TAO kinase 3; TBL2, transducin beta like 2; UQCRFS1, ubiquinol-cytochrome C reductase, Rieske iron-sulfur polypeptide 1; VIP36, lectin, mannose binding 2; WIPF1, WAS/WASL interacting protein family member 1; ZC3HAV1, zinc finger CCCH-type containing, antiviral 1; ZYX, zyxin.

Table 4. List of proteins differentially expressed at post-intervention versus baseline.

UniProt	Gene	Number of unique peptides	Log2FC change	FDR (q-value)	Related to immune system	Expression at post-intervention compared to baseline
P10412	HIST1H1E	8	5.613	<.001	No	↑
P20701	ITGAL	5	4.200	.009	Yes	↑
Q92522	H1FX	5	3.784	<.001	No	↑
Q16777	HIST2H2AC	3	3.588	.008	No	↑
Q92878	RAD50	5	3.531	.008	No	↑
P16402	HIST1H1D	5	3.213	<.001	No	↑
P55809	OXCT1	3	3.107	<.001	No	↑
P06454	PTMA	3	2.710	<.001	No	↑
P31943	HNRNPH1	3	2.667	<.001	No	↑
P34897	SHMT2	3	2.666	.008	No	↑
P13073	COX4I1	5	2.621	<.001	No	↑
P62244	RPS15A	4	2.594	<.001	No	↑
Q9UH99	SUN2	6	2.568	<.001	No	↑
Q92888	ARHGEF1	6	2.397	<.001	No	↑
P30273	FCER1G	3	2.338	.008	Yes	↑
P22307	SCP2	3	2.301	<.001	No	↑
P62805	HIST1H4A	9	2.265	<.001	No	↑
Q9BUJ2	HNRNPUL1	7	2.142	<.001	No	↑
P16104	H2AFX	4	2.080	<.001	No	↑
Q9UKM9	RALY	8	2.069	<.001	No	↑
P23634	ATP2B4	3	2.045	<.001	No	↑
P00505	GOT2	7	2.022	<.001	No	↑
Q96KP4	CNDP2	3	-2.103	<.001	No	↓
O15400	STX7	5	-2.110	<.001	No	↓
P60174	TPI1	6	-2.137	<.001	No	↓
O43290	SART1	3	-2.161	<.001	No	↓
Q01813	PFKP	3	-2.225	<.001	No	↓
Q15007	WTAP	3	-2.311	<.001	No	↓
P18669	PGAM1	3	-2.332	<.001	Yes	↓
Q9NTJ5	SACM1L	9	-2.348	<.001	No	↓
P12931	SRC	7	-2.352	<.001	Yes	↓
O15511	ARPC5	5	-2.388	<.001	Yes	↓
Q0VD83	APOBR	6	-2.389	<.001	No	↓
Q9HBI1	PARVB	4	-2.402	<.001	No	↓
P24557	TBXAS1	5	-2.407	<.001	No	↓
Q13418	ILK	9	-2.409	<.001	No	↓
P78417	GSTO1	4	-2.457	<.001	Yes	↓
P18754	RCC1	3	-2.470	<.001	No	↓
P09960	LTA4H	10	-2.531	<.001	Yes	↓
Q86UE4	MTDH	7	-2.536	<.001	No	↓
Q9H4B7	TUBB1	5	-2.565	<.001	Yes	↓
P12814	ACTN1	9	-2.596	<.001	No	↓
O60763	USO1	7	-2.658	<.001	No	↓
P32455	GBP1	3	-2.689	<.001	Yes	↓
Q5JTV8	TOR1AIP1	7	-2.754	<.001	No	↓
Q14766	LTBP1	17	-2.812	<.001	No	↓
P24534	EEF1B2	3	-2.930	<.001	No	↓
P16615	ATP2A2	5	-2.934	<.001	No	↓
P54577	YARS	10	-2.975	<.001	No	↓
P19878	NCF2	5	-2.977	<.001	Yes	↓
P09326	CD48	4	-3.020	<.001	No	↓
Q02218	OGDH	8	-3.049	<.001	No	↓
Q9NYU2	UGGT1	10	-3.206	<.001	No	↓
Q7Z2W4	ZC3HAV1	6	-3.252	<.001	No	↓
P33176	KIF5B	5	-3.257	<.001	Yes	↓
O60488	ACSL4	5	-3.288	<.001	No	↓
P57737	CORO7	5	-3.296	<.001	No	↓
O60264	SMARCA5	10	-3.373	<.001	No	↓
Q13464	ROCK1	8	-3.419	<.001	Yes	↓
P30153	PPP2R1A	4	-3.456	<.001	Yes	↓
P07814	EPRS	7	-3.531	<.001	No	↓
P31948	STIP1	7	-3.587	<.001	No	↓
Q9H299	SH3BGRL3	3	-3.613	<.001	No	↓
P17301	ITGA2	8	-3.676	<.001	No	↓
Q14764	MVP	10	-3.697	<.001	Yes	↓
Q04917	YWHAH	6	-3.953	<.001	No	↓
P31150	GDI1	5	-4.063	<.001	No	↓
P04275	VWF	14	-4.066	<.001	Yes	↓
P11413	G6PD	9	-4.159	<.001	No	↓
P51858	HDGF	8	-4.321	<.001	No	↓
Q9Y6C2	EMILIN1	9	-4.942	<.001	No	↓
P30041	PRDX6	5	-5.199	<.001	Yes	↓
P40925	MDH1	4	-5.888	<.001	No	↓
Q13043	STK4	3	-6.160	<.001	No	↓
P52209	PGD	5	-6.619	<.001	No	↓
P12081	HARS	3	-26.319	<.001	No	↓

