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Current advances in exercise immunology

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Current advances in exercise immunology

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Acute exercise mobilizes NKT-like cells with a cytotoxic transcriptomic profile but does not augment the potency of cytokine-induced killer (CIK) cells

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$CD3^+/CD56^+$ Natural killer (NK) cell-like T-cells (NKT-like cells) represent <5% of blood lymphocytes, display a cytotoxic phenotype, and can kill various cancers. NKT-like cells can be expanded *ex vivo* into cytokine-induced killer (CIK) cells, however this therapeutic cell product has had mixed results against hematological malignancies in clinical trials. The aim of this study was to determine if NKT-like cells mobilized during acute cycling exercise could be used to generate more potent anti-tumor CIK cells from healthy donors. An acute exercise bout increased NKT-like cell numbers in blood 2-fold. Single cell RNA sequencing revealed that exercise mobilized NKT-like cells have an upregulation of genes and transcriptomic programs associated with enhanced anti-tumor activity, including cytotoxicity, cytokine responsiveness, and migration. Exercise, however, did not augment the *ex vivo* expansion of CIK cells or alter their surface phenotypes after 21-days of culture. CIK cells expanded at rest, during exercise (at 60% and 80% VO_{2max}) or after (1h post)

were equally capable of killing leukemia, lymphoma, and multiple myeloma target cells with and without cytokine (IL-2) and antibody (OKT3) priming *in vitro*. We conclude that acute exercise in healthy donors mobilizes NKT-like cells with an upregulation of transcriptomic programs involved in anti-tumor activity, but does not augment the *ex vivo* expansion of CIK cells.

KEYWORDS

exercise immunology, cell therapy, physical activity, cancer, hematological malignancies, single cell RNA sequencing, cytotoxicity, donor lymphocyte infusions

Introduction

Natural killer (NK) cell-like T-cells (NKT-like cells) are a heterogenous subset of effector T-cells ($CD3^+CD56^+$) that comprise ~1-5% of the peripheral blood lymphocyte compartment (1–3). These effector cells share phenotypic properties with NK-cells and can kill various cancer types in a non-MHC restricted manner (1, 2, 4), making them attractive candidates for allogeneic cell therapy. Blood NKT-like cells can be expanded *ex vivo* into a therapeutic cell product known as cytokine-induced killer (CIK) cells (2, 3), which have potent cytotoxic effects against multiple tumor cell lines *in vitro* (1, 5–7). The adoptive transfer of CIK cells *in vivo* reduces tumor burden by efficient homing mechanisms and long-term persistence (5, 7). Several clinical trials have demonstrated the feasibility and therapeutic efficacy of CIK cell adoptive therapy against both hematologic and solid malignancies, without treatment-induced severe adverse effects (8).

CIK cells are typically manufactured from patients or donors through lymphocytoapheresis or cord blood by the time-sensitive addition of an anti-CD3 monoclonal antibody and the cytokines IFN-γ and IL-2 *in vitro* (1, 3, 4). This expansion protocol generates a CIK cell population that is >90% $CD3^+$ and 20-35% $CD56^+$ within 14-28 days (6). However, CIK cells are sometimes difficult to expand due to the low numbers of $CD3^+CD56^+$ cells in peripheral blood (9). Moreover, despite moderate success in clinical trials to date, many patients with hematological malignancies do not enter remission after therapy or relapse (10, 11). Therefore, identifying a more feasible method to produce a superior CIK cell product could increase their utilization in the clinic.

A single bout of dynamic cardiovascular-based exercise (e.g. running, cycling, rowing) is an effective way to transiently increase the numbers of effector lymphocytes in peripheral blood 2-5-fold (12) evoking a preferential mobilization of effector lymphocytes that exhibit enhanced anti-tumor function (e.g. NK-cells, $CD8^+$ T-cells, TCR-γδ T-cells) (12). In murine cancer models, these exercise-mobilized lymphocytes quickly travel to and infiltrate tumors and play a cytotoxic role in reducing tumor burden with exercise (13). We have previously shown that exercise-mobilized lymphocytes allow for the generation of superior therapeutic cell products including viral-specific T-cells, tumor antigen specific T-cells and TCR-γδ T-cells (14–16). Although exercise is known to mobilize large numbers of precursor $CD3^+CD56^+$ NKT-like cells into the circulation (17), it is not known if these mobilized cells display distinct anti-tumor transcriptomic signatures or if they can be used to improve the manufacture and potency of CIK cells.

The aim of this study was to characterize transcriptomic changes in exercise-mobilized NKT-like cells at the single cell level and determine if the mobilized cells can be used to enhance the *ex vivo* generation of CIK cells from healthy donors. We found that exercise-mobilized NKT-like cells have an upregulation of multiple transcriptomic programs associated with anti-tumor immune activity, including cytotoxicity, cytokine production and responsiveness, antigen binding and processing, and chemotactic capacity; however, the phenotype and potency of the expanded CIK cells against a range of hematological cancer cell lines *in vitro* was unaffected by exercise.

Methods

Participants

Ten healthy participants (five females, five males) aged 21-45 (31 ± 6.9) years were recruited for this study. Prior to their enrollment, each participant was screened and identified as 'low-risk' for cardiovascular disease in accordance with American Heart Association-American College of Sports Medicine criteria (18), were not taking medications with the exception of oral contraceptives, were physically active (score of >4 on the physical activity rating questionnaire) (19) and non-users of tobacco products. Participants were asked to abstain from alcohol, caffeine, and physical activity 24 h prior to each laboratory visit, as well as complete an 8-12 h overnight fast when only water was consumed. This was confirmed verbally upon their arrival to the laboratory. Additionally, participants were only permitted to consume water until all experimental procedures were completed during each visit. All participants provided written informed consent and the study was

approved by the International Review Board at the University of Arizona. All laboratory procedures were performed between 6:00am-9:00am local time to minimize diurnal variation.

Experimental design

Participants firstly completed a maximal graded exercise test on a cycling ergometer (Velotron, Quarq Technology, San Diego, CA) with real-time collection of respiratory gas exchange and heart rate (Quark CPET, COSMED, Pabona di Albona Laziale, Italy) to determine maximal oxygen uptake (VO_{2max}). After a 5 min warm-up at 50 watts (W), resistance was increased by 15W every minute until the participant reached volitional exhaustion. Participants maintained a consistent cycling cadence throughout the entire exercise bout (≥60rpm) and rating of perceived exertion (RPE; Modified BORG scale (0–10)) was recorded during the final 15 s of each incremental stage. Linear regression plots were produced for each participant to determine cycling powers corresponding to 50%, 60%, 70%, and 80% VO_{2max} for the subsequent laboratory visit.

Participants returned to the laboratory 1-3 weeks later to perform the main exercise trial. An indwelling catheter (BD, Franklin Lakes, NJ, USA) was placed inside an antecubital vein so that serial blood draws could be collected before, during, and after exercise. The catheter was flushed with a sterile isotonic saline solution after each blood draw and 3mL blood volume was drawn and discarded prior to collection of blood samples used for analysis. Blood samples were collected from each subject into a 6mL vacutainer collection tube containing acid-citrate dextrose (ACD) (BD Vacutainer® blood collection tubes) for PBMC isolations or K_2EDTA (BD Vacutainer® blood collection tubes) for whole blood phenotyping. After collecting the resting blood sample, participants completed a 5 min warm-up at 50W and were then asked to cycle continuously for 20 min. The trial consisted of four incremental 5 min stages with power outputs corresponding to 50%, 60%, 70%, and 80% of the predicted VO_{2max}. Participants again were asked to maintain a consistent cycling cadence throughout the entire exercise session (≥60rpm) and blood samples were collected from the IV catheter at the 60% and 80% cycling stages. Heart rate and oxygen uptake were measured continuously throughout the exercise trial and RPE was recorded during the final 15 s of every exercise stage. A final intravenous blood sample was collected 1 h (+1H) after exercise cessation. Overall, a schematic of the experimental design is shown in **Figure 1**.

Immunophenotyping and lymphocyte subset enumeration

Whole blood samples were labeled with directly conjugated antibodies for multi-parameter flow cytometry to enumerate $CD45^+CD14^+$ monocytes and $CD45^+CD14^-$ lymphocytes subsets, as previously described (20). Briefly, 100µL of EDTA whole blood was incubated with the following antibodies CD8-VioBlue, CD14-VioGreen, CD3-FITC, CD4-PE, CD62L-PE, CD20-PerCP, CD45RA-PerCPVio770, CD45-APC, and CD56-APC-Vio770 (Miltenyi Biotec Inc., Gernany) for 30 min at room temperature and then lysed (RBC lysis buffer; eBioscience, San Diego, CA) for 20 min at room temperature followed by three wash cycles. For lymphocyte and monocyte enumeration, whole blood samples were labeled with CD14 and CD45 only, underwent a lyse-no-wash procedure and adjusted for the dilution factor. The total cell numbers of each lymphocyte subset were determined by multi-parameter flow cytometry (MACSQuant 10; Miltenyi Biotec Inc. Bergisch Gladbach, Germany) by multiplying the percentage of all lymphocytes expressing the surface markers of interest by the total lymphocyte count.

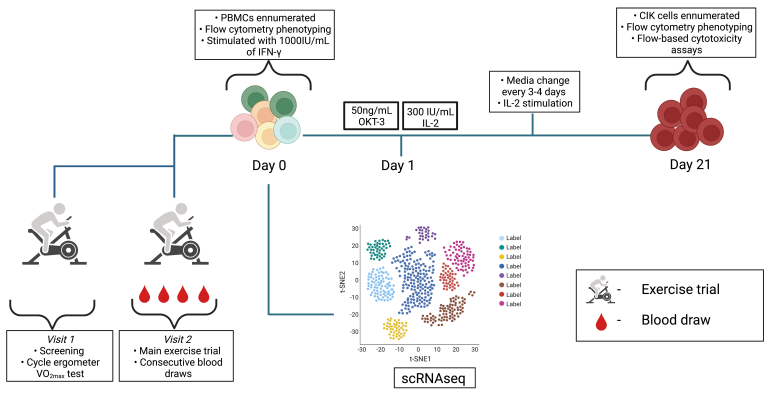

FIGURE 1
Schematic detailing the experimental design. Visit 1 and 2 consisted of exercise trials and serial blood draws were performed at rest, 60% VO_{2max}, 80% VO_{2max}, and +1H. PBMCs previously isolated from whole blood samples followed the CIK cell expansion protocol until day 21. scRNAseq was performed on resting PBMCs (n = 3). Diagram was created with BioRender.com.

Expansion of CIK cells

Peripheral blood mononuclear cells (PBMCs) were isolated from 6mL of ACD whole blood collected at rest, 60%, 80%, and +1H by density gradient centrifugation (Cytiva Ficoll-Paque™, Fisher Scientific, Hampton, NH) and cryopreserved until expansion. On Day 0, PBMCs were thawed and seeded at a concentration of 2-5 x 10^6 cells/mL in a 6-well plate with RPMI-1640 (Sigma-Aldrich) consisting of 10% FBS (Sigma-Aldrich) and 1% penicillin streptomycin (Sigma-Aldrich). Additionally, the generation of CIK cells was primed with 1000IU/mL of IFN-γ and incubated at 37°C in a 5% CO_2 humidified incubator for 24 h. The following day, 50ng/mL anti-CD3 (Miltenyi) and 300IU/mL IL-2 (Miltenyi) were added to the culture media to induce proliferation. Media was then changed every 3-4 days with the addition of 300IU/mL IL-2 and cells were seeded at a concentration of 1 x 10^6 cells/mL. After 21 days, cells were harvested to determine number, phenotype, and function by flow cytometry. Expanded CIK cells were enumerated, and 2 x 10^5 cells were labeled with appropriate combinations of the antibodies shown in **Supplementary Table 1**.

CIK cell cytotoxicity assays

Flow cytometry-based cytotoxicity assays were performed to examine functionality of the expanded CIK cells. 3 x 10^6 expanded CIK cells were stimulated for 1 h in media alone (RPMI-1640 with 10% FBS and 1% penicillin streptomycin) or media supplemented with 300IU/mL IL-2, 5ng/mL OKT-3, or a combination of IL-2 +OKT-3 at 37°C in a 5% CO_2 humidified incubator. CIK cells were then washed and recounted by flow cytometry. The leukemia cell line K562 (ATCC: CCL-243), the HLA-expressing (group 1 HLA-C *0304,*0702) multiple myeloma cell line U266 (ATCC: TIB-196), and the Daudi (ATCC: CCL-213) Burkitt's lymphoma cell line were used as target cells in the assays and maintained as previously described (20). 1 x 10^6 target cells were labeled with anti-CD71-FITC for 30 minutes and subsequently counted by flow cytometry. 300IU/mL of IL-2 was added to washed CIK cells and co-cultured with CD71-labeled target cells at a 0:1 (spontaneous lysis control) or 5:1 (CIK cell: target cell) ratio in a 96-well plate in duplicate for 4 h. Flow cytometry was used to determine CIK cell cytotoxic activity by identifying propidium iodide (PI) positive cells among the CD71+ targets following methods we have previously described (21). Lytic activity was calculated as specific lysis (% total lysis - % spontaneous lysis).

Single cell RNAsequencing (scRNAseq)

Isolated PBMCs from blood collected at rest, 80%, and +1H were resuspended in a PBS/RNAlater solution and delivered to the University of Arizona Genetics Core for single cell RNA sequencing (scRNAseq) analysis using the 10x Genomics platform. 5' RNA whole transcriptome libraries were generated using the "10xGenomics Chromium Next GEM Single Cell 5' reagents kit v2", following recommended guidelines. The gene expression libraries were quantified, normalized, pooled, and sequenced on an Illumina NextSeq500 sequencer. FastQ files were converted into expression matrices using the "cellranger count" function provided by Cell Ranger (10x Genomics Cell Ranger 6.0.1) and unfiltered matrices were imported into R, version 4.1.0. Empty droplets were identified and removed using the emptyDrops function found in the DropletUtils package (22). Reads with a high percentage of mitochondrial content were identified and removed using the perCellQCMetrics function provided by scuttle (23). After these QC steps were performed, 9,323 genes remained for downstream analysis. In order to analyze and visualize gene expression on a per cell basis, Principle component analysis (PCA) and uniform manifold approximation and projection (UMAP) clustering was performed using Seurat, version 4.0.5, to identify NKT-like cells (24). Differentially expressed genes were then detected using the FindMarkers function, with a log2 fold cutoff of 0, in Seurat. For each differential expression analysis comparison, gene set enrichment analysis (GSEA), with a false discovery rate of (0.25), was performed and annotated to both Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) terms.

Statistical analysis

All statistical analyses were completed using GraphPad Prism 8.0. All data are represented as the mean ± SD unless otherwise stated. Repeated measures ANOVA (RMANOVA) were used to analyze all cell number and flow cytometry surface expression levels (percentage or mean fluorescent intensity) across timepoints. RMANOVAs were used to analyze significant differences between resting and exercise-expanded CIK cells and their ability to kill target cell lines within stimulated conditions. Bonferroni test was applied to assess multiple comparisons for all RNAOVAs.

Results

Acute exercise mobilizes NKT-like cells with differentially expressed genes associated with cytotoxicity and tissue migration

As anticipated, numerous leukocyte subsets were mobilized to the peripheral blood compartment during exercise, including NKT-like cells which were elevated at both the 60% ($p = 0.02$) and 80% ($p = 0.006$) intensities compared to rest (**Table 1**). The numbers of NKT-like cells in blood at +1H fell below resting values ($p = 0.01$,

TABLE 1 The total number (cells/μL) of lymphocytes, CD3+ T-cells, CD4+ T-cells, CD8+ T-cells, 'double negative' T-cells, NK-cells, NKT-like cells, B-cells, and monocytes present in peripheral blood before (rest), during (at 60% and 80% VO_{2max}), and 1-hour post (+1H) exercise.

Leukocyte Subsets (cells/μL)	Rest	60%	80%	+1H
Lymphocytes	1745.02 ± 264.48	2381.62 ± 450.59***	3158 ± 606.80***	1463.44 ± 501.88
$CD3^+$ T-cells	1231.52 ± 283.79	1564.41 ± 438.77*	1892.15 ± 584.70**	1108.79 ± 508.73
$CD4^+$ T-cells	711.67 ± 244.42	802.21 ± 21*	920.48 ± 309.52**	633.10 ± 279.17
Naïve	280.47 ± 150.01	303.25 ± 170.79	341.97 ± 189.44*	210.48 ± 122.53
CM	167.17 ± 67.71	196.03 ± 74.33*	220.49 ± 139.47*	216.39 ± 118.67
EM	220.42 ± 107.65	256.51 ± 122.87	297.12 ± 139.47*	216.39 ± 118.67
EMRA	43.61 ± 45.79	46.42 ± 48.06	60.87 ± 63.44	54.93 ± 58.49
$CD8^+$T-cells	407.67 ± 118.77	541.09 ± 211.68**	683.69 ± 290.51**	338.80 ± 136.17
Naïve	116.36 ± 54.56	134.35 ± 56.32	157.18 ± 65.03*	100.48 ± 48.53
CM	37.50 ± 18.15	48.88 ± 19.98***	56.32 ± 22.67***	33.62 ± 18.09
EM	184.03 ± 117.91	240.81 ± 193.72	323.34 ± 257.81*	142.36 ± 88.62*
EMRA	69.77 ± 66.55	207.07 ± 101.00*	146.87 ± 137.38*	62.36 ± 73.87
$CD4^-CD8^-$ T-cells	100.64 ± 60.00	140.22 ± 88.49**	173.47 ± 105.99**	78.12 ± 48.34*
NK-cells	317.57 ± 158.23	591.13 ± 336.13**	935.79 ± 495.16**	210.48 ± 113.89***
NKT-like cells	147.34 ± 67.31	208.48 ± 118.77*	254.21 ± 139.81**	106.04 ± 55.34*
B-cells	112.12 ± 30.06	147.58 ± 46.12	188.17 ± 74.49**	111.34 ± 52.11
Monocytes	332.731 ± 70.80	482.12 ± 117.73***	596.65 ± 135.09***	331.32 ± 108.75

uL – microliter, CM – central memory, EM – effector memory, NK – natural killer cells, NKT – natural killer cell-like T-cells.
CD4+ and CD8+ T-cell subsets are also provided. Differentiated T-cells are phenotyped as follows: naïve ($CD45RA^+CD62L^+$), central memory (CM; $CD45RA^-CD62L^+$), effector memory (EM; $CD45RA^-CD62^-$), terminally differentiated effector memory (EMRA; $CD45RA^+CD62L^-$). Significant differences compared to rest indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

Table 1). We then aimed to understand if acute exercise altered gene expression in NKT-like cells at the single cell level. NKT-like cells were identified on UMAP clusters by the NCAM1 cluster within the CD3 clusters of PBMCs (Figure 2A). Differentially expressed genes (DEGs) (25) within the NKT-like cell cluster were then compared among three timepoints (Rest, 80%, and +1H) (Figure 2, Supplementary Table 2). We found seven genes to be significantly upregulated and six to be significantly downregulated at the 80% intensity compared to rest (Figure 2B). Cytotoxic DEGs, such as GZMB, GZMH, PRF1 were upregulated at 80% compared to Rest (Figure 2B). Interestingly, KLRC1 which encodes for the inhibitory receptor NKG2A, was downregulated with exercise (26) (Figure 2B). Similarly, DEGs associated with cytotoxic function, including GZMK, TNFSF10, and NKG7 were upregulated when comparing 80% and +1H (Figure 2D, Supplementary Table 1). There were fewer enriched DEGs between Rest and +1H timepoints, however, most upregulated genes were associated with migratory potential (e.g. CXCR1, CCL4, CXCR4) (Figure 2C). Overall, upregulated genes in exercise-mobilized NKT-like cells were closely related to cytotoxicity and increased migratory potential.