Table footnote. Symbols: ↑ upregulation at post-intervention compared to baseline; ↓ downregulation at post-intervention compared to baseline. Abbreviations (other than protein names): FDR, false discovery rate; Log2FC, log 2 fold change. Abbreviations (proteins): ACSL4, acyl-CoA synthetase long chain family member 4; ACTN1, actinin alpha 1; APOBR, apolipoprotein B Receptor; ARHGEF1, Rho guanine nucleotide exchange factor 1; ARPC5, actin related protein 2/3 complex subunit 5; ATP2A2, ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} Transporting 2; ATP2B4, ATPase plasma membrane Ca^{2+} transporting 4; CD48, CD48 molecule; CNDP2, carnosine dipeptidase 2; CORO7, CORO7-PAM16 readthrough; COX4I1, cytochrome C oxidase subunit 4I1; EEF1B2, eukaryotic translation elongation factor 1 beta 2; EMILIN1, elastin microfibril interfacer 1; EPRS, glutamyl-prolyl-tRNA synthase 1; FCER1G, Fc fragment of IgE receptor Ig; G6PD, glucose-6-phosphate dehydrogenase; GBP1, guanylate binding protein 1; GDI1, GDP dissociation inhibitor 1; GOT2, glutamic-oxaloacetic transaminase 2; GSTO1, glutathione S-transferase omega 1; H1FX, H1.10 linker histone; H2AFX, H2A.X variant histone; HARS, histidyl-tRNA synthetase 1; HDGF, heparin binding growth factor; HIST1H1D, histone cluster 1 H1 family member D; HIST1H1E, histone cluster 1 H1 family member E; HIST1H4A, H4 clustered histone 1; HIST2H2AC, H2A clustered histone 20; HNRNPH1, heterogeneous nuclear ribonucleoprotein H1; HNRNPUL1, heterogeneous nuclear tibonucleoprotein U Like 1; ILK, integrin linked kinase; ITGA2, integrin subunit alpha 2; ITGAL, integrin subunit alpha L; KIF5B, kinesin family member 5B; LTA4H, leukotriene A4 hydrolase; LTBP1, latent transforming growth factor beta binding protein 1; MDH1, malate dehydrogenase 1; MTDH, metadherin; MVP, major vault protein; NCF2, neutrophil cytosolic factor 2; OGDH, oxoglutarate dehydrogenase; OXCT1, 3-oxoacid CoA-transferase 1; PARVB, parvin beta; PFKP, phosphofructokinase, platelet; PGAM1, phosphoglycerate mutase 1; PGD, phosphogluconate dehydrogenase; PPP2R1A, protein phosphatase 2 scaffold subunit alpha; PRDX6, peroxiredoxin 6; PTMA, prothymosin alpha; RAD50, RAD50 double strand break repair protein; RALY, RALY heterogeneous nuclear ribonucleoprotein; RCC1, regulator of chromosome condensation 1; ROCK1, Rho associated coiled-coil containing protein kinase 1; RPS15A, ribosomal protein S15a; SACM1L, SAC1 like phosphatidylinositide phosphatase; SART1, spliceosome associated factor 1, recruiter of U4/U6.U5 tri-SnRNP; SCP2, sterol carrier protein 2; SH3BGRL3, SH3 domain binding glutamate rich protein like 3; SHMT2, serine hydroxymethyltransferase 2; SMARCA5, SWI/SNF related, matrix associated, actin dependent regulator of Chromatin, subfamily A, member 5; SRC, SRC proto-oncogene, non-receptor tyrosine kinase; STIP1, stress induced phosphoprotein 1; STK4; serine/threonine kinase 4; STX7, syntaxin 7; SUN2, Sad1 and UNC84 domain containing 2; TBXAS1, thromboxane A synthase 1; TOR1AIP1, torsin 1A interacting protein 1; TPI1, triosephosphate isomerase 1; TUBB1, tubulin beta 1 class VI; UGGT1, UDP-glucose glycoprotein glucosyltransferase 1; USO1, USO1 vesicle transport factor; VWF, Von Willebrand factor; WTAP, WT1 associated protein; YARS, tyrosyl-tRNA synthetase 1; YWHAH, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein Eta; ZC3HAV1, zinc finger CCCH-type containing, antiviral 1

Among the candidate proteins, 16 proteins are known to be potentially related to immune function (**Figure 4**):

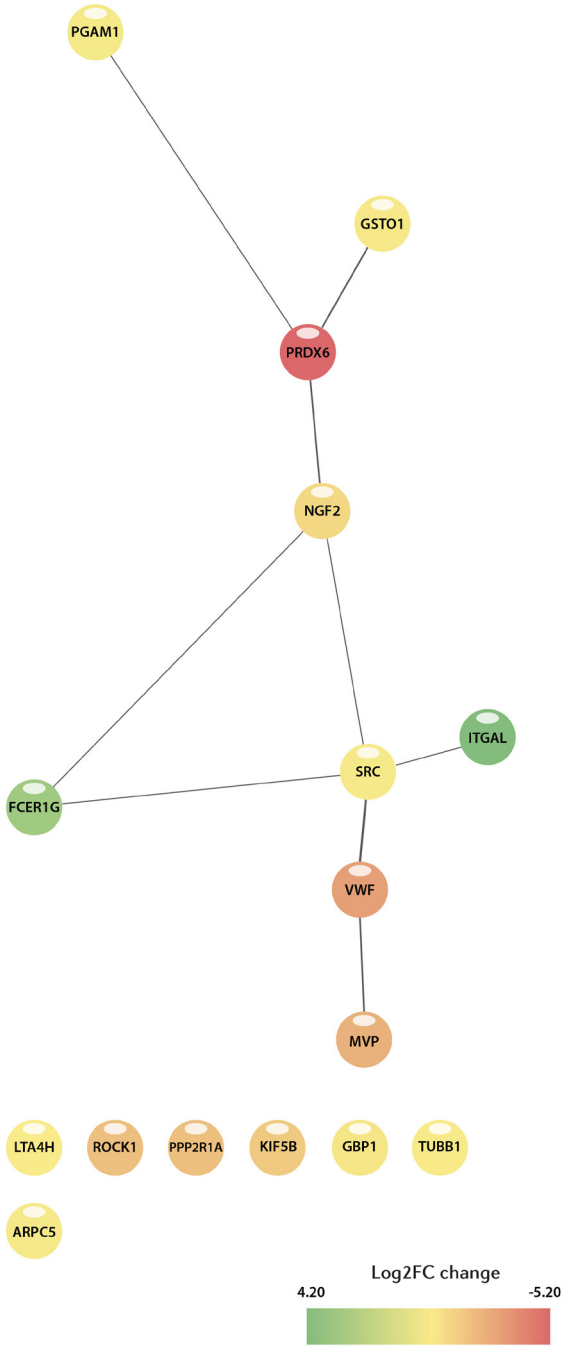

Figure 4. Visual protein interaction network of the proteins related to the immune system that were differentially expressed (upregulated or downregulated) at post-intervention compared to baseline.

Proteins upregulated at post-intervention (versus baseline): integrin subunit alpha L (ITGAL); and Fc fragment of IgE receptor Ig (FCER1G).

Proteins downregulated at post-intervention (versus baseline): ARPC5 actin related protein 2/3 complex subunit 5 (ARPC5); guanylate binding protein 1 (GBP1); glutathione S-transferase omega 1 (GSTO1); kinesin family member 5B (KIF5B); leukotriene A4 hydrolase (LTA4H); major vault protein (MVP); neutrophil cytosolic factor 2 (NCF2); phosphoglycerate mutase 1 (PGAM1); protein phosphatase 2 scaffold subunit alpha (PPP2R1A); peroxiredoxin 6 (PRDX6); Rho associated coiled-coil containing protein kinase 1 (ROCK1); SRC proto-oncogene, non-receptor tyrosine kinase (SRC); tubulin beta 1 class VI (TUBB1); and Von Willebrand factor (VWF).

Post-intervention versus mid-intervention. The expression of 106 plasma proteins was significantly different between mid- and post-intervention (FDR q-value < 0.01) (**Table 5**), with 26 and 80 upregulated and downregulated, respectively, at post-intervention. Among the candidate proteins, 24 proteins are known to be potentially related to immune function (**Figure 5**):

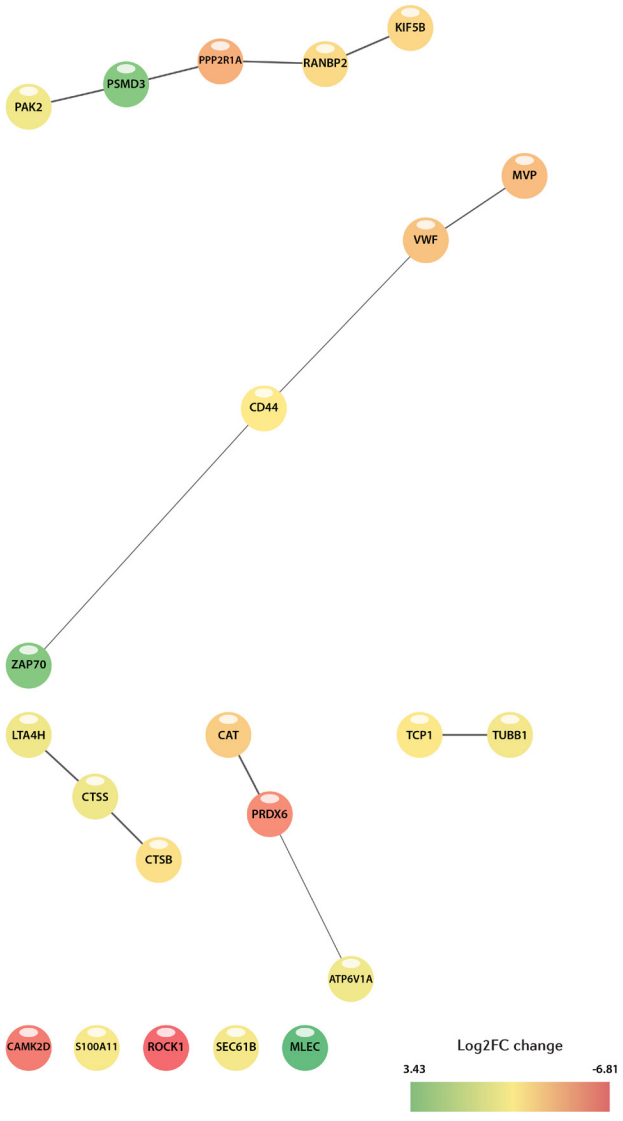

Figure 5. Visual protein interaction network of the proteins related to the immune system that were differentially expressed (upregulated or downregulated) at post-intervention compared to mid-intervention.

Proteins upregulated at post-intervention (versus mid-intervention): malectin (MLEC); proteasome 26S subunit (PSMD3); and zeta chain of T cell receptor associated protein kinase 70 (ZAP70).

Proteins downregulated at post-intervention (versus mid-intervention: ATPase H^+ transporting V1 subunit A (ATP6V1A); calcium/calmodulin dependent protein kinase II delta (CAMK2D); catalase (CAT); CD44 molecule (CD44); cathepsin B (CTSB); cathepsin S (CTSS); kinesin family member 5B (KIF5B); leukotriene A4 hydrolase (LTA4H); major vault protein (MVP); nucleoporin 88 (NUP88); P21 (RAC1) acti-

Table 5. List of proteins differentially expressed at post-intervention versus mid-intervention.