NKT-like cells mobilized with exercise display gene sets enriched to cytotoxic, anti-viral, and cytokine signaling functions

To identify biological processes associated with our differential gene expression data, we performed functional annotation and gene set enrichment analysis (GSEA) using both GO and KEGG terms. We found 1,207 GO terms and 92 KEGG terms to be significantly (FDR < 0.25) enriched among our three sample time points (Rest, 80%, and +1H). We therefore selected the 20 most relevant terms in each comparison. Notably, from Rest to 80%, we found a significant upregulation of gene sets enriched to GO and KEGG terms associated with lytic function, including overall leukocyte mediated cytotoxicity, NK-cell mediated cytotoxicity, and FcγR-mediated phagocytosis (Figures 3A and 4A). Gene sets associated with anti-viral responses were also upregulated, including defense response to viruses, regulation of interferon-alpha production, antigen presentation and processing, and antigen binding (Figures 3A and 4A). Downregulated pathways were mainly enriched to intracellular processing mechanisms such as amino acid metabolism, calcium signaling, and ribosomal processes (Figure 4A). When comparing 80% to +1H, enriched gene sets indicated enhanced responses to stimuli, such as cytokine receptor activity, cytokine-mediated signaling, cytokine-cytokine receptor interaction, and cellular response to cytokine stimulus (Figures 3B and 4B). Those that were downregulated included more intracellular processes such as ATPase activity, GDP binding, ribosome, and axon guidance (Figures 3B and 4B). Similar processes such as enhanced cytotoxicity and overall lymphocyte signaling and activation were also observed when comparing Rest *vs.* +1H, with the additional upregulation of pathways in cancer (Figures 3C and 4C). A summary of the leading-edge genes driving the gene set enrichment analyses for upregulation in NK-cell mediated cytotoxicity, cell adhesion molecules, regulation of leukocyte mediated cytotoxicity, defense response to virus, and

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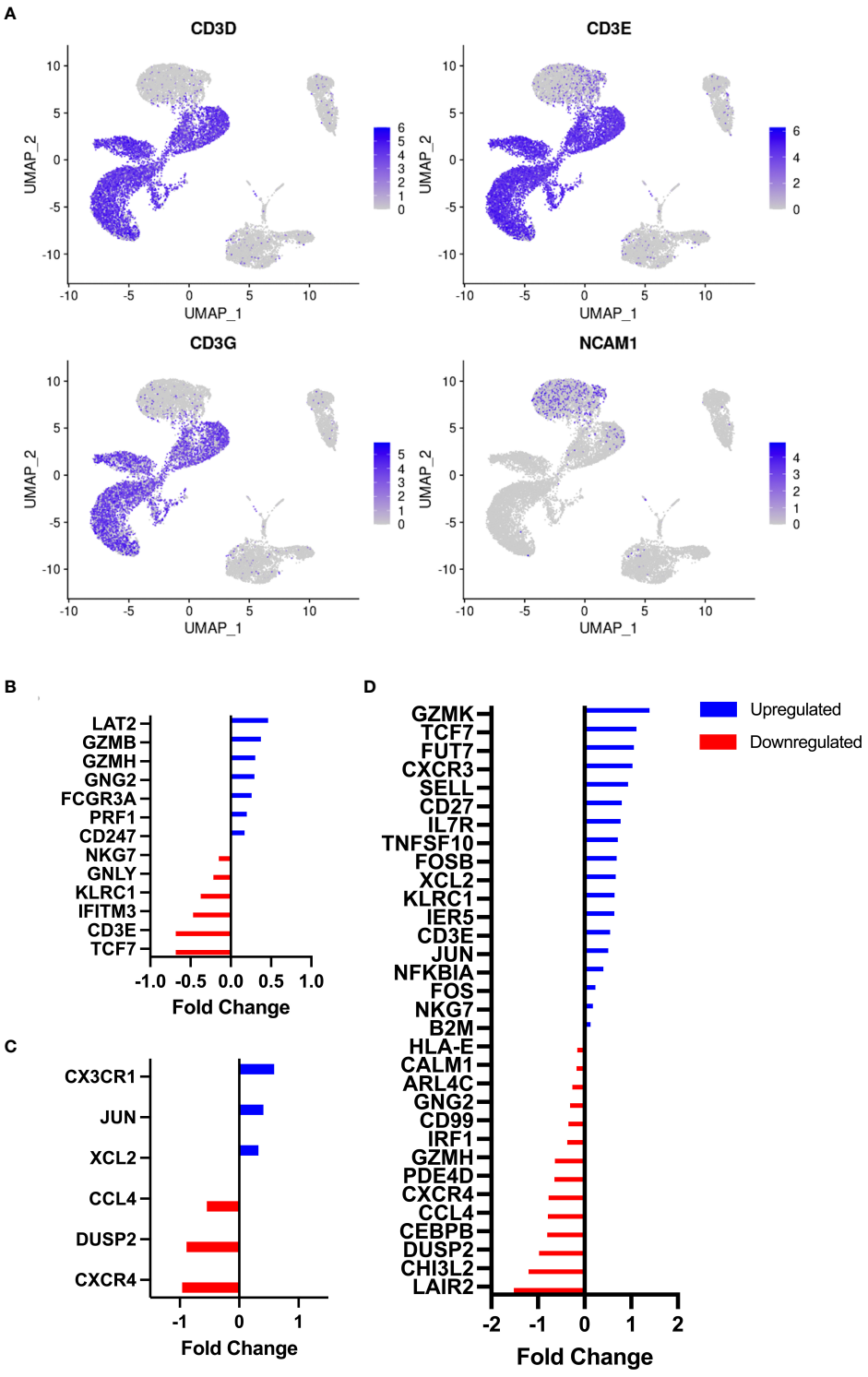

FIGURE 2
Identification of NKT-like cells by RNAseq and associated DEG analysis. **(A)** UMAP plots demonstrate clustering of CD3D, CD3E, and CD3G on resting PBMCs (n = 3). NKT-like cells were identified by the overlap of NK-cell marker NCAM1 and CD3 clusters. Differentially expressed genes on NKT-like cells were identified between timepoints, **(B)** Rest *vs* 80%, **(C)** Rest *vs* +1H, and **(D)** 80% *vs* +1H. Blue and red bars denote the upregulation and downregulation of DEGs, respectively. All are statistically significant ($p<0.05$).

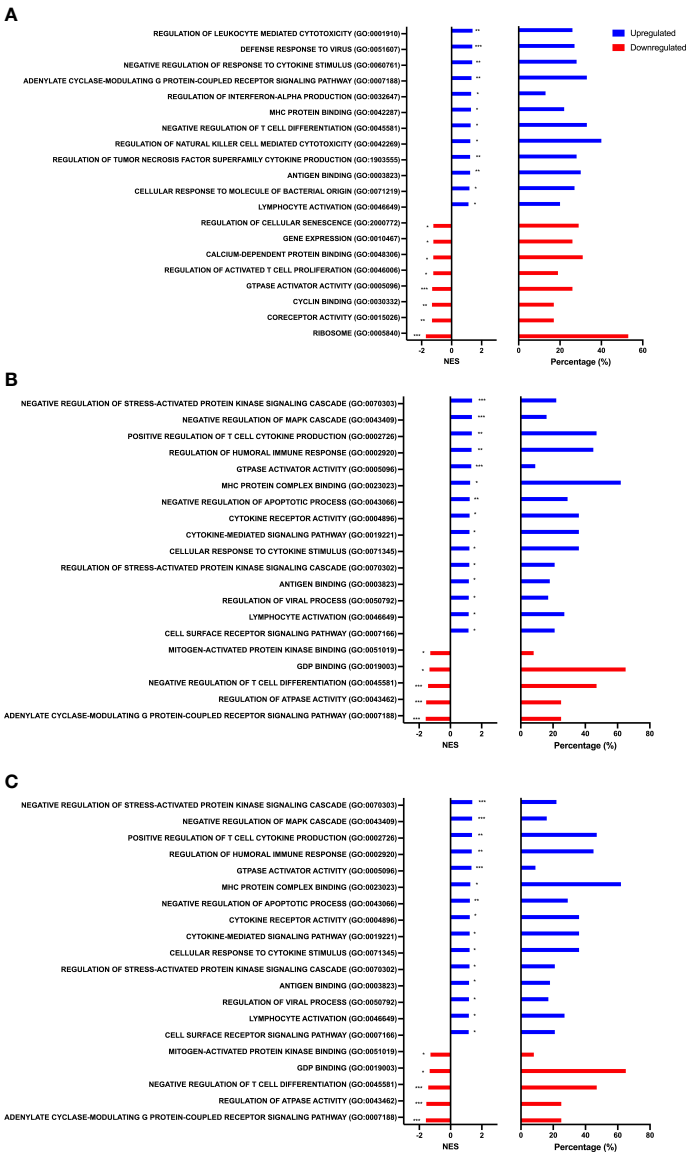

FIGURE 3
Gene set enrichment analysis performed using GO terms. Graphs show enriched upregulated (blue) and downregulated (red) pathways as well as the percentage of DEGs contributing to each mechanism between **(A)** Rest *vs* 80% **(B)** 80% *vs*. +1H, and **(C)** Rest *vs* +1H. Significance is indicated by * ($p < 0.25$), ** ($p < 0.10$), *** ($p < 0.05$).

cytokine receptor activity are also identified (Figure 5). Altogether, these analyses indicate that NKT-like cells mobilized during exercise exhibit transcriptomic profiles associated with greater cytotoxicity, anti-viral defense, and cytokine stimulus interaction.

Acute exercise does not augment the *ex vivo* expansion of CIK cells

To determine if exercise mobilized lymphocytes would result in enhanced proliferation and cytotoxic activity of CIK cells, we expanded CIK cells from PBMCs collected at rest, during exercise (60% and 80%) and during exercise recovery (+1H) (Figure 6). Following the described 21-day expansion protocol, CIK cells increased 50-100-fold across all exercise timepoints (Figure 6A). Fewer total CIK cells, however, were generated on day 21 utilizing PBMCs collected at 60% and 80% compared to both Rest and +1H, although not significantly different (Figure 6A). Comparably, CIK cells generated after 21 days relative to the number of PBMCs on day 0 was lower from PBMCs collected at 60% and 80% compared to Rest and +1H (Figure 6B). Additionally, CIK cells generated after 21 days relative to the number of NKT-like cells in PBMC

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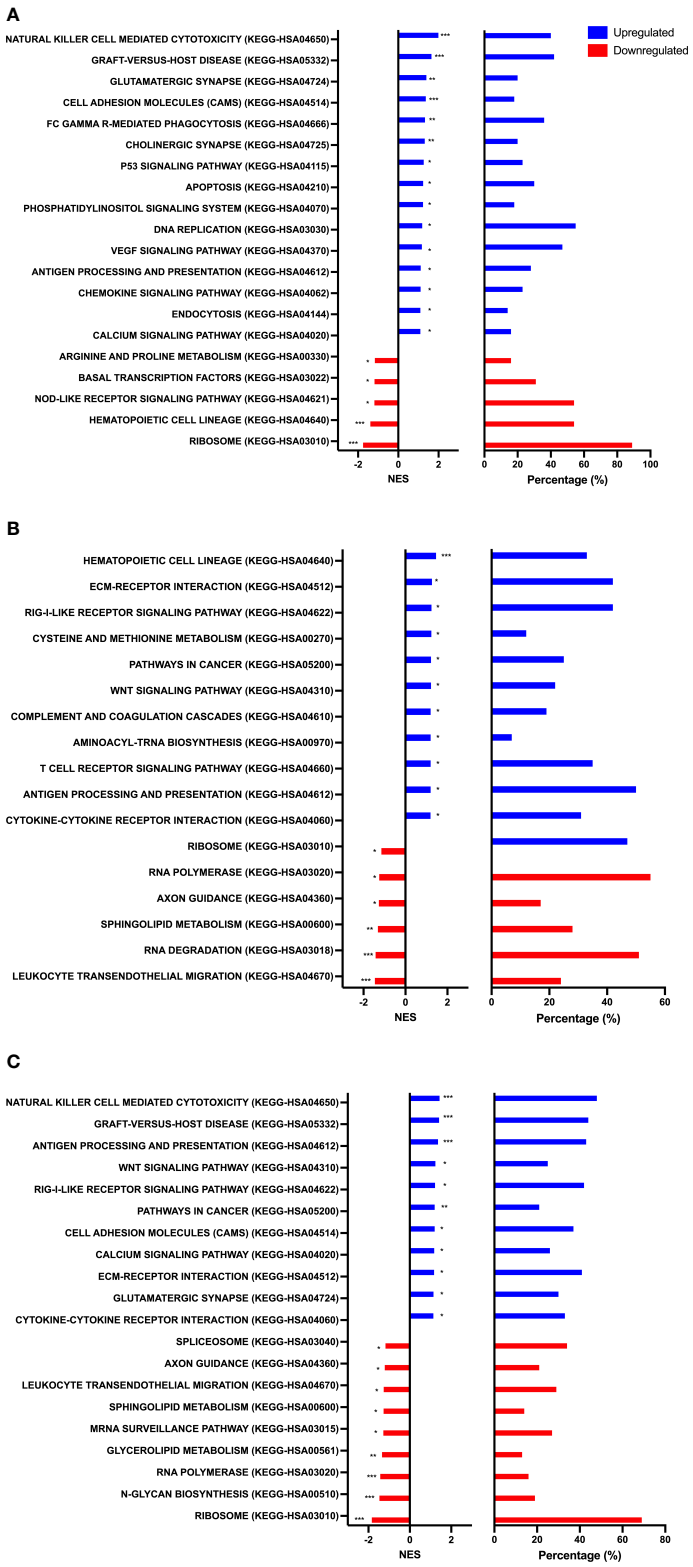

FIGURE 4
Gene set enrichment analysis performed using KEGG terms. Graphs show enriched upregulated (blue) and downregulated (red) pathways as well as the percentage of DEGs contributing to each mechanism between **(A)** Rest *vs* 80% **(B)** 80% *vs*. +1H, and **(C)** Rest *vs* +1H.Significance is indicated by * ($p < 0.25$), ** ($p < 0.10$), *** ($p < 0.05$).

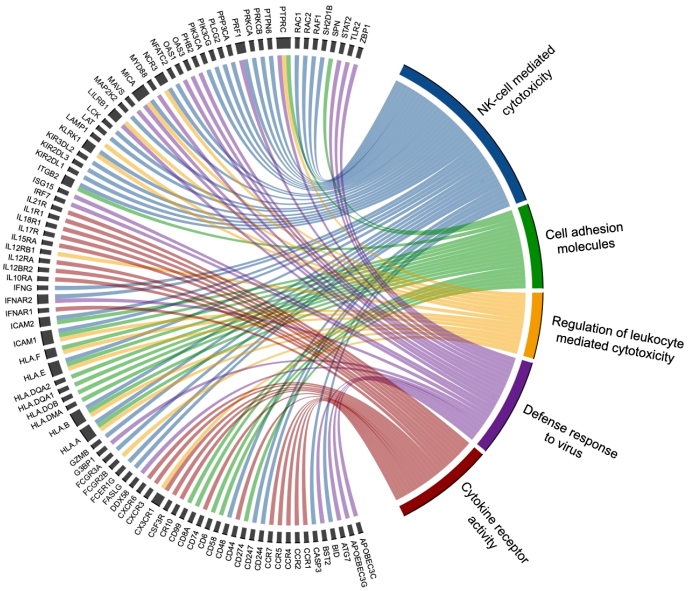

FIGURE 5
Leading edge genes are displayed in this chord diagram to indicate those that significantly drive the top selected GSEA pathways upregulated by exercise. The five biological processes with associated driving genes include NK-cell mediated cytotoxicity (blue), cell adhesion molecules (green), regulation of leukocyte mediated cytotoxicity (yellow), defense response to virus (purple), and cytokine receptor activity (red). Various genes are found to drive multiple pathways (e.g. perforin 1 [PRF1] drives NK-cell mediated cytotoxicity and defense to virus), while other genes are associated with one biological process.

fractions on day 0 was significantly lowest at 80% compared to Rest ($p = 0.01$, **Figure 6C**). These results indicate that exercise-mobilized lymphocytes do not have enhanced proliferation *in vitro* and thus do not result in a higher yield of *ex vivo* generated CIK cells from healthy donors.

Acute exercise does not affect the phenotype of *ex vivo* expanded CIK cells

Although exercise mobilized lymphocytes did not support enhanced *ex vivo* expansion of CIK cells, it was important to determine if the expanded products differed phenotypically. We performed a comprehensive phenotypic analysis on the expanded CIK cell products, focusing on surface markers related to cytotoxicity, homing, and activation (**Figure 6E**, **Supplementary Figure 1**). It has been shown previously that the percentage of $CD56^+$ cells among expanded CIK cells is positively associated with their potency against leukemic targets (27–29). Although the proportion of $CD56^+$ cells among our expanded CIK cell products (19-22%) is consistent with previous studies, exercise did not alter the composition of CD56+ cells among the expanded cell products. Furthermore, we found no differences in the expression of surface markers associated with CIK cell function, including NKG2D, DNAM-1, NKp30, and CD178 (FasL). We did, however, find that the terminal differentiation marker $CD57^+$ was significantly elevated ($p > 0.03$) on CIK cells expanded from PBMCs collected during the 60% exercise intensity when compared to all other exercise timepoints (**Figure 6E**) (4, 30). Overall, these findings indicate that exercise does not modify the surface phenotypes of expanded CIK cell products.

Acute exercise does not augment *in vitro* cytotoxicity of expanded CIK cells

Despite no discernible phenotypic differences between CIK cells expanded using resting and exercise mobilized lymphocytes, it was important to test for potential differences in cytotoxic function. The cytotoxic activity of expanded CIK cells was determined *in vitro* against three tumor cell lines, K562, U266, and Daudi (**Figure 7**). We also examined the effect of adding IL-2, OKT-3, or both (IL-2 + OKT-3) *in vitro* (**Figures 7B–D**), as they can augment the cytotoxic function of CIK cells (28, 31). Overall, CIK cells seemed to exhibit higher killing against K562 compared to U266 and Daudi cells (**Figure 7A**). However, CIK cell lytic activity against all three tumor cell lines was similar across the exercise time points (**Figure 7A**). Adding IL-2 or OKT-3 to the cultures did not increase the potency of the CIK cytolytic activity in cells expanded during or after exercise ($p>0.05$). These findings indicate that, in addition to not altering the phenotype of the expanded products, CIK cells expanded using exercise mobilized lymphocytes do not differ in their ability to kill hematologic target cells *in vitro*.

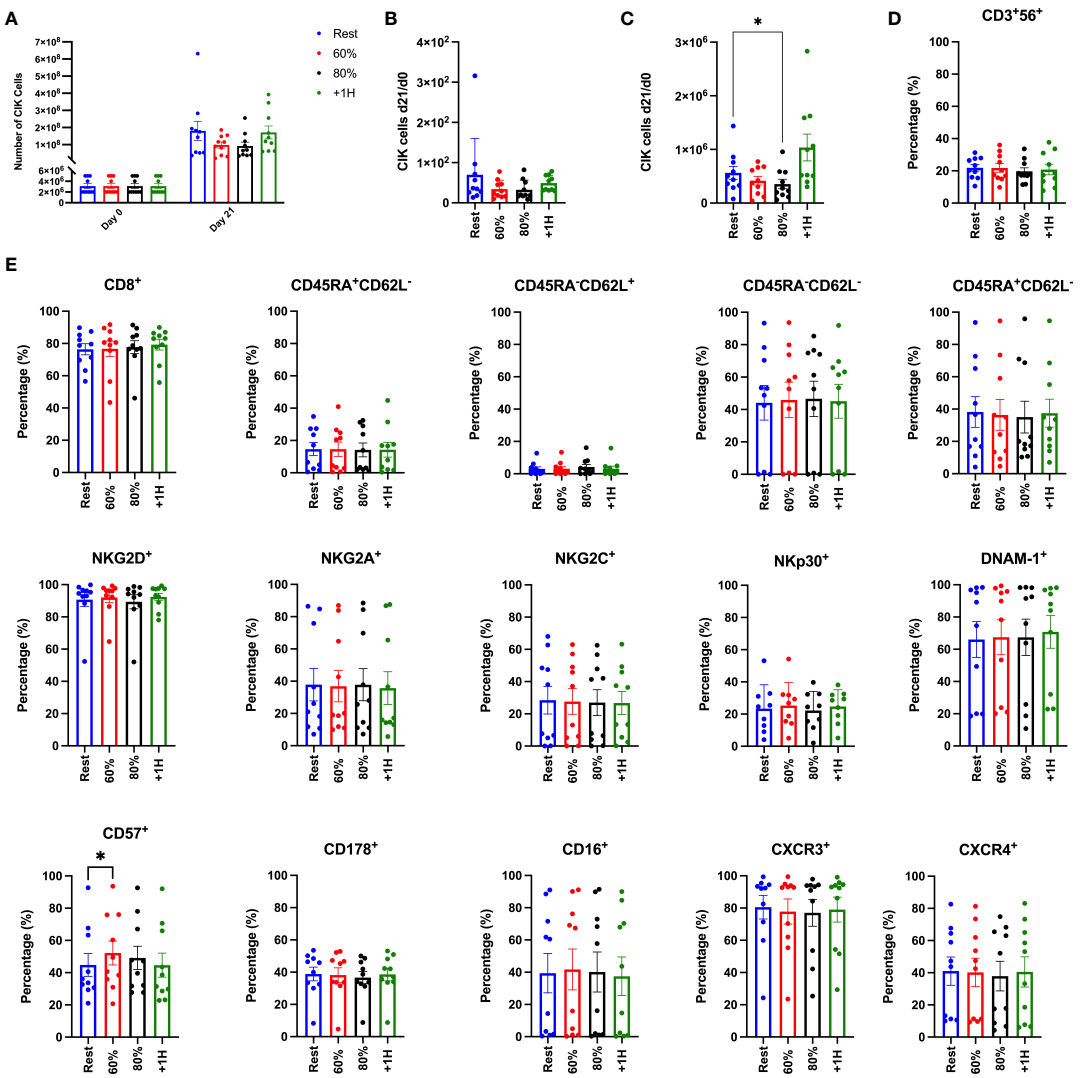

FIGURE 6
Enumeration and associated phenotypes of *ex vivo* expanded CIK cells. **(A)** The total number of PBMCs utilized for expansion on Day 0 and the total number of CIK cells generated in the expanded cell products after 21 days (n = 10). **(B)** The number of CIK cells generated at Day 21 divided by the number of CIK cells in the PBMC fraction at Day 0. **(C)** The number of CIK cells generated at Day 21 divided by the number of CIK cells in the NKT-cell fraction at Day 0. **(D)** The proportion of $CD3^+CD56^+$ expression on the total CIK cell population. **(E)** The percentage of surface markers expressed on the $CD3^+CD56$ CIK cell population on day 21. Differentiated T-cells are phenotyped as follows: naïve ($CD45RA^+CD62L^+$), central memory (CM; $CD45RA^-CD62L^+$), effector memory (EM; $CD45RA^-CD62^-$), terminally differentiated effector memory (EMRA; $CD45RA^+CD62L^-$). Surface expression was determined by flow cytometry and error bars are represented as mean ± SEM. Significance is indicated by *($p < 0.05$).

Discussion

CIK cells are candidates for adoptive immunotherapy due to their accessibility from donors, manufacturing simplicity, high proliferative capacity, and non-MHC-restricted anti-tumor function. While CIK cells have been met with mixed results in clinical trials (10, 11), several groups have attempted to optimize their expansion and potency (32, 33). We attempted to use acute exercise as a simple intervention to augment the manufacture of CIK cells from healthy donors. We report, for the first time, that NKT-like cells, considered precursors to CIK cells, mobilized to the blood with exercise display transcriptomic profiles associated with enhanced cytotoxicity, anti-viral defense, and cytokine responsiveness. Despite this, exercise mobilized lymphocytes did not alter the phenotype or cytotoxic function of CIK cells expanded in culture over a 21-day period.

Exercise-mobilized leukocytes have been purported to offer a better source of therapeutic cell products for the treatment of both

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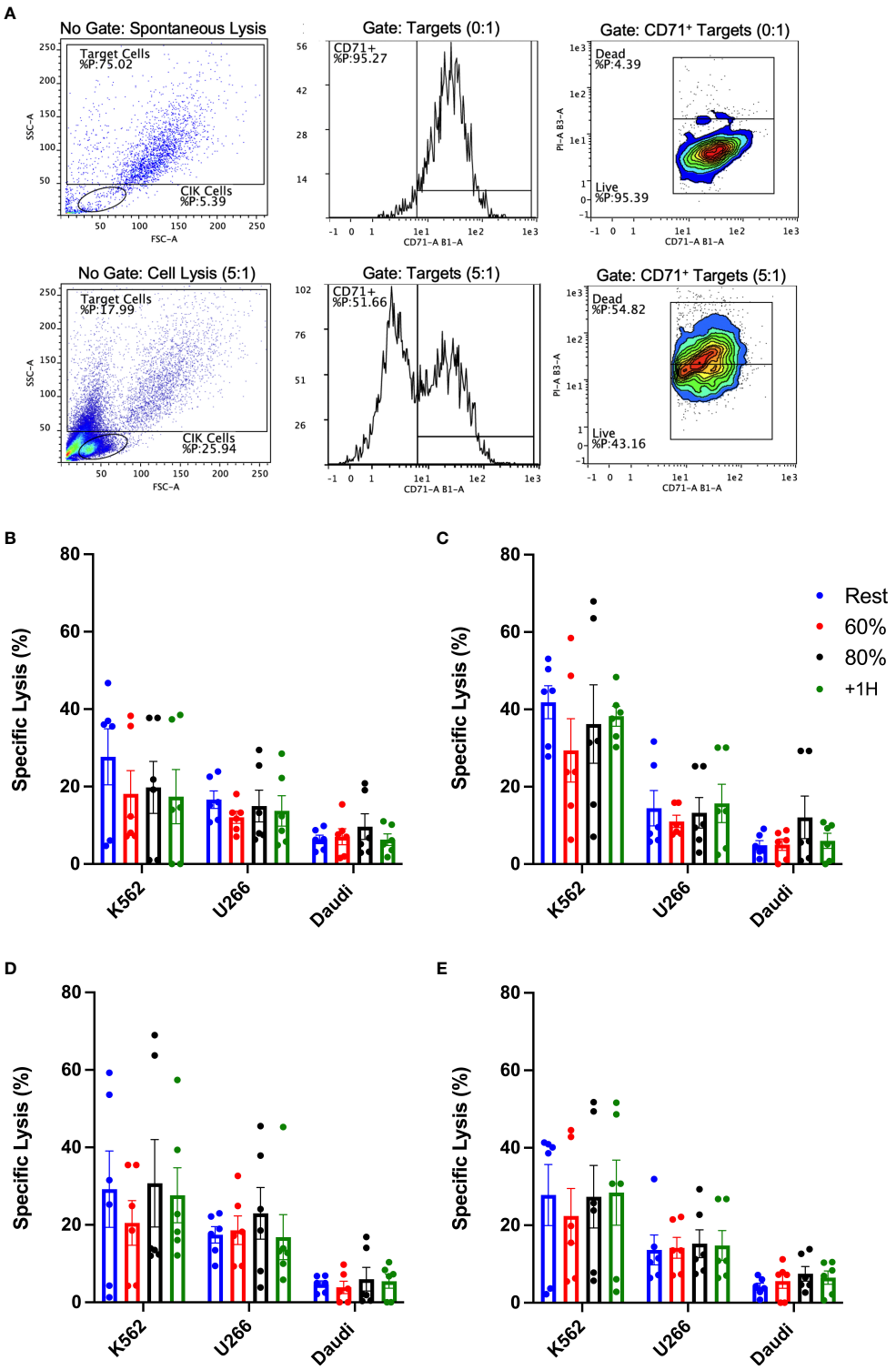

FIGURE 7
Exercise and cytokine stimulation does not augment anti-tumor activity of CIK cells against various cell targets *in vitro*. Cytotoxicity of CIK cells was assessed *via* flow cytometry-based assays against K562, U266, and Daudi cells at a E:T ratio of 5:1 (n = 6). **(A)** Representative flow cytometry plots illustrate the gating strategy utilized to determine specific lysis ($CD71^+/PI^+$). Expanded CIK cells were stimulated in **(B)** media alone or media supplemented with **(C)** 300IU/mL IL-2, **(D)** 5 ng/mL OKT-3, or **(E)** a combination of IL-2 and OKT-3. Error bars are represented as mean ± SEM. There were no significant differences found in lytic function between exercise timepoints.

cancers and viral infections (34). Several studies, mostly from our group, have found that exercise-mobilized cells allow for improvements in the *ex vivo* manufacture of viral-specific T-cells, tumor-antigen specific T-cells and TCR-γδ T-cell products (14–16). As exercise preferentially mobilizes effector lymphocytes, including T-cells with a $CD3^+CD56^+$ phenotype (35), we hypothesized that exercise would also have adjuvant effects in manufacturing CIK cells that could be utilized more effectively in treating blood cancers. We found that NKT-like cells were mobilized to blood during both moderate (60% VO_{2max}) and vigorous (80% VO_{2max}) intensity exercise, falling below resting values 1 h later. As lymphocytes mobilized with exercise have phenotypes associated with cytotoxicity, migration, and antigen experience (16, 20), we performed a deep transcriptomic analysis of NKT-like cells mobilized with exercise at the single cell level. Various genes associated with cytotoxicity such as GZMB, GZMH, and PRF1 were upregulated in NKT-like cells mobilized with exercise. Similarly, expression of genes involved in effector lymphocyte inhibition such as KLRC1 were downregulated. These findings indicate that NKT-like cells mobilized with exercise have the potential for enhanced cytotoxic function compared to those in resting blood. Indeed, gene set enrichment analysis to GO and KEGG terms revealed an upregulation in processes associated with lytic function, anti-viral responses, and cytokine receptor activation. These findings indicate that exercise could be used to enhance both the function and redistribution of NKT-like cells, which could have implications for cancer patients that exhibit distinct functional impairments within this immune cell population (36–38). Indeed, dysfunction in NKT-like cells have been correlated with poor prognosis and patient outcomes in gastric cancer (39) as well as CLL progression (39, 40). The use of therapeutics such as tyrosine kinase inhibitors and immune checkpoint inhibitors have been proposed to restore functionality of NKT-like cells and increase cytotoxicity (37, 38). Whether exercise, either alone or in combination with other therapeutics, can increase the function of NKT-like cells in cancer patients that elicit improvements in prognosis or outcomes remains to be determined.

The adoptive transfer of donor-derived CIK cells alone or in combination with other immunotherapies have been found to significantly improve survival and quality of life in patients with solid tumors (8). Conversely, their effectiveness against hematological malignancies such as AML and CML have been less successful (10, 41). NKT-like cells mobilized to blood during exercise displayed transcriptomic profiles indicative of increased anti-tumor function. Despite this, we did not find improvements in phenotype or potency when exercise mobilized lymphocytes were used as the source of our expanded CIK cell products. Overall, CIK cells expressed CD8 and were characterized as effector memory or terminally differentiated effector memory cells, corroborating previous findings (27). Interestingly, CIK cells expanded from the 60% exercise intensity stage had greater $CD57^+$ surface expression compared to all other time points. CD57 expression reflects NK-cell maturation and typically increases with age or chronic infections, such as CMV (42). While cells that express CD57 still sustain their cytotoxic abilities, proliferative capacity and cytokines responsiveness is reduced (42). This may explain the slight decrease in the number of CIK cells we were able to expand when using exercise-mobilized lymphocytes. Moreover, our functional annotation and enrichment analysis revealed a downregulation of gene sets associated with activated T-cell proliferation on NKT-like cells mobilized with exercise, indicating that CIK precursor cells mobilized with exercise, despite having increased cytotoxic potential, may have reduced proliferative capacity. Although NKT-like cells mobilized to blood with exercise exhibited an upregulation of individual genes and enriched gene sets associated with cytotoxicity, the *in vitro* cytotoxicity of expanded CIK cells against three hematological target cell lines was unaffected by exercise. Why acute exercise can boost the *ex vivo* manufacture of certain lymphocyte products (e.g. TCR-γδ T-cells, VSTs) but not CIK cells is yet to be determined. It is possible that consistent stimulation with high concentrations of cytokines over an extended period may override any beneficial effect of using exercise-mobilized lymphocytes with altered phenotypic and transcriptomic profiles. Despite this, the *in vivo* effects of exercise on NKT-like cells and other lymphocytes could have adjuvant effects for 'untouched' adoptive cell therapies, including standard donor lymphocyte infusions whereby lymphocytes are collected from donors and transferred to patients without any *ex vivo* manipulation (43), but this has yet to be determined.

A limitation of the present study includes the use of only one protocol to expand CIK cells. Although expansions were successful across all exercise timepoints, several studies have modified culture conditions to improve the proliferation and functional capacity of CIK cell products (32, 33, 44–46). For example, previous groups have determined that activated CIK cells stimulated with IL-15 exhibit enhanced cytotoxic potential against ALL and lymphoma cell lines (33, 46), therefore there is a possibility that exercise could have boosted CIK cell manufacture using different stimuli and cell culture conditions. Additionally, other groups have hypothesized that while $CD3^+CD56^+$ cells maintain expression and functionality after expansion, outgrowth of CIK cells may stem from $CD3^+CD56^-CD4^-CD8^+$ and $CD3^+CD56^-CD4^-CD8^-$ precursors (3, 27). Consequently, the exercise-induced transcriptomic shifts observed in NKT-like cells reported here may not have translated to the performance of the expanded CIK cell products. Moreover, it is possible that our small sample size prevented us from observing an exercise effect on the numbers and potency of expanded CIK cells. However, given that a

statistical power analysis estimated a sample size of 280 individuals, any positive effect of exercise is likely to be small. It is also possible that exercise expanded CIK cells will be more potent against other hematologic cancer cell lines not used in this study.

In summary, this is the first study to provide a detailed single cell transcriptomic analysis of NKT-like cells mobilized to blood with exercise, revealing that exercise mobilized cells display transcriptomic profiles associated with enhanced lytic function, migration, cytokine signaling interaction, and overall activation. These findings add to the large body of evidence indicating that exercise mobilizes effector lymphocytes with potent anti-tumor potential (34). While exercise did not alter the phenotype or increase the potency of *ex vivo* generated CIK cells, future studies may consider the effects of exercise on NKT-like cells that may increase the effectiveness of unmanipulated donor lymphocyte infusion products for patients with hematological malignancies.

Data availability statement

The data presented in the study are deposited in the GEO repository, accession number GSE212740.

Ethics statement

The studies involving human participants were reviewed and approved by University of Arizona IRB. The patients/participants provided their written informed consent to participate in this study.