UniProt	Gene	Number of unique peptides	Log2FC change	FDR (q-value)	Related to immune system	Expression at post-intervention compared to mid-intervention
P00505	GOT2	7	6.137	<.001	No	↑
P16402	HIST1H1D	5	5.700	<.001	No	↑
Q00325	SLC25A3	5	4.547	<.001	No	↑
P06454	PTMA	3	4.112	<.001	No	↑
Q16777	HIST2H2AC	3	3.769	<.001	No	↑
O00479	HMGN4	3	3.695	<.001	No	↑
Q92522	H1FX	5	3.669	<.001	No	↑
P84243	H3F3A	3	3.615	<.001	No	↑
Q6DD88	ATL3	4	3.512	<.001	No	↑
Q14165	MLEC	3	3.434	<.001	Yes	↑
Q9Y2W1	THRAP3	7	3.380	<.001	No	↑
P22307	SCP2	3	3.163	.008	No	↑
P46781	RPS9	4	3.066	<.001	No	↑
P13073	COX4I1	5	3.030	<.001	No	↑
P17844	DDX5	4	2.634	<.001	No	↑
Q9H9B4	SFXN1	4	2.408	<.001	No	↑
Q13151	HNRNPA0	4	2.396	<.001	No	↑
P62805	HIST1H4A	9	2.356	<.001	No	↑
Q99623	PHB2	9	2.334	.008	No	↑
P10412	HIST1H1E	8	2.294	<.001	No	↑
P46063	RECQL	6	2.281	<.001	No	↑
P08758	ANXA5	3	2.151	<.001	No	↑
P49368	CCT3	6	2.123	<.001	No	↑
P43403	ZAP70	3	2.070	<.001	Yes	↑
P16104	H2AFX	4	2.069	<.001	No	↑
O43242	PSMD3	4	2.061	<.001	Yes	↑
Q9NTI5	PDS5B	9	-2.006	<.001	No	↓
P61981	YWHAG	5	-2.011	<.001	No	↓
P09960	LTA4H	10	-2.025	<.001	Yes	↓
P51659	HSD17B4	6	-2.037	<.001	No	↓
Q15404	RSU1	3	-2.041	<.001	No	↓
Q13177	PAK2	6	-2.071	<.001	Yes	↓
P25774	CTSS	4	-2.074	<.001	Yes	↓
P38606	ATP6V1A	5	-2.102	<.001	Yes	↓
Q15717	ELAVL1	3	-2.103	<.001	No	↓
Q8TCU6	PREX1	3	-2.143	<.001	No	↓
Q13428	TCOF1	17	-2.147	<.001	No	↓
Q9H4B7	TUBB1	5	-2.172	<.001	Yes	↓
Q86UE4	MTDH	7	-2.197	<.001	No	↓
P60468	SEC61B	3	-2.201	<.001	Yes	↓
Q9UGI8	TES	10	-2.224	<.001	No	↓
P09326	CD48	4	-2.271	<.001	No	↓
P05387	RPLP2	3	-2.280	<.001	No	↓
P31949	S100A11	3	-2.287	<.001	Yes	↓
P46782	RPS5	4	-2.307	<.001	No	↓
Q12906	ILF3	7	-2.340	<.001	No	↓
O95831	AIFM1	6	-2.366	<.001	No	↓
O60832	DKC1	3	-2.377	<.001	No	↓
O75533	SF3B1	9	-2.387	<.001	No	↓
P60174	TPI1	6	-2.396	<.001	No	↓
P13489	RNH1	10	-2.397	<.001	No	↓
Q8TC12	RDH11	3	-2.421	<.001	No	↓
O60763	USO1	7	-2.424	<.001	No	↓
P16070	CD44	4	-2.489	<.001	Yes	↓
P17987	TCP1	6	-2.498	<.001	Yes	↓
Q01813	PFKP	3	-2.537	<.001	No	↓
Q13620	CUL4B	3	-2.590	<.001	No	↓
Q9NTJ5	SACM1L	9	-2.625	<.001	No	↓
P16615	ATP2A2	5	-2.628	<.001	No	↓
Q02218	OGDH	8	-2.669	<.001	No	↓
Q0VD83	APOBR	6	-2.677	<.001	No	↓
Q96T37	RBM15	3	-2.754	<.001	No	↓
P07858	CTSB	3	-2.804	<.001	Yes	↓
Q9NZB2	FAM120A	3	-2.845	<.001	No	↓
P05198	EIF2S1	5	-2.889	<.001	No	↓
Q13098	GPS1	4	-2.896	<.001	No	↓
P80723	BASP1	7	-2.906	<.001	No	↓
Q9NYU2	UGGT1	10	-2.922	<.001	No	↓
P52209	PGD	5	-2.985	<.001	No	↓
P07814	EPRS	7	-3.029	<.001	No	↓
Q9UHD8	SEPTIN9	8	-3.030	<.001	No	↓
P49792	RANBP2	5	-3.056	<.001	Yes	↓
Q8WYJ6	SEPTIN5	4	-3.101	<.001	No	↓
P33176	KIF5B	5	-3.108	<.001	Yes	↓
Q9H3N1	TMX1	4	-3.139	<.001	No	↓
96KP4	CNDP2	3	-3.145	<.001	No	↓
Q14766	LTBP1	17	-3.164	<.001	No	↓
Q15942	ZYX	9	-3.236	<.001	No	↓
Q3KQU3	MAP7D1	3	-3.254	<.001	No	↓
O60488	ACSL4	5	-3.298	<.001	No	↓
P11413	G6PD	9	-3.355	<.001	No	↓
P04040	CAT	7	-3.486	<.001	Yes	↓
P04275	VWF	14	-3.802	<.001	Yes	↓
P31948	STIP1	7	-3.819	<.001	No	↓
Q14764	MVP	10	-3.957	<.001	Yes	↓
P24534	EEF1B2	3	-4.280	<.001	No	↓
P31150	GDI1	5	-4.288	<.001	No	↓
P30153	PPP2R1A	4	-4.464	<.001	Yes	↓
P17301	ITGA2	8	-4.588	<.001	No	↓
Q04917	YWHAH	6	-4.940	<.001	No	↓
Q9Y6C2	EMILIN1	9	-5.342	<.001	No	↓
P51858	HDGF	8	-5.433	<.001	No	↓
P30041	PRDX6	5	-5.545	<.001	Yes	↓
P40925	MDH1	4	-5.759	<.001	No	↓
Q13557	CAMK2D	3	-6.154	<.001	Yes	↓
Q13464	ROCK1	8	-6.805	<.001	Yes	↓
Q13043	STK4	3	-7.982	<.001	No	↓
Q9UBS4	DNAJB11	3	-27.921	<.001	No	↓
P27986	PIK3R1	3	-27.050	<.001	Yes	↓
Q9UNS2	COPS3	3	-27.023	<.001	No	↓
Q9P258	RCC2	3	-26.768	<.001	No	↓
Q99567	NUP88	3	-26.728	<.001	Yes	↓
Q9H2K8	TAOK3	3	-26.724	<.001	No	↓
O75150	RNF40	3	-26.628	<.001	No	↓
Q8TD19	NEK9	3	-26.468	<.001	No	↓
Q9Y4P3	TBL2	3	-26.246	<.001	No	↓

Table footnote. Symbols: ↑ upregulation at post-intervention compared to mid-intervention; ↓ downregulation at post-intervention compared to mid-intervention. Abbreviations (other than protein names): FDR, false discovery rate; Log2FC, log 2-fold change. Abbreviations (proteins): ACSL4, acyl-CoA synthetase long chain Family member 4; AIFM1, apoptosis inducing factor mitochondria associated 1; ANXA5, annexin A5; APOBR, apolipoprotein B receptor; ATL3, atlastin GTPase 3; ATP2A2, ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2; ATP6V1A, ATPase H^{+} transporting V1 subunit A; BASP1, brain abundant membrane attached signal protein 1; CAMK2D, calcium/calmodulin dependent protein kinase II delta; CAT, catalase; CCT3, chaperonin containing TCP1 subunit 3; CD44, CD44 molecule; CD48, CD48 molecule; CNDP2, carnosine dipeptidase 2; COPS3, COP9 signalosome subunit 3; COX4I1, cytochrome C oxidase subunit 4I1; CTSB, cathepsin B; CTSS, cathepsin S; CUL4B, cullin 4B; DDX5, DEAD-box helicase 5; DKC1, dyskerin pseudouridine synthase 1; DNAJB11, DnaJ heat shock protein family (Hsp40) member B11; EEF1B2, eukaryotic translation elongation factor 1 beta 2; EIF2S1, eukaryotic translation initiation factor 2 subunit alpha; ELAVL1, ELAV like RNA binding protein 1; EMILIN1, elastin microfibril interfacer 1; EPRS, glutamyl-prolyl-tRNA synthetase 1; FAM120A, family with sequence similarity 120A; G6PD, glucose-6-phosphate dehydrogenase; GDI1, GDP dissociation inhibitor 1; GOT2, glutamic-oxaloacetic transaminase 2; GPS1, G protein pathway suppressor 1; H1FX, H1.10 linker histone; H2AFX, H2A.X variant histone; H3F3A, H3.3 histone A; HDGF, heparin binding growth factor; HIST1H1D, H1.3 linker histone, cluster member; HIST1H1E, H1.4 linker histone, cluster member; HIST1H4A, H4 clustered histone 1; HIST2H2AC, H2A clustered histone 20; HMGN4, high mobility group nucleosomal binding domain 4; HNRNPA0, heterogeneous nuclear ribonucleoprotein A0; HSD17B4, hydroxysteroid 17-beta dehydrogenase 4; ILF3, interleukin enhancer binding factor 3; ITGA2, integrin subunit alpha 2; KIF5B, kinesin family member 5B; LTA4H, leukotriene A4 hydrolase; LTBP1, latent transforming growth factor beta binding protein 1; MAP7D1, MAP7 domain containing 1; MDH1, malate dehydrogenase 1; MLEC, malectin; MTDH, metadherin; MVP, major vault protein; NEK9, NIMA related kinase 9; NUP88, nucleoporin 88; OGDH, oxoglutarate dehydrogenase; PAK2, P21 (RAC1) activated kinase 2; PDS5B, PDS5 cohesin associated factor B; PFKP, phosphofructokinase, platelet; PGD, phosphogluconate dehydrogenase; PHB2, prohibitin 2; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PPP2R1A, protein phosphatase 2 scaffold subunit alpha; PRDX6, peroxiredoxin 6; PREX1, phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1; PSMD3, proteasome 26S subunit, non-ATPase 3; PTMA, prothymosin alpha; RANBP2, RAN binding protein 2; RBM15, RNA binding motif protein 15; RCC2, regulator of chromosome condensation 2; RDH11, retinol dehydrogenase 11; RECQL, RecQ like helicase; RNF40, ring finger protein 40; RNH1, ribonuclease/angiogenin inhibitor 1; ROCK1, Rho associated coiled-coil containing protein kinase 1; RPLP2, ribosomal protein lateral stalk subunit P2; RPS5, ribosomal protein S5; RPS9, ribosomal protein S9; RSU1, Ras suppressor protein 1; S100A11, S100 calcium binding protein A11; SACM1L, SAC1 like phosphatidylinositide phosphatase; SCP2, sterol carrier protein 2; SEC61B, SEC61 translocon beta subunit; SEPTIN5, septin 5; SEPTIN9, septin 9; SF3B1, splicing factor 3b subunit 1; SFXN1, sideroflexin 1; SLC25A3, solute carrier family 25 member 3; STIP1, stress induced phosphoprotein 1; STK4, serine/threonine kinase 4; TAOK3, TAO kinase 3; TBL2, transducin beta like 2; TCOF1, treacle ribosome biogenesis factor 1; TCP1, T-complex 1; TES, testin LIM domain protein; THRAP3, thyroid hormone receptor associated protein 3; TMX1, thioredoxin related transmembrane protein 1; TPI1, triosephosphate isomerase 1; TUBB1, tubulin beta 1 class VI; UGGT1, UDP-glucose glycoprotein glucosyltransferase 1; USO1, USO1 vesicle transport factor; VWF, Von Willebrand factor; YWHAG, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma; YWHAH, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein Eta; ZAP70, zeta chain of T cell receptor associated protein kinase 70; ZYX, zyxin.

vated kinase 2 (PAK2); phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1); protein phosphatase 2 scaffold subunit alpha (PPP2R1A); peroxiredoxin 6 (PRDX6); Ran binding protein 2 (RANBP2); Rho associated coiled-coil containing protein kinase 1 (ROCK1); S100 calcium binding protein A11 (S100A11); SEC61 translocon beta subunit (SEC61B); T-complex 1 (TCP1); tubulin beta 1 class VI (TUBB1); and Von Villebrand Factor (VWF).