Author contributions

TMZ, RJS and EK developed the theoretical framework, working hypotheses, and designed the study. TMZ, FLB, KAS and HB performed laboratory experiments. TMZ, FLB, and BL analyzed results. TMZ, RJS, FLB, and EK interpreted data. TMZ and RJS wrote the manuscript with contributions from FLB, KAS, HB, BL, MPG, and EK. The statistical analyses were performed by TMZ, BL and FLB. The overall study was supervised by RJS. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.938106/full#supplementary-material

SUPPLEMENTARY TABLE 1
List of directly conjugated antibodies used to label expanded CIK cells on day 21 *via* flow cytometry.

SUPPLEMENTARY TABLE 2
Differentially expressed genes in NKT-like cells in response to exercise as determined by single cell RNAseq. GeneCards®: the human gene database was utilized to identify gene name and function (26). Upwards arrows (↑) indicate differentially expressed gene was upregulated between timepoints; downwards arrow (↓) indicates differentially expressed gene was downregulated between timepoints; double-sided arrows (↔) indicate there was no significant change in gene expression between timepoints.

SUPPLEMENTARY FIGURE 1
All graphs represent percentage of surface marker expression on the $CD3^+CD56^+$ CIK cell population on day 21 (n = 10). Expression was determined by flow cytometry and error bars are represented as mean ± SEM. Significance is indicated by * ($p < 0.05$).

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Aerobic exercise alleviates pyroptosis-related diseases by regulating NLRP3 inflammasome

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Pyroptosis plays a crucial role in a variety of human diseases, including atherosclerosis, obesity, diabetes, depression, and Alzheimer's disease, which usually release pyroptosis-related cytokines due to inflammation. Many studies have demonstrated that aerobic exercise is a good option for decreasing the release of pyroptosis-related cytokines. However, the molecular mechanisms of aerobic exercise on pyroptosis-related diseases remain unknown. In this review, the effects of aerobic exercise on pyroptosis in endothelial cells, adipocytes and hippocampal cells, and their potential mechanisms are summarized. In endothelial cells, aerobic exercise could inhibit NOD-like receptor protein 3 (NLRP3) inflammasome-mediated pyroptosis by improving the endothelial function, while reducing vascular inflammation and oxidative stress. In adipocytes, aerobic exercise has been shown to inhibit pyroptosis by ameliorating inflammation and insulin resistance. Moreover, aerobic exercise could restrict pyroptosis by attenuating microglial activation, neuroinflammation, and amyloid-beta deposition in hippocampal cells. In summary, aerobic exercise alleviates the pyroptosis-related diseases by regulating the NLRP3 inflammation si0067naling.

KEYWORDS

aerobic exercise, pyroptosis, pyroptosis-related diseases, mechanisms, NLRP3 inflammasome

Introduction

Pyroptosis, a type of lytic programmed cell death caused by inflammasomes, is an important natural immune response in our body (Kovacs and Miao 2017). Pore formation in the plasma membrane, swelling and rupture of cells, massive leakage of cytoplasmic contents, and release of inflammatory factors are typical features of pyroptosis (Man et al., 2017). Pyroptosis is induced by the NOD-like receptor protein 3 (NLRP3) inflammasome, and triggered by Caspase-1 (Tang et al., 2020), which controls the N-terminal domain of gasdermin D (GSDMD) by assembling channels in the cell membrane and activates interleukin (IL)-1β and IL-18 (Schroder and Tschopp 2010) (Figure 1).

Pyroptosis occurs in multiple cell types (Shi et al., 2017), including endothelial cells, adipocytes and hippocampal cells. Many studies have suggested that pyroptosis

takes an important role in the development of human diseases, including obesity (Mardare et al., 2016), diabetes (Vandanmagsar et al., 2011), atherosclerosis (Hong et al., 2021), Alzheimer's disease (AD) (Liang et al., 2020), and depression (Liu et al., 2015). Aerobic exercise exhibits an obvious anti-inflammatory effect and is closely related to pyroptosis (Kar et al., 2019). As it is known, aerobic exercise could reduce chronic inflammation and effectively inhibit the expression of inflammatory factors, thereby increasing the release of anti-inflammatory cytokines. Previous studies have found that aerobic exercise could decrease the expression of NLRP3 inflammasome and markedly inhibit the activation of ASC, Caspase-1, IL-1β, and IL-18 (Kar et al., 2019; Lee et al., 2020). Although aerobic exercise can regulate cell pyroptosis, its specific effects on pyroptosis-related diseases and potential mechanisms still need further clarification.

The present review aimed to identify the relationship between aerobic exercise and NLRP3 inflammasome-mediated pyroptosis in endothelial cells, adipocytes, and hippocampal cells, and to investigate the potential mechanism of the effect of aerobic exercise on pyroptosis-related diseases.

Aerobic exercise and endothelial cell pyroptosis-related diseases

Endothelial cell's pyroptosis is among the major causes of cardiovascular diseases (Zhang L. et al., 2019). Aerobic exercise is an important strategy to control the endothelial cell's pyroptosis, and inhibiting the development of cardiovascular diseases.

Endothelial cell's pyroptosis and its related diseases

Endothelial cells are considered to be an important modulator in vascular homeostasis, regulated by various paracrine factors, and they play a critical role in maintaining normal vascular tension and blood flow and in inhibiting vascular inflammation and oxidative stress. Endothelial dysfunction is a classical symbol and predictor of cardiovascular diseases (Bai et al., 2020), and pyroptosis confers a decisive contribution to vascular endothelial dysfunction during the development of related diseases. Previous studies have suggested that endothelial cell's pyroptosis was associated with cardiovascular diseases,

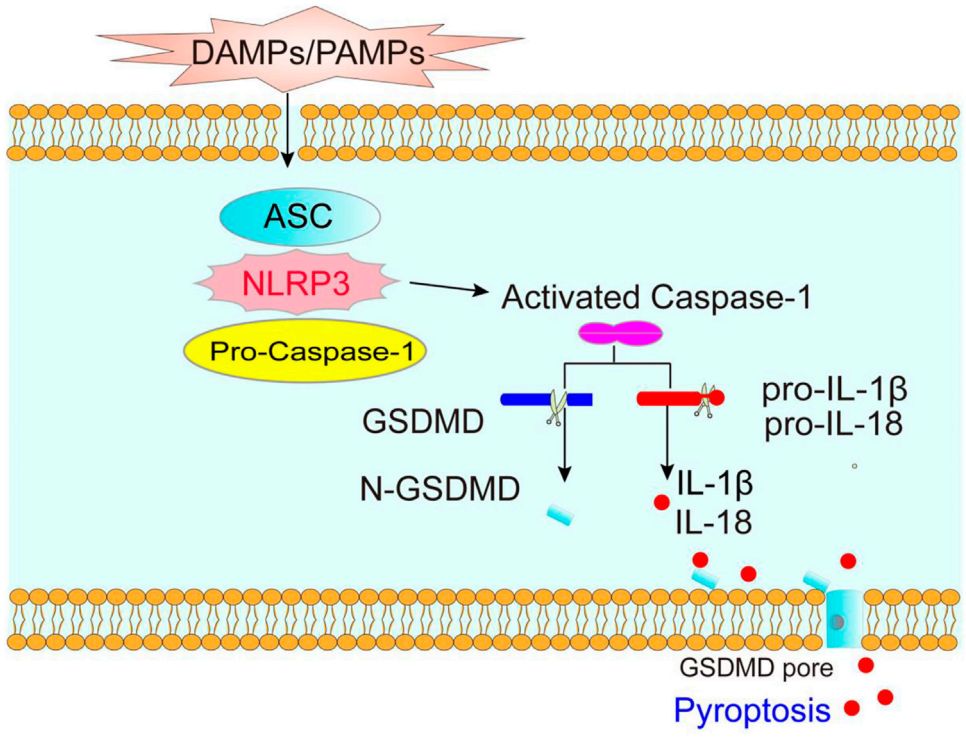

FIGURE 1
The molecular mechanism of pyroptosis. DAMPs (danger-associated molecular patterns) and PAMPs (pathogen-related molecular patterns) activate NLRP3 inflammasome, promotes Caspase-1 activation, which cleavages GSDMD and the precursor of IL-1β and IL-18, forming mature IL-1β and IL-18, thereby causing pyroptosis.

including atherosclerosis (Zhang L. et al., 2019) and hypertension (Wu et al., 2022). Besides, the activation of NLRP3, ASC, Caspase-1, and GSDMD is increased significantly in atherosclerotic endothelial cells (Zhang et al., 2018). Furthermore, NLRP3 inflammasome, Caspase-1, and IL-1β trigger inflammation in the blood vessel wall, thereby leading to atherosclerosis (Karasawa and Takahashi 2017). Oxidized low-density lipoprotein (ox-LDL) and cholesterol crystals are abundant in atherosclerotic lesions (Zhang et al., 2015; Keping et al., 2020). Ox-LDL (Keping et al., 2020) and cholesterol crystals (Duewell et al., 2010; Zhang et al., 2015) could also promote NLRP3 inflammasome and Caspase-1 activation, leading to the release of IL-1β and IL-18 in immune cells. Especially, NLRP3 inflammasome promotes plaque formation and contributes to the development of atherosclerosis by affecting several targets, including signal transducer and activator of transcription (STAT), mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinase (JNK), microRNA network, reactive oxygen species (ROS), and protein kinase R (PKR) (Hoseini et al., 2018). Thus, endothelial cell's pyroptosis contributes to atherosclerosis formation and development by accelerating the release of inflammatory cytokines and increasing the vascular permeability (Zhaolin et al., 2019).

Additionally, NLRP3-dependent pyroptosis mediates endothelial dysfunction, which provides an impetus for hypertension (Pasqua et al., 2018), cardiovascular complications of coronary heart disease, and atherosclerosis in endothelial cells. The study highlighted that pyroptosis is a significant mediator of vascular dysfunction and injury in hypertensive patients (De Miguel et al., 2021). The serum level of IL-1β was higher in patients with essential hypertension than in healthy persons (Zeng et al., 2019). Besides, the research shows that the downregulation of the expression of key components of the NLRP3 inflammasome can delay the development of hypertension (De Miguel et al., 2021). The study found that microcrystals, and high levels of extracellular ATP and ROS could activate the NLRP3 inflammasome in the hypertensive patients (Krishnan et al., 2014). Overall, the endothelial cell's pyroptosis is closely associated with the development of cardiovascular diseases. Aerobic exercise is an ideal non-drug management to inhibit endothelial cell's pyroptosis and takes an essential role in treating cardiovascular diseases.

Effect of aerobic exercise on endothelial cell's pyroptosis

Aerobic exercise is beneficial for maintaining the function of vascular endothelial cells (Kourek et al., 2021). Notably, aerobic exercise could significantly alleviate the endothelial dysfunction and reduce the risk of cardiovascular diseases (Neunhäuserer et al., 2021). The study also showed that aerobic exercise could increase the blood flow and laminar shear stress as well as reduce leukocyte adhesion (You et al., 2013), and risk of inflammation, thereby improving the antioxidant system of enzymes and immune responses. Many studies have found that endothelial cell's pyroptosis can be inhibited by aerobic exercise (Lee et al., 2018; Lee et al., 2020). Lee et al. (2018) have proved that voluntary running could reduce the activation of NLRP3 inflammasome in the endothelial cells of the coronary arteries. Their findings further suggested that aerobic exercise improves the vascular function by inhibiting NLRP3 inflammasome signaling (Lee et al., 2020). Other studies also reported that treadmill exercise of >12 weeks could reduce the endothelial cell's pyroptosis in arteriosclerosis (Hong et al., 2018; Hong et al., 2021) (as shown in Table 1).

Aerobic exercise reduces vascular inflammation

Accumulating evidence has demonstrated that NLRP3 inflammasome plays a vital role in vascular inflammation (Wang L. et al., 2016). In endothelial cells, NLRP3 inflammasomes could be activated in response to multiple stimuli and are involved in vascular pathology (Lee et al., 2020).

Stimuli, including oxidative stress, mitochondrial dysfunction and lysosomal rupture have been demonstrated to activate the NLRP3 inflammasomes (Hoseini et al., 2018), which are important initiators in the development of vascular diseases. Moreover, ox-LDL and cholesterol crystals stimulate nuclear factor-κB (NF-κB) activation and TNF-α secretion (Steyers and Miller 2014). Then, the activated NF-κB further affects the NLRP3 signaling and contributes to the development of atherosclerosis (Hoseini et al., 2018). Studies have demonstrated that 12 weeks of treadmill exercise could down-regulate NF-κB protein expression and inhibit NF-κB-mediated aortic inflammation in participants (Wu et al., 2017).

Additionally, NLRP3 inflammasomes can be activated by the thioredoxin-interacting protein (TXNIP), which plays a crucial role in inflammatory response (Byon et al., 2015). The TXNIP/NLRP3 inflammasome signaling is closely associated with the development and progression of atherosclerosis (Hoseini et al., 2018). The activated NLRP3 inflammasome could increase the expression and release of the high-mobility histone box-1 (HMGB1) in endothelial cells (Lee et al., 2020), promoting endothelial hyperpermeability and leading endothelial dysfunction (Wang L. et al., 2016; Wang et al., 2016a). Several studies have demonstrated that aerobic exercise can significantly reduce vascular inflammation by inhibiting NLRP3 inflammasome, HMGB1, and its downstream effects (Goh and Behringer 2018; Kar et al., 2019; Lee et al., 2020).

TABLE 1 Effects of aerobic exercise on pyroptosis-related factors in different cells.

Cell types	Object	Diseases	Exercise pattens	Effect of exercise	References
Endothelial cells	Mouse	Atherosclerosis	Treadmill training (12 weeks)	eNOS↑, Caspase-1↓	Hong et al. (2018)
Aortic endothelial	Mouse	Obesity	Voluntary wheel running (12–14 weeks)	IL-1β↓, NLRP3↓, Caspase-1↓, Oxidative stress↓	Lee et al. (2020)
Endothelial cells	Mouse	Atherosclerosis	Treadmill training (12 weeks)	NADPH↓, TXNIP/NLRP3↓, Oxidative stress↓	Hong et al. (2021)
Adipose tissue	Human	T2DM	Calorie restriction and exercise (1 year)	IL-1β↓, NLRP3↓, Caspase-1↓, IL-18↓	Vandanmagsar et al. (2011)
Adipose tissue	Human	T2DM and coronary artery disease	Endurance training combined with resistance training (1 year)	IL-18 = , Caspase1 = , NLRP3 = , Circulating IL-18↓	Zaidi et al. (2019)
Adipose tissue	Mouse	Obesity	Treadmill training (10 weeks)	IL-1β↓, IL-18↓, TNF-α↓	Mardare et al. (2016)
Adipose tissue	Mouse	HFD rats	Treadmill training (8 weeks)	NLRP3↓, FGF2↓	ZhuGe et al. (2020)
Prefrontal cortex	Mouse	Depression	Swimming (4 weeks)	NLRP3↓, Leptin↑	Liu et al. (2015)
Hippocampus	Mouse	Depression like behavior rats	Treadmill training (4 weeks)	IL-1β↓, NLRP3↓, Caspase-1↓, IL-18↓, Body weight↓	Wang et al. (2016b)
Hippocampus	Mouse	HFD-induced obese rats	Treadmill training (8 weeks)	IL-1β↓, NLRP3↓, Nrf2/Ho-1↑, BDNF↑	Cai et al. (2016)
Hippocampus	Mouse	T2DM rats	Treadmill training (4 weeks)	IL-1β↓, NLRP3↓, PI3K/AKT/mTOR↑, AMPK/Sirt↑, NF-κB/NLRP3/IL-1β↓	Li et al. (2019)
Prefrontal cortex	Mouse	Diabetic Rats	Treadmill training (4 weeks)	NLRP3↓, PI3K/AKT↑, NF-κB↓	Wang et al. (2019)
Hippocampus	Mouse	Alzheimer disease	Treadmill training (12 weeks)	NLRP3↓, IL-1β↓, Caspase-1↓, ASC↓	Liang et al. (2020)
Hippocampus	Mouse	Post-stroke drepression	Treadmill training (4 weeks)	NLRP3↓, TLR4↓, NF-κB↓	Li et al. (2020)
Hippocampus	Mouse	Alzheimer disease	Treadmill training (4 weeks)	NLRP3↓, TXNIP↓, Caspase-1↓, ASC =	Rosa et al. (2021)
Brains	Mouse	Parkinson's disease	Treadmill training (6 weeks)	IL-1β↓, NLRP3↓, Caspase-1↓, Oxidative stress↓, TLR4↓, NF-κB↓, ASC↓	Wang et al. (2021)
Neuronal tissue	Mouse	Hyperlipidemia	Swimming (12 weeks)	NLRP3↓, IL-18↓, Caspase-1↓	Bai et al. (2021)

Note: "↓" indicates that its expression can be downregulated by exercise, "↑" indicates that it can be enhanced by exercise, "=" indicates that the change is not obvious by exercise.

Aerobic exercise improves endothelial cell function

Vascular elasticity is regulated by generating many potent vasoactive substances, including vasodilator nitric oxide (NO) and contractile factor endothelin-1 in endothelial cells (Haybar et al., 2019). NO is a vasomotor factor produced and released by vascular endothelial cells, which has an important protective effect on the vascular wall and endothelial function (Ferentinos et al., 2022). NO bioavailability refers to the production and utilization of NO in endothelial cells, which is closely related with endothelial dysfunction. The reduction of NO bioavailability reportedly resulted from oxidative stress and expression of inflammatory factors (Chen et al., 2018). Similarly, a previous study has found that NO inhibits NLRP3 activation, thereby preventing pyroptosis in endothelial cells (Jiang et al., 2020).

Aerobic exercise is a promising non-medical treatment for preventing early endothelial dysfunction and redox imbalance by increasing NO bioavailability and reducing chronic inflammation (Gao et al., 2021). Moreover, aerobic exercise can effectively increase the NO content and enhance the diastolic function of vascular endothelial cells (Gao et al., 2021). NO can further increase the blood flow in the body during aerobic exercise.