Selection of the ten best candidate proteins. The proteins that showed a higher differential expression (all with a Log-2FC > 10 and FDR q-value < 0.001) were as follows: COP9 signalosome subunit 3 (COPS3); DnaJ heat shock protein family (Hsp40) member B11 (DNAJB11); histidyl-TRNA synthetase 1 (HARS); NIMA related kinase 9 (NEK9); NUP88; PIK3R1, regulator of chromosome condensation 2 (RCC2); TAO kinase 3 (TAOK3); transducin beta like 2 (TBL2); and ring finger protein 40 (RNF40). All of them were upregulated with MICT versus baseline, except HARS, which was downregulated with MICT versus baseline. Of these ten candidate proteins only two, PIK3R1 and NUP88, are currently known to play a documented role in immune function.

Gene set enrichment analysis. A total of four enriched pathways (FDR < 25%) were found, of which two related to transmembrane transport and cellular composition were downregulated at mid-intervention versus baseline, and two related to oxidation-reduction reactions were upregulated at post-intervention vs baseline (**Table 6**).

DISCUSSION

Our study reports several novel findings. We found that the first 4-week phase of the program (MICT) increased the number of PBMC and NK cells in blood over baseline, and this was further increased by the second 4-week phase (HIIT). However, the second part of the intervention did not lead to additional improvements over the first one for NK cell function with respect to cytotoxicity (or 'killing capacity'). In fact, a ceiling effect was observed, where both training phases induced a comparable and remarkable (~10-fold) improvement in NK cell cytotoxicity over the untrained, baseline state. Of note, such a noticeable benefit on NK function was observed after only four weeks of regular exercise (MICT in this case). Importantly, because immune function was assessed after a 48-hour rest upon termination of both MICT and HIIT phases, controlling for an acute exercise effect, our results point to a chronic benefit of regular exercise training on NK function.

Flow cytometry analysis showed that exercise training benefits do not necessarily involve upregulation of NK activating receptors. A trend towards a decrease in CXCR3 expression (determined as mean fluorescence intensity) was found at both mid- and post-intervention compared with baseline, yet there were no significant differences in post hoc pairwise comparisons. Using proteomics coupled to systems biology, we identified some candidate proteins and pathways (or 'protein networks') enriched by the training intervention. Thus, compared with baseline, the MICT phase downregulated the expression of proteins related to transmembrane transport and cellular composition, whereas the expression of proteins related to redox reactions was upregulated after the subsequent HIIT phase. Another interesting finding was that while the increment in NK cytotoxicity was similar at both mid- and post-intervention, the candidate proteins and pathways involved were different between the two time points. Exercise studies like the one described here might contribute to identify new proteins with a previously unreported role on NK function.

Given the increasingly important role of NK cells, it is fundamental to unravel the effects of regular exercise on NK cell function and the potential mechanisms involved. Indeed, NK cells, which share features of both innate and adaptive immunity, have a powerful killing function against diseased target cells that can compromise organismal homeostasis. NK cells are constantly on high alert for malignant cell-transformation and bacterial/viral infection, and monitor target cells for surface expression of stress-regulated self-molecules that are ligands for NK cell activating receptors (37). Engagement of these receptors triggers the exocytic release by NK cells of granules with cytotoxic mediators – granzymes and perforins – to destroy such abnormal cells. A high presence of NK cells in tumor microenvironments has been reported to be a positive prognostic factor for patients with a variety of malignancies, including during metastatic disease (30). However, despite the rapid and efficient capacity of NK cells to recognize and kill

Table 6. Processed differentialy expressed after endurance exercise training (moderate-intensity continuous training followed by high-intensity interval training) compared with baseline.

Gene set	FDR (q-value)	Nominal p-value	Related process	Expression
GO_OXIDOREDUCTASE_ACTIVITY	.017	<.001	Catalysis of oxidation-reduction reaction	↑ at post-intervention *vs* baseline
GO_OXIDATION_REDUCTION_PROCESS	.060	<.001	Oxidation-reduction reaction	↑ at post-intervention *vs* baseline
GO_TRANSMEMBRANE_TRANSPORT	.091	<.001	Transmembrane transport	↓ at mid-intervention *vs* baseline
GO_CYTOPLASMIC_REGION	.198	.006	Cellular composition	↓ at mid-intervention *vs* baseline

tumor cells, their function is impaired by the tumor microenvironment, even leading to their dysfunction or exhaustion (78). Thus, numerous strategies to improve NK cell cytotoxic capacity have been attempted, including activation with cytokines or analogs (78). In this context, our results showing an increase in NK cytotoxicity of one order of magnitude with both training programs provide support to the notion that a non-pharmacological intervention – regular exercise – could be considered as a coadjuvant to cancer immunotherapies and cancer treatment in general (6, 7, 60).

Our findings that MICT enhances NK cell cytotoxicity in healthy adults is in agreement with several prior studies in adults (15, 38, 45, 52), although others did not report an improvement (41, 42, 56). In this regard, a novelty of our study was that, at least in healthy non-immunocompromised adults, the addition of heavy exercise (HIIT) following MICT is essentially as beneficial as prior MICT in terms of improving NK function. In fact, a further increase in the release of NK cells to the bloodstream was observed at post-intervention compared with mid-intervention. Whether this result is due to an additional benefit of HIIT per se (i.e., higher work intensities) or to the accumulation of more exercise bouts in general (i.e., longer duration of the intervention) remains to be determined. That addition of HIIT did not negatively affect NK cell function and might actually considerably improve the number and function of these cells is an important finding because intense exercise has been traditionally viewed as a stressor to immune function that could potentially lead to a certain state of immunosuppression with subsequent increase in the risk of infections, at least of the upper respiratory tract (8). In fact, one month of heavy exercise training during the pre-competition season was reported to reduce NK cytotoxicity in female volleyball players (65), and another study found reductions in resting NK cell numbers and proportions after a 7-month training season in elite swimmers (20). It remains to be determined, however, whether a HIIT intervention with no previous MICT in untrained subjects elicits similar adaptations to those observed here.

The mechanisms by which exercise might increase NK number or function remain to be clearly elucidated. A preclinical murine study by Pedersen et al. found that exercise training did not enhance NK cell cytotoxicity per se, but rather 'prepared' the tumor microenvironment for their infiltration by enhancing the expression of ligands for NK cell-activating receptors such as NKG2D and NKp46 (51). Of note, no training effects were noted in our study for surface expression of NKG2D or NKp46 in flow cytometry analyses. NK cells – especially $CD56^{dim}$ cells, which make up 90% of the total cell population and are classified as cytotoxic (68) – can respond to exercise-induced epinephrine (51). In this context, the Pedersen et al. study showed that NK cells were mobilized by exercise-induced epinephrine and were redistributed to tumors in an IL6-dependent manner (51). More recently, another mechanistic study showed that the acute mobilization of NK cells in response to a 30-minute exercise bout at an intensity above the lactate threshold – and thus also above a typical MICT session – in cyclists was largely dependent on epinephrine signaling through β_2-adrenergic receptors (21). In this regard, our proteome analyses failed to find significant changes in NK b_2-adrenergic or IL-6 receptors at mid- or post-intervention, suggesting that, if exercise were able to change the expression levels of these receptors in humans, it would be more an acute than a chronic effect. Indeed, increases in the secretion of epinephrine and of the myokine IL-6 occur during (and in the hours following) an acute exercise bout whereas in the present study we aimed at assessing the chronic effects of regular exercise bouts, and as such we studied the participants' NK cells under resting conditions (after a previous 48-hour rest).

Transcriptomic (53) and epigenetic (79) modifications have been reported in NK cells after an acute exercise session. Just two-minute bouts of MICT (cycle ergometer exercise at 77% of VO_2peak) interspersed with one-minute rest result in the upregulation of genes related to pathways involved in cancer and cell communication (p53 signaling pathway, melanoma, glioma, prostate cancer, adherens junction, and focal adhesion) in healthy young men (53). Also, running a half marathon was found to induce global histone modifications in NK cells and a subsequent increase in the expression of the activating NK cell receptor NKG2D in patients with cancer and their controls (80). Another study found that acute exercise can provoke epigenetic modifications in NK cells, in this case affecting the balance between the activating immunoglobulin-like receptor KIR2DS4 and the inhibiting KIR3DL1 receptor, with potential benefits on NK cell function (57). Finally, acute exercise could also have a stimulating effect on NK cell cytotoxicity through intracellular signaling, for instance by mediating an increase in perforin levels inside these cells (23), with perforin-mediated cytolysis playing a key role in the control of acute viral infections by NK cells (64).

The effects of chronic exercise on NK cells at the molecular level are much less clear. Dias et al. (12) found that 18 weeks of aerobic endurance training changed the expression of 211 gene transcripts involved in cell cycle regulation, proliferation, and development of immune cells in PBMC. Similarly, a study comparing young endurance athletes and non-athlete controls identified 72 candidate transcripts in PBMC involved in encoding ribosomal proteins and oxidative phosphorylation (29). It should be borne in mind, however, that PBMC are a heterogeneous mix of immune cells, and changes in gene expression over time may be driven by alterations in immune cell proportions and not necessarily in NK cells per se. In contrast to the aforementioned studies in PBMC, a recent study showed that a 12-week resistance training intervention had negligible effects on the NK cell transcriptome (48). Slight increases were found for some candidate gene transcripts, of which only one of them, ten-eleven translocation methylcytosine dioxygenase 1 (TET1, involved in DNA demethylation) is actually known to play a relevant role with regard to NK cell function.

The ten candidate proteins that showed a higher differential expression in the present study were COPS3, DNAJB11, HARS, NEK9, NUP88, PIK3R1, RCC2, TAOK3, TBL2 and RNF40 (all FDR values < 0.001). All of them were upregulated at mid-intervention versus baseline, except HARS, which was downregulated at mid-intervention versus baseline. Of the ten candidate proteins two – PIK3R1(p85a) and NUP88 – have bona fide roles in immune function. The PI3K signaling pathway is involved in a broad range of cellular processes, including growth, metabolism, differentiation, proliferation,

motility, and survival (46). The PI3Kδ enzyme complex is primarily present in the immune system and comprises a catalytic (p110δ) and regulatory (PIK3R1/p85α) subunit. Dynamic regulation of PI3Kδ activity is required to ensure normal function and differentiation of immune cells, including NK cells. PI3K signaling plays indeed an important role in multiple key aspects of NK cell biology, including development/maturation, homing, priming, and function of these cells (33). PI3K activation leads to mobilization of intracellular calcium stores, which is required for NK cell migration and granule exocytosis (33). In addition to activating cytotoxicity, PI3K plays a pivotal role in signaling downstream (through PI3K-mammalian target of rapamycin [mTOR] pathway) of cytokine activation, particularly of IL-15 – the critical NK cell development and survival cytokine (32) – thereby coupling the metabolic sensor mTOR to NK cell anti-viral responses (39). Importantly, inhibition of the PI3K-signaling pathway blocks this priming effect and attenuates the antitumor response of these cells (39, 72).The other protein related to immune function, NUP88, is a structural constituent of the nuclear pore complex (nucleoporins), which are large protein complexes residing in the nuclear envelope. Nucleoporin 88 kDa (Nup88) selectively mediates the nucleocytoplasmic transport of NF-kappaB, an ubiquitous transcription factor involved in immune responses, apoptosis, and cancer (66).