In summary, aerobic exercise could regulate NO production and bioavailability to improve the endothelial cell's function. Firstly, aerobic exercise increased the NO bioavailability by enhancing phosphorylated eNOS expression and reversing aortic endothelial dysfunction. In the vascular endothelium, aerobic exercise improves the NO bioavailability by enhancing endothelial NO synthase (eNOS) expression and eNOS/NO signaling (Lee et al., 2018), decreasing oxidative stress and inflammatory pathways. Aerobic exercise can enhance the heart's pumping function, increase the heart's output, accelerate the blood flow and blood shear stress, thereby stimulating the NO synthesis by vascular endothelial cells (Inoue et al., 2020). Secondly, aerobic exercise could improve the NO production by aggrandizing adiponectin (APN) and AdipoR1 levels (Lee et al., 2020). Thirdly, aerobic exercise can elevate the expression of junction proteins zonula occludin-1 (ZO-1) and ZO-2 (these are associated with endothelial permeability and dysfunction (Wang L. et al., 2016)) in endothelial cells, thereby facilitating NO production (Lee et al., 2020). Lastly, aerobic exercise induces the activity of

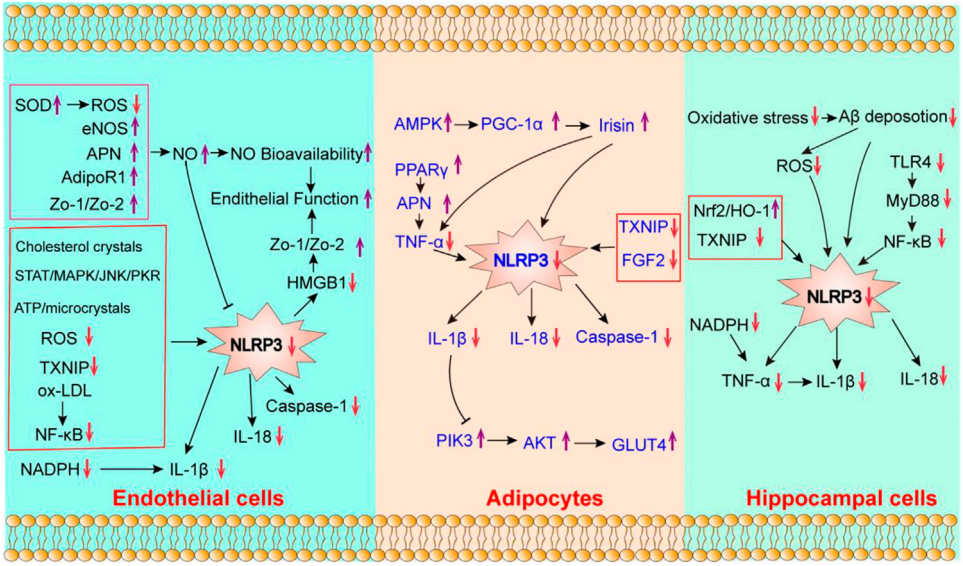

FIGURE 2
The potential effect of aerobic exercise on the pyroptosis of endothelial cells, adipocytes and hippocampal cells. "↓" indicates that its expression can be downregulated by aerobic exercise; "↑" indicates that it can be enhanced by aerobic exercise.

superoxide dismutase (SOD), which results in the decrease in ROS production and ultimately improves the generation of NO (Cao et al., 2020). Studies have indicated that aerobic exercise could improve the endothelial cell's function by downregulating TXNIP/NLRP3 inflammasome signaling (Hong et al., 2021). Overall, aerobic exercise could inhibit endothelial cell's pyroptosis by improving the vascular endothelial cell's function.

Aerobic exercise decreases oxidative stress

Nicotinamide adenine dinucleotide-phosphate (NADPH) oxidases take a vital role in oxidative stress. NADPH oxidases could produce superoxides (O^{2-}), which induce reactive free radicals (Gjevestad et al., 2015) and act as the main source of ROS in blood vessels. The ROS-dependent activation of NLRP3 inflammasome can induce endothelial impairment (Rovira-Llopis et al., 2018) and oxidative stress. Previous studies have shown that NADPH subunit p22phox decreased the expression of IL-1β (Liao et al., 2020). Aerobic exercise could inhibit superoxide production and NADPH oxidases activity in coronary arteries (Hong et al., 2021), thereby reducing ROS production and oxidative stress (Cunha et al., 2017). Treadmill exercise reportedly could suppress ROS production by reducing the activity of NADPH oxidases (Jeong et al., 2018). As shown in Figure 2, the potential mechanisms of aerobic exercise modulating endothelial cell's pyroptosis are as follows: 1) reduces vascular inflammation by inhibiting the expression of NLRP3 inflammasome. 2) improves endothelial function by enhancing NO bioavailability, and 3) decreases oxidative stress by reducing the activity of NADPH oxidase and IL-1β.

Aerobic exercise and adipocyte pyroptosis-related diseases

Adipocytes are closely related to metabolism, and aerobic exercise plays an important role in improving metabolic diseases by regulating adipocyte's pyroptosis. Studies have shown that targeting the NLRP3 inflammasome would reduce diet-induced metabolic abnormalities in mice (Chiazza et al., 2016; Ding et al., 2019).

Adipocyte's pyroptosis and its related diseases

Adipose tissue is the largest endocrine organ of the human body capable of storing lipids, secreting a large amount of adipokines, and it takes an essential role in the metabolism of human nutrients (Carbone et al., 2019). Chronic inflammation and adipose tissue dysfunction usually occur in individuals or mice with diabetes or obesity (Šimják et al., 2018). Adipocyte's pyroptosis is an important upstream event in metabolism-related diseases

including obesity (Giordano et al., 2013) and diabetes (Vandanmagsar et al., 2011). The expressions of Caspase-1, NLRP3, and other related factors of adipocyte's pyroptosis were abundantly present in obese patients and mice (Giordano et al., 2013), which are involved in systematic inflammation and glucose homeostasis of adipose tissues (Ding et al., 2019; Wu et al., 2020). Additionally, the elevated expressions of the NLRP3 inflammasome, IL-1β, and IL-18 in adipose tissues are directly associated with insulin resistance and severity of diabetes (Esser et al., 2013). Mitochondria are reportedly involved in regulating NLRP3 inflammasome activation in adipocytes (Zhang et al., 2021). Moreover, a previous study found that high-fat diet induced overactivation of NLRP3 inflammasome in mice, the protein expression of genes related to mitochondrial biogenesis decreased, suggesting that mitochondrial damage caused by glucose and lipid metabolism disorders may activate the NLRP3 inflammasomes (Zhang et al., 2021). Therefore, the adipocyte's pyroptosis is mainly related to metabolic diseases. Further, aerobic exercise is an effective strategy to prevent metabolic diseases by limiting the adipocyte's pyroptosis.

The potential mechanism of aerobic exercise on adipocyte's pyroptosis

Lipids have important biological functions, in fact, fat is the energy provider in our body. The prominent roles of adipose tissue are to sequester fatty acids in times of energy excess and to release fatty acids via the process of lipolysis during times of high-energy demand, such as during an exercise. (Tsiloulis and Watt 2015). Several studies demonstrated that aerobic exercise could improve the function of adipocytes (Stanford et al., 2015), alter the expression of adipokines (Stinkens et al., 2018), and decrease adipocyte's inflammation. Aerobic exercise training has been reported to inhibit the expression of pro-inflammatory factors in adipocytes, promotes the balance of the oxidative and antioxidant systems, and improves the inflammatory state. Researches have also demonstrated that 10 weeks of aerobic exercise ameliorates HFD-induced complications through the reduction of NLRP3, IL-18, TNF-α, TLR4 and IL-1β activation in adipocytes (Mardare et al., 2016). Therefore, aerobic exercise training is an effective strategy to reduce the expression of pyroptosis-related factors in adipocytes. As shown in Table 1, previous studies have shown that treadmill exercise training for >8 weeks can decrease the release of pyroptosis-associated factors in the adipocytes of obese or HFD rats (Mardare et al., 2016; ZhuGe et al., 2020). Nevertheless, the molecular mechanism of the effect of aerobic exercise on adipocyte's pyroptosis remains unclear.

Aerobic exercise reduces adipocyte inflammation

Inflammation in adipocytes plays a vital role in metabolic diseases, as it increases the expression of NLRP3 and its related inflammatory factors (Vandanmagsar et al., 2011). TXNIP (Wu et al., 2020) and FGF2 (ZhuGe et al., 2020) can exacerbate the inflammatory response in adipocytes by activating NLRP3 inflammasomes and Caspase-1. A previous study has shown that 8 weeks of treadmill training effectively inhibited the NLRP3 expression and reduced the FGF2 levels in adipose tissues (ZhuGe et al., 2020). Moreover, it has been reported that TNF-α is responsible for regulating the transcription of NLRP3 inflammasome components and inflammatory molecules in cryopyrinopathies (McGeough et al., 2017). Similar studies confirmed that the expression of NLRP3 was positively correlated with the release of TNF-α in adipose tissues (Bauernfeind et al., 2016). The increase in peroxisome proliferator-activated receptor-γ (PPARγ) levels raises the expression of APN as well as inhibits TNF-α release (Xia 2015). As it is known, PPARγ is responsible for regulating adipocyte differentiation (Ahmadian et al., 2013). In adipose tissues, the expression levels of PPARγ and APN could be increased significantly after aerobic exercise, while that of TNF-α was decreased (Xia 2015). Moreover, after 10 weeks of treadmill training, the significantly decreased expressions of NLRP3, TNF-α, and IL-1β were observed in adipose tissues (Mardare et al., 2016). The above mentioned results suggest that aerobic exercise training may inhibit adipocyte's pyroptosis by reducing adipocyte inflammation.

Irisin, also known as fibronectin domain-containing protein 5 (FNDC5), is an exercise-inducing factor; it is not only a muscle factor but also an adipocytokine. A previous study has shown that irisin is a promising therapeutic agent that inhibits NLRP3-mediated pyroptosis of cardiomyocytes (Yue et al., 2021). AMP-activated protein kinase (AMPK) is essential for maintaining peroxisome proliferator-activated receptor-coactivator-1α (PGC-1α) (Gholamnezhad et al., 2020) and irisin (Lally et al., 2015) expressions. Irisin could inhibit the ROS/NLRP3 inflammatory signaling (Peng et al., 2017), TNF-α (Clark and Vissel 2019), and pyroptosis (Yue et al., 2021). Aerobic exercise has been demonstrated to activate AMPK and PGC1-α (Lally et al., 2015), increasing irisin expression in adipose tissues (Sanchez-Delgado et al., 2015) and inhibiting the NLRP3 related signaling.

Aerobic exercise ameliorates insulin resistance

Phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (AKT) signaling has been regarded as a key signaling pathway in glucose homeostasis, lipid metabolism and insulin

resistance (Abeyrathna and Su 2015). The activation of the NLRP3 inflammasome could enhance the expression of IL-1β, IL-18, and interferonγ (IFNγ), while it inhibits IRS-1/PI3K/AKT signaling (Sun et al., 2017) (Vandanmagsar et al., 2011), thereby leading to insulin resistance. Vandanmagsar et al. (2011) proved that aerobic exercise ameliorated insulin resistance in the adipose tissues of T2DM patients by inhibiting the expression of NLRP3 and IL-1β. Another study found that the decreased expression of IL-1β and NLRP3 was positively associated with decreased blood glucose levels and improved insulin resistance index (Vandanmagsar et al., 2011). Moreover, aerobic exercise could enhance the expression of PI3K and AKT, and sequentially activate the PI3K/AKT/glucose transporter 4 (GLUT4) signaling pathway in adipose tissues, thereby improving insulin sensitivity (Yi et al., 2020). Taken together, ameliorating adipocyte's inflammation and insulin resistance are the potential molecular mechanisms of the effects of aerobic exercise on adipocyte's pyroptosis (Figure 2).

Aerobic exercise and hippocampal cell pyroptosis-related diseases

Hippocampal cell's pyroptosis is closely related to the development of neurodegenerative diseases (Han et al., 2020; Li et al., 2021), and aerobic exercise is an ideal regimen to inhibit pyroptosis of hippocampal cells, which is beneficial for patients with neurodegenerative diseases.

Hippocampal cell's pyroptosis and its related diseases

The hippocampal cells take a vital role in storing information associated with memory. Pyroptosis of hippocampal cells is closely associated with AD's pathogenesis (Han et al., 2020), depression (Li et al., 2021), and so on. Neuroinflammation mediated by hippocampal cells and microglia take a crucial role in AD, primarily owning to amyloid-beta (Aβ) deposition and pyroptosis. The inhibition of NLRP3 in AD mice reduced Caspase-1 expression and Aβ deposition, and improved the cognitive function (Dempsey et al., 2017). Moreover, the activation of IL-1β and GSDMD will induce neuronal pyroptosis, and plays a significant role in the pathogenesis of AD (White et al., 2017; Han et al., 2020).

Current evidence has demonstrated that the NLRP3-mediated pyroptosis was a key modulator in the development of depression (Li et al., 2021). Especially, the NLRP3 inflammasome promotes hippocampal neurons and depression-like behavior in the hippocampus in depressed rats (Herman and Pasinetti 2018; Yang et al., 2020).

In fact, the downstream cytokines of NLRP3, including IL-1β and TNF-α, were increased in the cerebral spinal fluid and serum of patients with depression (Herman and Pasinetti 2018). In brief, the hippocampal cell's pyroptosis is closely related to AD and depression. Aerobic exercise is an important way to suppress hippocampal cell's pyroptosis in patients with neurodegenerative diseases.

The molecular mechanism of the effect of aerobic exercise on hippocampal cell's pyroptosis

Emerging evidence indicates that aerobic exercise can improve the function of hippocampal cells (Zhang X. et al., 2019). The possible mechanism is that aerobic exercise effectively reduces Aβ deposition by regulating neuroinflammation and oxidative stress (Zhang X. et al., 2019). Some studies have proved that aerobic exercise can inhibit hippocampal cell's pyroptosis. As shown in Table 1, the studies indicated that aerobic exercise could inhibit NLRP3 inflammasome-related inflammatory cytokines, including Toll-like receptor 4 (TLR4), NF-κB, TXNIP, IL-1β, and IL-18. As mentioned above, these studies have suggested that aerobic exercise can reduce the expression of pyroptosis-related factors in the hippocampal cells. Aerobic exercise could inhibit the TLR4/NF-κB/NLRP3 signaling pathway in the dentate gyrus region of the hippocampus of post-stroke depression models (Li et al., 2020), which could prevent the activation of TXNIP and NLRP3 inflammasome pathways in AD rats (Rosa et al., 2021), and ameliorate depression-like behaviors by decreasing NLRP3, IL-1β, and IL-18 expressions in the hippocampal tissues (Wang et al., 2016c). These studies suggested that aerobic exercise could reduce hippocampal cell's pyroptosis.

Aerobic exercise reduces microglia activation

Microglia are the major source of inflammatory cytokines in the central nervous system (Habib and Beyer 2015) and coordinate the brain's inflammatory response (Andoh and Koyama 2020). Studies have demonstrated that TLR4 could activate microglia, which transmit downstream inflammatory signals through the adaptor protein MyD88 (Kang et al., 2016), then activate NF-κB and NLRP3 inflammasome. NLRP3 inflammasome has been demonstrated to activate the microglia (Freeman et al., 2017), and NLRP3 protein was preferentially expressed in the microglia (Xia et al., 2021). The NLRP3 complex secretes IL-1β and IL-18, leading to pro-inflammatory response and pyroptosis (Zhou et al., 2011).

Numerous studies have found that aerobic exercise upregulated the expression of anti-inflammatory cytokines, thereby inhibiting the activation of microglia (Andoh and Koyama 2020) and expression of the NLRP3 inflammasome

(Wang et al., 2016b). Aerobic exercise can inhibit microglial activation by decreasing the levels of IL-1β and TNF-α (Zhang X. et al., 2019), and regulating TLR signaling pathways (Mee-Inta et al., 2019). Long-term treadmill running could also reduce the expression of IL-1β and IL-18, inhibiting microglial activation caused by the activation of NLRP3 inflammasome in the hippocampal tissues (Wang et al., 2016b). Therefore, aerobic exercise can inhibit hippocampal cell's pyroptosis by reducing microglial activation.

Aerobic exercise protects neurons by decreasing neuroinflammation

Neuroinflammation is an immune response mediated by cytokines released from the microglia, which is related to increased expression of inflammatory cytokines, including NLRP3, IL-1β, and IL-18, in the hippocampal cells. Aerobic exercise has been shown to relieve neuroinflammation and protect neurons by decreasing the expression of NLRP3, IL-1β, and IL-18 (Wang et al., 2016b; Rosa et al., 2021). Wang et al. (2019) have shown that 4 weeks of treadmill exercise training inhibited neuroinflammation and played a neuroprotective role by suppressing the NF-κB/NLRP3 signaling pathway. The potential mechanism for aerobic exercise inhibits the expression of hippocampal NLRP3 inflammasome by reducing the TXNIP levels in the hippocampal dissection (Rosa et al., 2021). Moreover, TXNIP mediates the activation of NLRP3-related inflammatory signaling pathways through oxidative stress (Italiani et al., 2018). Moreover, aerobic exercise activates the Nrf2/HO-1 pathways, although it suppresses the NLRP3/IL-1β pathway (Cai et al., 2016), thereby inhibiting hippocampal cell's pyroptosis.

Multiple studies have demonstrated that aerobic exercise could inhibit upstream signaling of hippocampal cell's pyroptosis. Specifically, Qu et al. have demonstrated that 8 weeks of aerobic exercise training inhibited the TLR4/myeloid differentiation 88 (MyD88)/NF-κB signaling pathway in the hippocampal tissue (Qu et al., 2020). Li et al. (2020) found that 28 days of running training inhibited the TLR4/NF-κB/NLRP3 inflammatory signaling pathway, which mediates the hippocampal neurons' protective effect in post-stroke depressed mice. Qu et al. (2019) identified that 8 weeks of moderate-intensity treadmill exercise significantly reduced the expression of TLR4 in the hippocampal tissue of mice, and activated the TLR4/miR-223/NLRP3 pathway axis, thereby improving the hippocampal function and promoting the repair of the damaged hippocampal tissue. Furthermore, Li et al. (2019) proved that 4 weeks of treadmill exercise could modulate the NF-κB/NLRP3/IL-1β signaling pathways in the hippocampal proteins. Moreover, long-term running wheel exercise training inhibited the expression of NADPH oxidase, and release of TNF-α and IL-1β, and induced the antioxidant and protective effects of microglia on nerves (Simioni et al., 2018). In other words, aerobic exercise could inhibit hippocampal cell's pyroptosis by reducing neuroinflammation.