The candidate proteins COPS3, DNAJB11, HARS, NEK9, RCC2, TAOK3, TBL2 and RNF40, have not yet been assigned a role related to NK function per se. COPS3 is a subunit of the COP9 signalosome (CSN), which regulates protein degradation (de-ubiquitination) and protein kinase activities in a variety of processes (59, 74). DNAJB11 is a member of the heat shock protein (HSP) family, whose main function is to facilitate folding of other proteins. HSPs are generally stress-inducible as they play a particularly important cytoprotective role in cells exposed to stress conditions (81). They are generally regarded as danger signaling biomarkers that prompt the immune system to react to prevailing adverse cellular conditions. Accordingly, DNAJB11 might also stimulate immune responses, for instance through activation of bone-marrow derived dendritic cells (77). In turn, HARS can mediate deleterious adaptive immune responses, contributing to the disease phenotype of the anti-synthetase syndrome (1). TAOK3 may be involved in T cell receptor signaling as well as in the regulation of early signaling from receptors that utilize Src kinases in cells other than T lymphocytes (47). NEK9 might play an important role in cell cycle control, contributing to the establishment of the microtubule-based mitotic spindle (17). RCC2 belongs to the so-called 'interactome', connecting integrins with the cell-migration machinery (11). TBL2 is an endoplasmic reticulum-localized transmembrane protein that is involved in cell survival and in translation of activating transcription factor 4 through its association with mRNA (69). Finally, RNF40 is a RING finger protein that is known to be involved in protein-protein and protein-DNA interactions, and has been documented to exert pro-tumorigenic functions in colorectal cancer in vitro by increasing clonogenic potential as well as by suppressing apoptosis (58).

Systems biology analysis highlighted four enriched pathways (FDR <25%), of which two related to transmembrane transport and cellular composition were downregulated at mid-intervention (after MICT) compared with baseline. It is possible that MICT could temporarily block endocytic signaling of NK cell membrane surface receptors, thereby keeping activating receptors – with a preserved cytotoxic capacity – at the membrane for a longer time with no need for receptor upregulation. This would explain the flow cytometry results (Table 2), where no major changes were found in activating NK surface receptors compared to baseline, perhaps reflecting a longer duration of biological signals due to the aforementioned decrease in endocytosis. Further knowledge of the role that transmembrane transport and cellular composition have with regard to activating/inhibitory membrane surface receptors in NK cells and other lymphocytes will be important to understand how regulation of receptor function within the endocytic compartments relates to the functional status of these cells. The same analysis showed that redox reactions were upregulated at post-intervention compared with baseline. In this regard, oxidants play a role in the regulation of NK cell function. Of note, Evans et al. (14) reported a crucial role of oxidant production in cancer cell killing or sensitization by NK cells. A possible mechanism underlying the sensitizing effect of oxidants to NK-mediated killing of cancer cell may be the upregulation of NK cell activating molecules on the surface of cancer cells (22).

We are aware of several limitations in our study. In addition to the relatively small sample size, we did not use a counterbalanced design with regard to the two training modalities, with the MICT program preceding the HIIT phase for all the participants. However, we felt it more appropriate for individuals who were untrained at baseline and thus not familiarized with exercise training programs to start with the less stressful/demanding MICT intervention. Furthermore, owing to the short resting period between MICT and HIIT, we could not discern whether the results found at post-intervention are attributable to HIIT alone and/or to a potential carry-over effect of the previous MICT phase. Implementation of a more controlled design would have strengthened our study (i.e., with a longer 'washout' period between MICT and HICT and also using a quasi-experimental design, with all the participants assessed at an additional 'control' time point, that is, after a long training cessation period following the intervention). In turn, we believe there are major strengths in our study, notably the use of a proteomics approach, which is a more rational strategy than transcriptomic approaches. Indeed, while the cellular concentration of proteins correlates with the abundance of their corresponding mRNAs, the correlation is not strong and, as such, relative abundances of proteins may not occur in proportion to their relative mRNA levels (71). The proteome is the final product of genome expression and comprises all the proteins present in a cell at a particular time. It supplements the other "omics" technologies to explain the identity of proteins in an organism, tissue or cell and to study the structure and functions of a particular protein (2). Further, proteomics analyses, especially if complemented with systems biology as here, allow to study the role of functional protein pathways in different conditions (2).

In conclusion, the training program induced a very remarkable improvement in NK function compared with the

untrained state although at the mechanistic level the pathways involved seem to differ over the intervention (i.e., between mid-intervention, which allowed to assess MICT-only effects, and post-intervention, which reflected HIIT effects as well as potential carry-over effects of previous MICT). The main findings of our study are summarized in **Figure 6**.

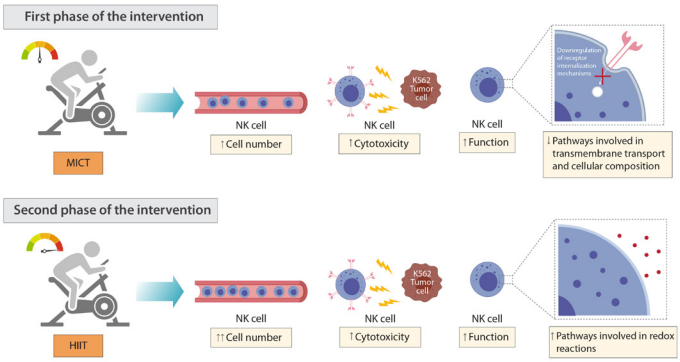

Figure 6. Summary of the main study results. Abbreviations: HIIT, high-intensity interval training; MICT, moderate-intensity continuous training.

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