Aerobic exercise decreases Aβ deposition

Aβ deposition is neurotoxic and can destroy the neurons, resulting in abnormal autophagy, blocking the clearance of Aβ, and affecting the cognitive function of neurodegenerative diseases. Aβ deposition promotes ROS production oxidative stress (Matěj et al., 2015) and activates the NLRP3 inflammasome in microglial cells *in vitro* and *in vivo* (Luciunaite et al., 2020). Aerobic exercise reduces microglia-mediated neuroinflammation, oxidative stress and Aβ deposition by inhibiting NLRP3 expression in the microglia (Zhang X. et al., 2019; Liang et al., 2020; Nakanishi et al., 2021). Together, these studies suggested that aerobic exercise could inhibit hippocampal cell's pyroptosis by decreasing Aβ deposition.

Summary and prospect

In summary, the review highlighted the close association between aerobic exercise and pyroptosis-related diseases, suggesting that aerobic exercise can alleviate the pyroptosis by regulating the NLRP3 inflammation signaling. Aerobic exercise inhibits endothelial cell's pyroptosis by improving the endothelial function, while reducing vascular inflammation and oxidative stress. Moreover, aerobic exercise affects adipocyte's pyroptosis by ameliorating adipocyte inflammation and insulin resistance. The potential mechanism of the effects of aerobic exercise on hippocampal cell's pyroptosis is the reduction of microglial activation, neuroinflammation and Aβ deposition.

Different patterns of exercise have varying effects on cell pyroptosis. For example, Khakroo Abkenar et al. (2019) and Comassi et al. (2018) have found that one-time acute and acute high-intensity exercises can promote the activation of pyroptosis-associated protein, which are related to exercise intensity. However, aerobic exercise, resistance training and chronic high-intensity intermittent exercise can inhibit the activation of pyroptosis. Thus, further studies are needed to define the optimal effects of different patterns of exercise on specific cell's pyroptosis and their molecular mechanism. Additionally, at present, animal experiments to investigate the effect of exercise on cell pyroptosis are more frequently performed, as compared to human experiments, which are scarce and more likely involved a small sample size. More in-depth research on the human body can provide a more scientific basis on the efficacy of exercise in regulating cell pyroptosis and promoting health. Therefore, more methodological, high-quality, and large-sized human studies are needed to determine the ideal patterns of exercise.

Author contributions

SH and XWW designed the study. SH and XXW drafted the manuscript. SH and XXW drew the figures and filled the table. XWW and XHL revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Lymphocyte and dendritic cell response to a period of intensified training in young healthy humans and rodents: A systematic review and meta-analysis

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Background: Intensified training coupled with sufficient recovery is required to improve athletic performance. A stress-recovery imbalance can lead to negative states of overtraining. Hormonal alterations associated with intensified training, such as blunted cortisol, may impair the immune response. Cortisol promotes the maturation and migration of dendritic cells which subsequently stimulate the T cell response. However, there are currently no clear reliable biomarkers to highlight the overtraining syndrome. This systematic review and meta-analysis examined the effect of intensified training on immune cells. Outcomes from this could provide insight into whether these markers may be used as an indicator of negative states of overtraining.

Methods: SPORTDiscus, PUBMED, Academic Search Complete, Scopus and Web of Science were searched until June 2022. Included articles reported on immune biomarkers relating to lymphocytes, dendritic cells, and cytokines before and after a period of intensified training, in humans and rodents, at rest and in response to exercise.

Results: 164 full texts were screened for eligibility. Across 57 eligible studies, 16 immune biomarkers were assessed. 7 were assessed at rest and in response to a bout of exercise, and 9 assessed at rest only. Included lymphocyte markers were $CD3^+$, $CD4^+$ and $CD8^+$ T cell count, NK cell count, NK Cytolytic activity, lymphocyte proliferation and CD4/CD8 ratio. Dendritic cell markers examined were CD80, CD86, and MHC II expression. Cytokines included IL-1β, IL-2, IL-10, TNF-α and IFN-γ. A period of intensified training significantly decreased resting total lymphocyte (d= −0.57, 95% CI −0.30) and $CD8^+$ T cell counts (d= −0.37, 95% CI −0.04), and unstimulated plasma IL-1β levels (d= −0.63, 95% CI −0.17). Resting dendritic cell CD86 expression significantly increased (d = 2.18, 95% CI 4.07). All other biomarkers remained unchanged.

Conclusion: Although some biomarkers alter after a period of intensified training, definitive immune biomarkers are limited. Specifically, due to low

study numbers, further investigation into the dendritic cell response in human models is required.

KEYWORDS

immune biomarkers, intensified exercise, dendritic cells, altered immunity, humans, rodents

1 Introduction

Overloading the body whilst preventing inadequate recovery is a necessary process implemented within an athletes' training program to improve athletic performance (Whyte, 2006). If there is not an appropriate balance of stress and recovery, states of overtraining may occur. These states are functional overreaching (FOR), non-functional overreaching (NFOR) and the overtraining syndrome (OTS). When in a state of FOR a short-term decrement in performance may occur (Halson et al., 2002) but with sufficient recovery a "*supercompensatory*" effect on performance may be seen (Birrer et al., 2013). However, if recovery is not implemented at the appropriate moment, athletes may enter a state of NFOR (Kellmann et al., 2018) which could take weeks or months for full recovery to occur (Meeusen et al., 2013). If NFOR is left undiagnosed, and the training/recovery imbalance continues, athletes experience a heightened risk of suffering from the OTS, which can take months to years to fully recover (Meeusen et al., 2013). Symptoms of NFOR/OTS occur in individual (37%) and team (17%) sport athletes (Matos et al., 2011), with the incidence in an athletes' career ranging from 30% to 60% (Morgan et al., 1987; Birrer et al., 2013). Despite the high incidence of states of overtraining, little progress has been made on establishing objective and reliable biomarkers for identifying when an athlete may be entering the various states of overtraining following periods of intensified training (Armstrong and VanHeest, 2002).

Cortisol is a hormone that is synthesised and released in response to physical and mental stress *via* the hypothalamic pituitary adrenal (HPA) axis. The HPA axis consists of the hypothalamus, pituitary gland and adrenal cortex (Guilliams and Edwards., 2010). During periods of intensified training, it has been reported that there is a blunting, by 72%, of the cortisol response to a short duration (30 min), high-intensity cycle test when comparing before to after an 11-day intensified training period (Hough et al., 2013). This disrupted functioning of the HPA axis following an intensified training period has previously been highlighted. Meeusen et al. (2004) examined the hormonal responses to an exercise stress test composed of two maximal cycle tests separated by 4 h resting recovery in well-trained athletes before and after a 10-day intensified training period. They reported a 118% and 73% reduction in the response of cortisol and adrenocorticotrophic hormone (ACTH; a precursor hormone to cortisol) in the athletes in response to the second maximal cycling bout after the 10-day training period compared to before the training period (Meeusen et al., 2004). Meeusen et al. (2010) also reported that athletes in a state of OTS (classified according to the duration and severity of symptoms and underperformance experienced) show little or no exercise-induced increases in ACTH in response to the second maximal exercise bout in their exercise stress test. This suggests that the exercise-induced response of the HPA hormones, specifically cortisol and ACTH, may be lowered following periods of intensified training.

Cortisol plays an important role in the anti-inflammatory response of the immune system to exercise by increasing the phagocytic potential of neutrophils and monocytes (Blannin et al., 1996; Ortega et al., 1996), supressing pro-inflammatory mediators such as reactive oxygen species (ROS) (Franchimont, 2004) and inducing lymphocytopenia (Okutsu et al., 2005). Lymphocytopenia refers to the lowering of lymphocytes in the blood, and most likely is a reflection of their increased migration into the tissues for increased immune-surveillance (Kruger et al., 2007). Therefore, a temporary dysfunctional HPA axis caused by a period of intensified training may lead, in part, to an impaired immune response during intensified exercise.

The impact of heavy periods of training on the immune system remains unclear, with some evidence suggesting a decline in immunity after repeated arduous exercise bouts (Walsh, 2019). As debated in Simpson et al. (2020), it is suggested that the reduced post-exercise immunosurveillance that occurs after prolonged (>5 days) and intensive (>60% $\dot{V}$ O_{2max}) (Hoffman-Goetz et al., 1990) endurance training, in addition to the post-exercise decline in cytotoxic T cells (Steensberg et al., 2001) introduces a "window of opportunity" for infection. Repeated exposures to these acute declines in immunity bare additive negative consequences to infection risk (Pedersen and Ullum, 1994). In line with this, it has been reported that elite athletes that undergo heavy training regimes experience significantly higher episodes of upper respiratory tract infections (URIs) than recreational athletes (Spence et al., 2007), with a small proportion of athletes experiencing recurrent episodes at higher rates than the general population (Fricker et al., 2000). These recurring URIs have been associated with persistent fatigue that can hinder an athletes training (Reid et al., 2004). Moreover, it has been shown that elite endurance athletes prone to recurrent URIs i.e., more than 4 episodes per year, have an altered cytokine response, suggestive of impaired inflammatory regulation compared to healthy athletes (Cox et al., 2007). Similarly, a reversible defect in $CD4^+$ T cell IFN-γ secretion, a cytokine known to affect illness severity and

duration, has been associated with illness-prone athletes experiencing fatigue (Clancy et al., 2006). Furthermore, suppression of immune parameters can occur in elite athletes over years of training, which can result in reactivation of viruses (Gleeson et al., 2002; Reid et al., 2004). At a cellular level, studies have reported a reduced CD4+/CD8+ ratio in response to a 4 weeks strength training program involving progressive intensity increases each week from 75% to 85% heart rate maximum (HRmax) (Dongqing, 2013), a reduction in T cell proliferation immediately after a 30 min treadmill run at 80% $\dot{V}O_{2max}$ following a 3 weeks intensified training period (25% above normal training load) when compared to before the training (Verde T. J et al., 1992), and reduced natural killer (NK) cell cytotoxicity after 1 month of intense volleyball pre-season compared to before pre-season began (Suzui et al., 2004).

However, the "window of opportunity" theory is not accepted by all, with suggestions that reductions in immune cell function post-exercise could reflect the lowered number of immune cells in the circulation after exercise, which are redistributed into tissues for enhanced immunosurveillance at sites of infection risk (Kruger et al., 2007; Campbell et al., 2009). For example, Green et al. (2002) showed a significant decrease in lymphocyte proliferation–an important first step to create effector lymphocytes - after a 60 min, high-intensity run, but found no significant differences between the exercise and control groups when assessing lymphocyte proliferation in an NK cell depleted culture, or when adjusted per T cell. This suggests that the decreased proliferation found initially was likely due to an exercise-induced increase in NK cells within the sample, thus a reduction in the proportion of T cells that can be stimulated, rather than the exercise bout causing an actual reduction in T cell proliferation. Therefore, it is argued that studies reporting changes in immune cell function that coincide with changes in immune cell count cannot use lymphocytopenia as evidence for a decline in immunity. This is because the fall in cell number does not reflect mass apoptosis but a redistribution of highly functional T cells and NK cells from the bloodstream into the tissues and organs (Kruger et al., 2007; Campbell et al., 2009). This redistribution enhances the identification and eradication of tissue tumour cells; a clear benefit to the host. It has been shown that cancer cells incubated with exercised serum form less tumors when inoculated into mice (Hojman et al., 2018), and 4 weeks of voluntary wheel running prior to tumor cell inoculation reduced tumor growth by 61%, attributed to the redistribution of NK cells after exercise causing an increased infiltration of NK cells to tumor sites (Pedersen et al., 2016). Another commonly used measure of URI susceptibility in athletes is salivary immunoglobin A (sIgA). Although there are reports that the lowered sIgA seen with intense periods of training is associated with increased URIs in athletes (Fahlman and Engels, 2005), this has not been shown consistently (Antualpa et al., 2018; Gill et al., 2014; Pacque et al., 2007). Moreover, studies that do relate URI with decreased sIgA levels rarely consider confounding factors that may impact sIgA secretion and concentration, such as the profound intra-and inter-individual variation, likely due to oral health, psychological stress or sleep, and diurnal or seasonal-changes (Brandtzaeg, 2013). Finally, immune competency is also influenced by non-exercising factors, and without clinical confirmation that a URTI is present, symptoms could be due to allergy (Kennedy et al., 2016), or caused by variables such as psychological stress (Cohen et al., 1991), low energy availability (Bromley et al., 2018), or low sleep efficiency (Prather et al., 2015). Evidently the arguments for both an increased and reduced immune response post exercise are well supported and more definitive research is required to provide a firm conclusion.

The HPA axis is known to be involved in the regulation of important antigen presenting cells, involved in linking the innate and adaptive immune responses, known as dendritic cells (DC) (Liberman et al., 2018). Glucocorticoids, such as cortisol can regulate the maturation, survival, and migration toward the lymph nodes of DCs, but also can inhibit their immunogenic functions (Liberman et al., 2018). Cortisol itself has been shown to downregulate DC costimulatory molecules and dampen pro-inflammatory cytokine production, such as IL-6, IL-12 and TNF-α, which subsequently reduces the ability of the DCs to prime naïve $CD8^+$ T cells (Elftman et al., 2007). Given the importance of these cytokines in orchestrating the immune response, the measure of cytokines, such as, TNF- α, IFN- γ and IL-1 β, as pro-inflammatory orchestrators of a type 1 immune response, and IL-10 and IL-2, as key anti-inflammatory immune-regulators, can act as a measure of immune function. Specifically, these cytokines are released from and also activate T cells and DCs (Blanco et al., 2007; Shaw et al., 2018).

Upon engulfing and processing an extracellular antigen, or degrading and processing intracellular antigens, DCs mature and gain T cell stimulatory capacity *via* antigen processing and upregulation of the major histocompatibility complex (MHC), costimulatory molecules (CD80/86) and cytokines (i.e., IL-12) (Wehr et al., 2019). The MHC is located on the surface of a DC and is loaded with peptide fragments from a pathogen, which it then presents to the T cell receptor for recognition (Guermonprez et al., 2002). CD80/86 are co-stimulatory molecules which bind to CD28 on the T cell to amplify the initial activating signals provided to the T cell receptors by the antigen loaded-MHC (Magee et al., 2012). Finally, the cytokines are required in order to drive the differentiation and proliferation of the T cells. All 3 of these signals are therefore required for T cell stimulation. The matured DCs then migrate towards the lymph nodes to present the antigen to T cells. An upregulation of MHC I complex is required for presentation of intracellular antigens to $CD8^+$ T cells, whereas MHC II is loaded with extracellular antigens degraded *via* the endocytic pathway, for presentation to $CD4^+$ T cells (British Society for Immunology, 2021). Cross presentation can also occur, meaning exogenous antigens can be presented by MHC I molecules. DCs also operate a bi-directional

link with NK cells; a lymphocyte functioning within the innate immune system (Thomas and Yang, 2016). DCs can induce NK cell proliferation and cytotoxicity *via* the release of cytokines such as IL-12, IL-15 and IL-18 (Ferlazzo and Morandi, 2014). Conversely, NK cells can induce DC maturation *via* the secretion of IFN-γ and TNF-α (Moretta et al., 2006), and eliminate DCs that do not mature properly, a process known as 'DC editing', through engagement with the activating receptor NKp30 (Moretta et al., 2006). To our knowledge, there are currently four reports investigating the DC response to exercise training, indicating that after chronic exercise training in rats, DC function; as a measure of the expression of co-stimulatory molecules and MHC II receptors, and IL-12 production, required for T-cell stimulation, remains unchanged (CD80) (Liao et al., 2006; Chiang et al., 2007; Mackenzie et al., 2016; Fernandes et al., 2019), increased (CD86) (Chiang et al., 2007; Mackenzie et al., 2016) or unclear (MHC II) (Chiang et al., 2007; Mackenzie et al., 2016). Despite the known DC changes with HPA axis alterations, the lack of evidence surrounding DCs leaves the question of how DCs respond to periods of intensified training unanswered.

With evidence that DCs are in part regulated by the HPA axis, and the knowledge that the HPA axis response to exercise stress may be blunted following a period of intensified exercise, it is important to examine the impact that intensified exercise has on DCs. As these cells orchestrate the immune response, specifically, the direct nature of the relationship DCs have with both T lymphocytes and NK cells, it is logical to review evidence surrounding all three immune cells, providing further direction towards a conclusion in the response of the immune system to intensified training.

Therefore, the aim of this systematic review is to assess the current literature examining the effects of a period of intensified training on lymphocyte (T cells and NK cells) and DC number and function, in both humans and rodents. This review focuses on the normal impact of high intensity training due to the difficulties surrounding confirmation of NFOR/OTS diagnosis. However, heavy training is a factor involved in the establishment of NFR/OTS, and as such, any highlighted immune biomarkers could potentially indicate NFOR/OTS has occurred. The main purpose being to highlight areas already studied, indicate potential gaps requiring further investigation, and assess if there is scope for the future use of immune biomarkers in the diagnosis of overtraining.

2 Methods

This review conforms to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009) and was registered with PROSPERO international prospective register for systematic reviews (CRD42021248776; 21 May 2020).

2.1 Inclusion and exclusion criteria

To develop the inclusion and exclusion criteria for this review a consideration of Population, Intervention, Comparison and Outcome (PICO) was used (Richardson et al., 1995).

2.2 Eligibility criteria

2.2.1 Population

Humans aged 18–50 years with a maximum oxygen uptake ($\dot{V}\,O_{2max}$) of fair or higher (>38.5 ml kg^{-1}. min^{-1}) according to ACSM guidelines for cardiorespiratory fitness (ACSM, 2017) or Rodents aged 6 weeks—5 months were included in this review.

Human studies using females must have controlled for menstrual cycle to be included in the review. The menstrual cycle is known to impact certain elements of the immune system e.g. lowered $CD4^+$ T cell numbers and increased type 1 cytokine production during the luteal phase compared to the follicular phase (Timmons et al., 2005; Oertelt-Prigione, 2012).

2.2.2 Intervention

Studies must include an increased training load compared to their regular training load, completed over multiple days.

2.2.3 Comparison

Studies included were required to have a comparative control. In human studies, participants were used as their own controls, comparing their pre-and post-training biomarker values. Where no pre-training values were given in rodent studies, the control group was used as a comparison.

2.2.4 Outcome

Studies must have measured at least one immunological biomarker relating to lymphocytes, DC, or cytokines before and after a period of training. The immune biomarkers could be measured at rest, or in response to an acute bout of exercise; this will be referred to as "exercise-induced" and indicates that the biomarker was measured immediately after an acute exercise bout both before and after a period of intensified training. Data must have been presented as mean and standard deviation to allow the calculation of the standardised mean difference (SMD) of the change in biomarker from pre-to post-training. A minimum of two studies measuring the same biomarker, using the same measurement units, were required to include that biomarker in the meta-analysis component. Where possible, differing units of measurements were converted into the same "gold standard" units for comparison. If this was not possible, it was excluded from the meta-analysis.

2.3 Search strategy for identification of studies

A literature search was conducted in the following databases on 26 May 2021: SPORTDiscus, PUBMED, Academic Search Complete, Scopus and Web of Science. Databases were searched from inception up until May 2021 for articles published in English. In addition to database searches, reference lists of relevant studies were screened for eligible studies. The search was re-run in June 2022 to identify any additional articles meeting the inclusion criteria.

Titles, abstract and keywords were searched using the following search terms:

1) "chronic exercise*" OR "training volume" OR "intensified training" OR "exercise training" OR "overtrain*" OR "endurance training*" OR "physical education and training" OR "high intensity training" OR "chronic exercise training" OR "physical conditioning, animal*" OR "Physical exertion"
2) "lymphocyte function" OR "immune response" OR "dendritic cell function" OR "immune function" OR "dendritic cell" OR "myeloid" OR "plasmacytoid" OR "t cell*" OR "cd4*" OR "cd8*" OR "T helper" OR "T cytotoxic" OR "lymphocyte*" OR "NK cell" OR "natural killer cell" OR "cd56*" OR "T regulatory" OR "cd25*" OR "lymphocyte proliferation" OR "T cell proliferation" OR "CD80*" OR "CD86*" OR "cd80*/86*" OR "NK-cell" OR "NKCA" OR "Natural Killer cell cytotoxic activity" OR "killer cells, natural" OR "cytotoxicity, immunologic" OR "lymphocyte activation" OR "antigen presenting cell*" OR "dendritic cells" OR "genes, mhc class i" OR "genes, mhc class ii" OR "interleukin"
3) "athlete*" OR "Mice" OR "animals"
4) "elderly" OR "Cancer" OR "Elder" OR "older" OR "geriatric" OR "aged"
5) AND 2 AND 3 NOT 4.

2.4 Study selection

Articles retrieved through the systematic search were exported to ProQuest RefWorks, a reference management software (RefWorks 3.0, Pro-Quest LLC, Michigan U.S.), and further exported to Excel (Microsoft 365, Microsoft, Washington, United States), whereby duplicates were removed and assessment for eligibility began. Two investigators (CB and JH) independently screened articles by title and abstract, and full text when necessary, against the inclusion criteria. Full texts from the eligible studies were then independently screened (CB and JH) for inclusion into the review.

2.5 Data extraction and management

Data extraction was conducted by one reviewer (CB) whereby the following data from all eligible articles were extracted into an Excel document: Title, publication details (year and author), participant characteristics (sex, age, number, $\dot{V}$ O_{2max}, age), intensified training period details (mode and duration) and assessed biomarker information (biomarker assessed, and method and units of measurement). Pre- and post-training values were extracted for each relevant biomarker in the form of mean and standard deviation. Where appropriate data was not presented, the authors were emailed, and were allocated 4 weeks to reply. If no reply was received after 4 weeks, the study was excluded. Any variables included in the search string that did not have sufficient studies to perform a meta-analysis were not included in the results. Where figures were used displaying the mean and standard deviation, data was extracted by eye.

2.6 Risk of bias

Risk of bias was assessed by one reviewer (CB) and independently verified by one member of the review team (JH). Three Cochrane Collaboration tools were used for assessing risk of bias; ROBINS-1 for non-randomised controlled trials, ROB-2 for randomised controlled trials and ROB-2 (Crossover) for randomised crossover trials (Cochrane Collaboration 2021; Oxford, United Kingdom). Specific study components assessed for risk of bias using the ROBINS-1 tool included confounding, selection of participants, classification of intervention, deviations from intended interventions, missing data, measurement of outcomes and reporting of results. Study components assessed using the ROB-2 tool included the randomisation process, deviations from intended interventions, missing outcome data, measurement of outcomes and reporting of results. The ROB-2 crossover tool assessed the same components as the ROB-2 tool with the addition of carryover effects.

2.7 Statistical analysis

Inverse variance, random effects meta-analysis was then conducted on immune biomarker data in Review Manager Software (RevMan, Version 5.3, Cochrane Collaboration, Oxford, United Kingdom). Hedge's *g* standardised mean difference (SMD) was calculated *via* the RevMan software (RevMan, Version 5.3, Cochrane Collaboration, Oxford, United Kingdom).

A separate meta-analysis was conducted for each biomarker where >2 studies measured the same biomarker using the same method and units of measurement. Human and rodent studies

TABLE 1 Risk of Bias assessment of included studies using Cochranes ROB-2, ROBIN-2 Cross-Over and ROBINS-1 tools. ROB-2 and ROB-2 Cross-Over: Low (✓), Some concern (~), High (X), Not enough Information (?). ROBINS-1: Low (✓), Moderate (~), Serious (S), Critical (X), Not enough information (?).

ROB-2

Study	Domain 1	Domain 2	Domain 3	Domain 4	Domain 5	Overall ROB
	Risk of Bias arising from tde randomisation process	Risk of Bias due to deviations from tde intended interventions	Risk of Bias due to missing outcome data	Risk of Bias in measurement of tde outcome	Risk of Bias in selection of tde reported result	
Croft et al. (2009)	✓	~	✓	✓	✓	~
Gholamnezhad et al. (2014)	✓	✓	✓	✓	✓	✓
Hack et al. (1997)	✓	✓	?	✓	✓	✓
Hasanli et al. (2021)	✓	✓	?	✓	✓	✓
Hoffman-Goetz. (1986)	✓	✓	?	✓	✓	~
Hoffman-Goetz et al. (1988)	✓	✓	?	✓	✓	✓
Hwang et al. (2007)	✓	✓	?	✓	✓	✓
Kaufaman et a (1994)	✓	✓	✓	?	✓	✓
Koyama et al. (1998)	✓	✓	?	✓	✓	✓
Kwak (2006)	✓	✓	?	✓	✓	✓
Louis et al. (2016)	✓	✓	?	✓	✓	✓
Mackenzie et al. (2016)	✓	✓	?	✓	✓	✓
Mitchell et al. (1996)	✓	✓	✓	✓	✓	✓
Peijie et al. (2003)	✓	✓	✓	✓	✓	✓
Poffe et al. (2019)	✓	?	✓	✓	✓	✓
Sheyklouvand et al. (2018)	✓	✓	?	✓	✓	✓
Wang et al. (2011)	✓	~	?	✓	✓	~
Wang et al. (2011)	✓	~	?	✓	✓	~
Watson (1986)	~	✓	X	✓	✓	x
Weng et al. (2013)	✓	✓	✓	✓	✓	✓
Zhang et al. (2019)	✓	?	✓	✓	✓	✓

ROB-2 Cross-Over

Study	Domain 1	Domain S	Domain 2	Domain 3	Domain 4	Domain 5	Overall ROB
	Risk of Bias arising from tde randomisation process	Risk of Bias arising from period and carryover effects	Risk of Bias due to deviations from tde intended interventions	Risk of Bias due to missing outcome data	Risk of Bias in measurement of tde outcome	Risk of Bias in selection of tde reported result	
Li et al. (2013)	✓	✓	✓	✓	~	✓	~
Meyer et al. (2004)	~	~	✓	✓	✓	✓	~
Pizza et al. (1995)	?	✓	~	✓	✓	✓	~

(Continued on following page)

TABLE 1 (*Continued*) Risk of Bias assessment of included studies using Cochranes ROB-2, ROBIN-2 Cross-Over and ROBINS-1 tools. ROB-2 and ROB-2 Cross-Over: Low (✓), Some concern (~), High (X), Not enough Information (?). ROBINS-1: Low (✓), Moderate (~), Serious (S), Critical (X), Not enough information (?).

ROBINS-1

Study	Domain 1 Bias due to confounding	Domain 2 Bias in selection of participants into the study	Domain 3 Bias in classification of interventions	Domain 4 Bias due to deviations from intended interventions	Domain 5 Bias due to missing data	Domain 6 Bias in measurement of outcomes	Domain 7 Bias in selection of the reported result	Overall ROB
Baj et al. (1994)	✓	✓	✓	✓	✓	✓	✓	✓
Baum et al. (1994)	~	✓	✓	?	?	~	✓	~
Blank et al. (1994)	✓	✓	✓	✓	✓	✓	✓	✓
Borges et al. (2012)	~	✓	✓	?	✓	~	✓	~
Borges et al. (2018)	✓	✓	✓	?	✓	✓	✓	✓
Bresciani et al. (2011)	✓	?	✓	?	?	~	✓	?
Bury et al. (1998)	~	?	✓	✓	✓	✓	✓	~
Chiang et al. (2007)	✓	✓	✓	✓	✓	✓	✓	✓
Chung et al. (2021)	✓	✓	✓	✓	?	✓	✓	✓
Córdova Martinez et al. (2015)	✓	✓	✓	✓	~	~	✓	~
Dongqing (2013)	✓	✓	✓	✓	?	✓	✓	~
Dressendorfer et (2002)	✓	✓	✓	✓	✓	~	✓	~
Ferry et al. (1990)	✓	✓	?	?	?	✓	✓	?
Fry et al. (1992)	✓	?	?	?	?	~	✓	?
Halson et al. (2003)	?	✓	✓	?	?	✓	✓	?
Heisterberg et al. (2013)	✓	✓	✓	?	✓	✓	✓	✓
Jurimae and Purge. (2021)	✓	✓	?	?	?	✓	✓	?
Kajiura et al. (1995)	✓	✓	✓	?	?	?	✓	?
Lancaster et al. (2004)	✓	✓	✓	?	✓	✓	?	✓
Leet al. al. (2021)	?	✓	✓	✓	?	✓	✓	✓
Main et al. (2010)	✓	✓	✓	?	?	~	?	?
Mueller (2001)	✓	✓	✓	?	?	?	✓	?
Mujika et al. (1996)	✓	✓	✓	?	✓	✓	✓	✓
Ndon et al. (1992)	✓	✓	✓	?	~	✓	✓	~
Nickel et al. (2011)	?	✓	✓	✓	✓	✓	✓	✓
Peake et al. (2003)	✓	✓	✓	?	?	✓	✓	✓
Rebelo et al. (1998)	✓	✓	✓	?	?	✓	✓	✓
Ronsen et al. (2001)	✓	✓	✓	✓	~	✓	~	~
Shing et al. (2007)	✓	✓	✓	?	?	✓	✓	✓
Smith and Myburgh. (2006)	✓	✓	✓	?	?	✓	✓	✓
Tanimura et al. (2009)	✓	✓	✓	?	?	✓	✓	✓
Verde T et al. (1992)	✓	✓	✓	?	?	✓	✓	✓
Witard et al. (2012)	✓	✓	✓	?	?	✓	✓	✓

were analysed together for all biomarkers, apart from 'lymphocyte proliferation' due to human studies measuring peripheral blood lymphocytes, and rodent studies measuring spleenocytes. Effect sizes were classified based on the magnitude of change from pre to post intervention. Classifications included very small (0.01–0.19), small (0.20–0.49), moderate (0.50–0.79), large (0.80–1.19), very large (1.20–1.99) and huge (>2.0) (Cohen, 1988; Turner and Bernard, 2006; Sawilowsky, 2009). Statistical heterogeneity was determined using the I^2 statistic; 0%–40% indicated non-important (low) heterogeneity, 40%–60% indicted moderate heterogeneity, 50%–75% indicated substantial heterogeneity and 75%–100% indicated considerable heterogeneity (Cochrane, 2021). All results were reported as Hedge's *g* with 95% confidence intervals (CI). Additional sub-group analysis was conducted on resting immune cell count biomarkers based on the duration of intensified training periods i.e. ≤ 7 days, 8 days-2 weeks, 15 days- 4 weeks or >4 weeks.

3 Results

3.1 Risk of Bias

A complete analysis of ROB is displayed in Table 1. For studies assessed with the ROBINS-1 tool, bias in "selection of participants to the study" was deemed as "not applicable" (n = 3) or "Low" (n = 30) because most studies followed a group of athletes over time or assessed the same group of participants before and after a period of intensified training. The bias arising from participant awareness of intervention encapsulated in ROBINS-1 domain 6; bias in measurement of outcome, was judged as being negligible in most studies (n = 24). It is difficult to blind participants from intervention when intensified training is the independent variable and training loads were often monitored or implemented by the investigators themselves, so knowledge of intervention was necessary. It could be argued that as objective immune biomarkers were measured, results are unlikely to be affected by knowledge of intervention, especially in the rodent studies.

Despite this, it has been suggested that anticipatory stress may cause alterations to the immune system, such as decreased lymphocyte counts and reduced lymphocyte proliferation (Ironson et al., 1990; Ader and Cohen, 1991; Lekander, 2001). However, studies investigating this phenomenon tend to use the anticipatory stress surrounding major life events such as cancer patients waiting for chemotherapy treatment (Lekander, 2001), and homosexual men waiting for HIV test results (Ironson et al., 1990). The evoked stress response caused by such serious events could be deemed as incomparable to the anticipation of undertaking exercise, especially when undertaken by trained athletes. Therefore, whilst we acknowledge that the anticipation of undertaking exercise may elicit a stress response to some extent, perhaps more so in untrained personnel, it is an unavoidable, and potentially non-significant bias. It is impossible to know the true effect anticipatory stress may have on the measured immunological outcomes without studies undertaking measures of stress scores.

Bias due to missing outcome data and attrition rate was mainly low (n = 22) or unclear (n = 31) in most studies, mainly because no information regarding excluded participants or reasons for missing data were highlighted. Only one study (Watson, 1986) was rated as "high" for bias due to missing data as table 4 only included n = 5 results for the placebo group's % T lymphocytes, when the placebo group consisted of 15 participants. A 'moderate' rating for bias due to missing outcome data was given for Córdova Martinez et al. (2015), as although they stated blood samples were collected before and after each stage of the cycling competition, unlike the other blood markers, only pre and post competition values were reported for cytokines. Ronsen et al. (2001) and Ndon et al. (1992) were also rated as "moderate" for bias due to missing outcome data as participants with incomplete data sets were still included in the final analysis (Ronsen et al., 2001) and participants were excluded from analysis by the investigators after final outcome measures were taken as they were perceived to be overtrained (Ndon et al., 1992).

3.2 Study outcomes

(Figure 1) Across the 57 included studies (Table 2), the variables used to assess immune cell changes included immune cell counts (Ferry et al., 1990; Fry et al., 1992; Ndon et al., 1992; Baj et al., 1994; Baum et al., 1994; Pizza et al., 1995; Mitchell et al., 1996; Mujika et al., 1996; Hack et al., 1997; Bury et al., 1998; Rebelo et al., 1998; Mueller, 2001; Ronsen et al., 2001; Dressendorfer et al., 2002; Halson et al., 2003; Peake et al., 2003; Lancaster et al., 2004; Meyer et al., 2004; Smith and Myburgh, 2006; Shing et al., 2007; Tanimura et al., 2009; Bresciani et al., 2011; Wang et al., 2011; Wang et al., 2011; Borges et al., 2012; Witard et al., 2012; Heisterberg et al., 2013; Li et al., 2013; Weng et al., 2013; Louis et al., 2016; Sheyklouvand et al., 2018; Poffe et al., 2019; Chung et al., 2021; Leal et al., 2021), lymphocyte proliferation (Hoffman-Goetz, 1986; Watson, 1986; Hoffman-Goetz et al., 1988; Verde T et al., 1992; Mitchell et al., 1996; Bury et al., 1998; Koyama et al., 1998; Peake et al., 2003; Peijie et al., 2003; Kwak, 2006; Hwang et al., 2007), CD4/CD8 ratio (Ferry et al., 1990; Fry et al., 1992; Verde T et al., 1992; Blank et al., 1994; Kaufman et al., 1994; Kajiura et al., 1995; Pizza et al., 1995; Hack et al., 1997; Mueller, 2001; Dressendorfer et al., 2002; Smith and Myburgh, 2006; Shing et al., 2007; Wang et al., 2011; Dongqing, 2013; Li et al., 2013; Weng et al., 2013; Poffe et al., 2019; Zhang et al., 2019; Leal et al., 2021), cytokine secretion (Dressendorfer et al., 2002; Halson et al., 2003; Shing et al., 2007; Croft et al., 2009; Main et al., 2010; Bresciani et al., 2011; Nickel et al., 2011; Gholamnezhad

TABLE 2 Return of relevant studies from the systematic search. Studies in Bold Italics were eligible for inclusion from the systematic search, but were not suitable for inclusion into the meta-analysis.

Study	Participants	Age	$\dot{V}$ O_{2max}	Training status	Intensified training	Duration	Measurement method	Biomarker
Anomasiri et al. (2002)	***Humans (Males)***	***21.1 ± 0.44***	***40.2 ± 8.7***	***Trained***	***Military Training, 5d.wk***	***8 weeks***	***Flow Cytometry***	***CD3+, CD4+, CD8+, NK cell, lymphocyte counts***
Baj et al. (1994)	Humans (males)	21 ± 1.5	74.0 ± 1.4	Trained	Cycling training and competition; 500 km.wk training & 12,000 km during competition	24 weeks	Flow cytometry	Lymphocyte, CD3+, CD4+, CD8+ and NK cell counts, CD3/ CD4 daysRatio, lymphocyte proliferatio^{+}, IL-$^{+}$
Baum et al. (1994)	Humans (males)	20.8 ± 3	No Information	Trained	Training 3 phases: endurance runs (60–160 km.wk), 8 weeks anaerobic km wke (intensive training- submax and max runs, uphill runs) & competition phase. (Pre and post phase 2 used)	8 weeks	Flow cytometry	Lymphocyte, CD3+, CD4+, CD8+ Counts
Blank et al. (1994)	Rodents (females)	8–10 weeks	Not applicable	Not applicable	Treadmill running 60 min.d, 5 d.wk at 12 m/min, 8 degree gradient	10 weeks	Automated cell counter	CD4/CD8 Ratio, NK cell, CD4+ andCD8+ counts
Blank et al. (1997)	***Rodents (females***	***9–10 + weeks***	***Not applicable***	***Not applicable***	***Treadmill running 60 min.d, 5 d.wk at 12 m/min, 8 degree gradient***	***10 weeks***	***51Cr-release assay in lytic units and Flow cytometry.***	***Spleen NK cell, CD4+, CD8+ counts and NK cytolytic activity.***
Borges et al. (2012)	Humans (males)	22 ± 4.2	61.2 ± 5.5	Trained	Kayak season (t0-t2 timepoint used)	26 Weeks	Automatic cell counter.	Lymphocyte count.
Borges° (2018)	Humans (males)	22 ± 4.3	61.2 ± 5.5	Trained	11 weeks high volume, 5 weeks high intensity; (kayaking, running, swimming & strenth).	26 Weeks	ELISA (unstimulated)	TNF- ± α , IFN- γ , IL-1 β .
Bresciani et al. (2011)	Humans (males)	22.3 ± 1.4	45.2 ± 2.3	Recreationaly Active	Running 3d.wk starting at 40, 30 ,30 min per session and increasing weekly volume by 5 mins each session every week. (T1-13 used). Intensity started at 42.5% and increased to 80% TRIMP.	9 weeks	ELSA (unstmulated)nd Automatic cell counter	Resting Lymphocyte ± count, T ± F- α
Bury et al. (1998)	Humans (males)	24.2 ± 2.6	62.8 ± 4.0	Trained	Football season	40 weeks	Resting PHA stimulated labelled thymidine incorporation via liquid scintillation (proliferation). Immunofluorescent staining and microscope (count)	Lymphocyte, CD4+, CD8+, CD56+ Counts, lymphocyte proliferation.
Cardoso et al (2018)	***Rod(males)***	***6–8 weeks***	***Not applicable***	***Not applicable***	***30 mins swimming d***	***15 days***	***ELISA (unstimulated)***	***IL-10, IL-1 β , TNF- α***
Chiang et al. (2007)	Rodents (males)	9 weeks	Not applicable	Not applicable	Treadmill endurance tranining 6d.wk^{+}(progressively increased from 10 mmin to 25 m/min and 5 min to 30 min sessions)	5 weeks	LPS stimulated, measured via. minw cytometry	BMD MHC II, Myeloid DC CD80 and CD86, DC IL12
Chung et al. (2021)	Rodents (males)	6 weeks	Not applicable	Not applicable	treadmill running; 15 m/min at a 5% day scope increasing by 3 m.min every 10 min until exhanstion. 1 session first 4 weeks and 2 sessions	8 weeks	Autmoated cell counter, ELISA (Unstimualted exercise induced)	Lymphocyte count, IL- β

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TABLE 2 (*Continued*) Return of relevant studies from the systematic search. Studies in Bold Italics were eligible for inclusion from the systematic search, but were not suitable for inclusion into the meta-analysis.

Study	Participants	Age	$\dot{V}$ O_{2max}	Training status	Intensified training	Duration	Measurement method	Biomarker
					for last 4 week 4 hr rest between sessions in last 4 weeks.			
Córdova Martinez et al. (2015)	Humans (males)	20.03 ± 0.9	73.2 ± 6.7	untrained	460 km cycling: 4 stages (Basal and post 3rd stage used)	3 days	ELISA (unstimulaed)	TNF- α , IFN- γ , 1L-1B
Croft et al. (209)	Humans (males)	20 ± 1	55.9 ± 6.8	untrained	High intensity interval running 4xwk: 10 mins at 70% $\dot{V}$ O_{2max} , 3 × 5 min at 90%, 1.5 min at 50%, then 10 ± min cooldown at 70%	6 weeks	Unstimulated Bead assay (flow cytometry)	TNF- α
Dongqing (2013)	Humans (males)	20.2 ± 1.3	No Inforation	Trained	Weightlifting; progressive incr ± ase each week@ 75%, 80%, 80%, 85% HRmax, 5.5 d.wk.	4 weeks	Fluo minnt double labeling × method using Imm noassay	Resting CD4, CD8+ counts, CD4/CD8 Ratio
Dos Santos Cunha et al. (2004)	***Rodents ((males)***	***6–8 weeks***	***Not applicable***	***Not applicable***	***Treadmill running, 5 d.wk at 60%–65% $\dot{V}$ O2max***	***5 weeks***	***CON A stimulated proliferation, Thymidine incor o wkon measured by liquid scintillation (dpm) and ELISA (PHA stimulated).***	***Resting and exercise induced spleen Lymphocyte proliferation, IL-2, IL-10 and TNF- α***
Dressendorfer et al. (2002)	Humans (males)	24.4 ± 2.1	59.3 ± 5.0	trained	HIIT at 100%HRmax 4d.wk on a bike, plus one wind tunnel cycle and one weight session per week. (Baseline to end of I phase used)	6.5 weeks	Flow cytometry.	Lymphocyte count Resting: CD3+, CD8+, CD4+ Counts. Exercising: CD3+, CD8+, CD4+ Counts
Fernandes et al. (2019)	***Rodents ((males)***	***6–8 weeks***	***Not applicable***	***Not applicable***	***Treadmill running 1 hr.d at 50% average max speed.***	***5 weeks***	***Flow cytometry.***	***pDC and mDC count, CD80 and) CD86 expression of lung ad lymph***
Ferry et al. (1990)	Humans (males)	20.1±2.9	63.2 ± 4.3	trained	Across a cycling training cycle	20 weeks	Flow cytometry	Resting: Lymphocyte, CD8+, CD4+, CD56+ Counts Exercising: Lymphocyte, CD8+, CD4+, CD56+ Counts. CD4/CD8 Ratio
Fery et al. (1992)	***Rodents (males)***	***12 weeks***	***Not applicable***	***Not applicable***	***Treadmill running 6(d.wk,) duration increased from 30–60 min and speed from 20–30 m.min***	***4 weeks***	***Flow cytometry***	***Spleen CD4+ (resting and +xerci+e) and+CD8+ (resting) counts***
Fry et al. (1992)	Humans (males)	3.6 ± 3.5 (No body mass (kg) provided to convert to $ml.kg^{-1}.min^{-1}$)	3.71 ± 0.14 ($L.min^{-1}$)	trained	Army training: 10 d treadmill intervals 2x day (15 x1 min exercise period 2 mins rest in AM, PM= 10 × 1 min intervals 1 min rest), 5 dactive recovery (Day1–10 used).	10 days	Flow cytometry	Lymphocyte, CD3+, CD8+, CD4+ CD56+ Counts, CD4/CD8 ratio, lymphocyte proliferation.
Gholamnezhad et al. (2014)	Rodents (males)	6–8 weeks	Not applicable	Not applicable	Treadmill running at 25 m.min, 60 min.d, 6 d.wk	11 weeks	ELISA (unstimulated)	IL-10, IFN- γ , TNF- α
Hacket al. (1997)	Humans (males)	23.4 ± 0.8	No Information	untrained	Anaerobicr, 60 min sessions 3 d.wk 2x sprint sessions (90%–110% $\dot{V}$ O_{2max} with 5–8 min recovery; 5 × 80-300 m), 1 × 60 min weights session	8 weeks	Automated cell counter and flow cytometry	Lymphocyte, CD3+, CD4+, C8+ and CD4+CD45RA+ counts, CD4/CD8 Ratio

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TABLE 2 (*Continued*) Return of relevant studies from the systematic search. Studies in Bold Italics were eligible for inclusion from the systematic search, but were not suitable for inclusion into the meta-analysis.

Study	Participants	Age	$\dot{V}$ O_{2max}	Training status	Intensified training	Duration	Measurement method	Biomarker
Halson et al. (2003)	Humans (males)	21.1 ±3.0	58.0 ± 1.7	trained	2 week normal cycling training, 2 week– intensified cycling eriod (7 d.wk 150% normal load)	4 weeks	ELISA (unstimulated) and flow cytometry	Lymphocyte count, TNF- α
Hasanli et al.(2021)	Rodents (male)	10 weeks	Not applicable	Not applicable	Treadmill running; 5 d.wk at 15 m.min in week 1, increasing tso 25 m.min by week 8. Duration started at 60 min.session in week days and increased to 60 min in week 5.	8 weeks	ELISA (unstimulated)	IFN- γ
Heisterberg et al., (2013)	Humans (males)	26.3 ± 1.1	62.5	trained	Professional soccer season (5–8 x wk, 1.5- 2 hr. session days)	24 weeks	Automated cell counter	Lymphocyte count
Weng et al. (2013)	Rodents (males)	12 weeks	Not applicable	Not applicable	Treadmill runing at 28 m.min, 6 d. wk at gradient 8 degree	6 weeks	CON A stimulated thymidine incorporation via liquid scintillation (CPM)	Spleen–lymphocyte –oliferation
Hoffman-Goetz et al. (1988)	Rodents (males)	8 weeks	Not applicable	Not applicable	Treadmill running (2 wks; 12-30 m-min, 0-8 degree gradient, 30 min. d, 5 d.wk and 6 wks; 30 m.min, 8 degree gradient, 20min.d, 5 d.wk)	8 weeks	LPS and PW*via*timulated thymidine incorporation via liquid scintillation (cpm)	Lymphocyte proliferation
Hoffman-Goetz et al. (1990)	***Humans (males)***	***24.5 ± 0.9***	***46.4 ± 6.4***	***untrained***	***Cyclin–30 m $\dot{V}$ O_{2ma-} , 1 hr.d***	***5 days***	***Flow cytometry***	***Resting and exercising CD3+, CD4+, CD8+ and NK cell counts***
Hwang et al. (2007)	Rodents (males)	6 weeks	Not applicable	Not applicable	Swimming 30 min.d, 5 d.wk in week 1, then extended by 10 min.d, up to 60 min.d, 5 d.wk	10 weeks	CON A and LPS induced thymidine incorporation via liquid scintillation (cpm)	Spleen lymphocyte proliferation
Jurimae and Purge (2021)	Humans (males)	25.0 ± 6.5	64.0 ± 3.5	trained	Rowing training, starting at 11.6 ± 1.4 hr.wk and increasing to 18.4 ± 1 hr.wk	24 weeks	ELISA (unstimulated)	IFN- γ , TNF- α , IL-1 β , IL-2
Kajiura et al. (1995)	Humans (males)	20.2 ± 1.8	60.1 ± 5.2	trained	High volume, high intensity running phase (100% increase in normal running load, with 1000 m intervals at 95%–100% $\dot{V}$ O_{2max} ever other day)	10 days	Flow cytomet ± y	Resting and exercising CD4+ count, CD4/CD8ratio
Kafman et l. (1994)	Rodents (males)	6 weeks	Not applicable	Not applicable	Swimming 15 min intervals, increased over 2.5 wks to 2 hr.d 5 d.wk, with an additional 1.5 wk at 2 hr.d 5 m.wk	4 weeks	Flow–cytometry	Spleen CD4/CD8 ratio
Kilgore et al. (2002)	***Humans (males)***	***28.3 ± 6.3***	***No Information***	***trained***	***Weightlifting; 57%–90% 1RM rang(ng fr)m 3–5 d.wk***	***6 weeks***	***Automated cell counter***	***Lymphocyte count***
Koyama et al. (1998)	Rodents (males)	7 days	Not applicable	Not applicable	Progressive wheel running; 1 wk (60–120 min.d), 3 wks (120 min.d, 6d.wk), average distance increased from 1500–2500 m.d	4 weeks	CON A induced thymidine incorporation via liquid scintillation (cpm)	Peripheral lymphocyte proliferation

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Study	Participants	Age	$\dot{V}$ O_{2max}	Training status	Intensified training	Duration	Measurement method	Biomarker
Kwak (2006)	Rodents (males)	6 weeks	Not applicable	Not applicable	Swimming 1 week; 30 min.d, increased by 10 min.wk up to 60 min.d	1 days weeks	CON A and LPS induce thymidine incorporation via liquid scintillation (cpm)	Spleen lymphoc*via* proliferation
Lancaster et al. (2004)	Humans (males)	30 ± 2	60.6 ± 1.5	trained	Cycling training every day at 150% normal volume with $\dot{V}$ O_{2max} tests before and after.	6 days	Flow cytometry and geometric mean fluorescence intensity	CD3+, CD4+, CD8+, *via*+CD45RO+, CD8+CD45RO+ and lymphocyte counts, IFN- γ
Leal et al. (2021)	Humans (males)	21 ± 5	59 ± 6	untrained	Running session repeated in the order of; 90 min continuous treadmill; 70 min @55% v $\dot{V}$ O_{2max} & 20 mn @75% v $\dot{V}$ O_{2max}; 5 km TT; 70 min treadmill at 12 RPE (borg) for 30 mins, 13 RPE for 3 mins &15 RP for 10 mins.	12 days	Flow cytometry and Automated cell counter	CD4+, CD3+, CD8+, NK cell counts, CD4/CD8 ratio, DC CD11c Expression
Leandro et al. (2006)	***Rodents (male)***	***No Information***	***Not applicable***	***Not applicabe***	***Treadmill running 5 d.wk, 60 min.d a(70% $\dot{V}$ O_{2max}***	***8 weeks***	***CON A induced minymidine incorporation via liquid scintillation***	***Lymphocyte proliferation***
Li et al.(2013)	Humans (male)	19.2 ± 1.6	No Information	trained	Military training	1 week	Flow cytometry	CD4/CD8 ratio, Resting NK cell, CD3+, CD4+ count
Liao et al. (2006)	***Rodents (male)***	***6–8 weeks***	***Not applicable***	***Not applicable***	***Treadmill running 6 d.wk, timing increased from 15-35 min.d, speed increased from 10-25 m.m(n. 2) incline increase in last week. 30% intensity every 3rd day of each week.***	***5 weeks***	***Flow cytometry***	***mDC CD80 and +D86 e+pression, mDC count***
Louis et al. (2016)	Humans (male)	31.0 ± 4.7	58.7 ± 5.6	trained	6 sessions over 4 consecutive days, 10-15 h.wk; High intensity afternoon session (8 × 5 min cycling @85% MAP and 6 × 5 min running at 10 km intensity); low intensity the next morning (60 min cycle at 65% MAP). Light sessions 3 d.wk.	3 weeks	Automated cell counter	Lymphocyte count
Mackenzie et al. (2016)	Rodents (males)	6–8 weeks	Not applicable	Not applicable	Treadmill running at 60% m × x velocity, 1hr.d, 5d.wk	4 weeks	Flow cytometry	Bone marrow dendritic cell count
Main et al. (2010)	Humans (males)	26.6 ± 4.1	65.0 ± 34 days	trined	14 sessions. Wk; 7d.wk^{-1} (10 x rowing, the rest weights, running and ergometer). 24 h.–k^{-1},80% endurance based, 20% at LT and max sprint efforts	8 weeks	low cytometry (unstimulated)	IL-1β , TNF- α , IL-12p70, IL-10
Meyer et al. (2004)	Humans (males)	24.8 ± 3.8	68.4 ± 10.0	trained	Cycling training 20 h.wk^{-1} (40% i days crease from normal training volume)	13 days	Flow cytometry	Lymphocyte and NK cell counts
	Humans (males)	23.4 ± 7.0	40.4 ± 0.1	untrained		12 weeks		

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Study	Participants	Age	$\dot{V}$ O_{2max}	Training status	Intensified training	Duration	Measurement method	Biomarker
Mitchell et al., 1996					Cycle ergoeter; 30 min.d^{-1}, 2 d.wk^{-1} at 75% $\dot{V}$ O_{2max}		PHA and PWM induced thymidine incorporation via liquid scintil ation	Lymphocyte count, lymphocyte proliferation
Mueller, 2001	Humans (males)	25.8—37.9	73.7 ± 4.7 (Ex) 44.8 ± 5.2 (Con)	trained	Season of cross country endurance skiing	8 weeks	ELISA, flow cytometry	Lymphocyte, CD3+, CD4+, CD8+ counts, CD4/CD8 ratio, IFN- γ , IL-10, IL-12
Mujika et al. (1996)	Humans (males)	21.1 ± 3.4	No Information	trained	Swimming season; 12 weeks traini– 4 weeks taper	16 weeks	Automated cell counter	Lymphocyte count
Ndon et al. (1992)	Humans (males)	25.6 ± 2.6	67.9 ± 2.3	trained	Cycling, swimming, running and weights training; 150% normal training duration	4 weeks	Automated cell counter	Lymphocyte count
Nickel et al. (2011)	Humans (males)	40 ± 7	No Information	trained	Continuous aerobic running and interval training, gradual increase in training and intensity (week 1: 38 ± 1 km.wk^{-1} – week 10: 54 ± 2 km.wk)	10 weeks	ELISA (unstimulated)	TNF- α
Peake et al. (2003)	Humans (males)	28 ± 7	No Information	trained	16% increase normal running training volume; 104±48 km average distance	4 weeks	Flow cytometer, CON A and PWM induced thymidine incorporation via liquid scintill ± t ion	Lymphocyte, CD3+, CD4+, CD8+, NK cell counts, lymphocyte proliferation
Peijie et al. (2003)	Rodents (males)	6 weeks	Not applicable	Not applicable	Swimming; Week 1 (1.4 m.s^{-1} for ± 0 in) increased by 5 mins.d^{-1} until 120 min.d^{-1}. intensity increased to 1.6 m.s^{-1} at week 2 *via* 1.8 m.s^{-1} at week 5.	6 weeks	CON A induced thymidine incorporation via liquid scintillation	Spleen lymphocyte proliferation
Pizza et al. (1995)	Humans (males)	34.8 ± 7.6	65.1 ± 4.9	trained	200% increased running training min than normal	10 days	Flow cytometry	Lymphocyte, CD +, CD3+, CD8+ and NK ce l counts, CD4/C8 Ratio
Poffe et al. (2019)	Humans (males)	21.2 ± 2.9	55.3 ± 6.1	trained	Cycling 6d.wk^{-1}; HIIT (30 s max sprint 100 rpm, 4.5 min active recovery at 50 W; sprints increased from 4–6 over 3 weeks); intermittent endurance (5 × 6 min 100-110% av. PO, 8 min 55%–85%$^{+}$recov^{+}ry periods); constant load endurance (70%–95% av. PO 30 min TT for 60-150 min)	3 weeks	Flow cytometry	CD3+, CD4+, CD days+ counts, CD4/CD8 ratio
Rebelo et al. (1998)	Humans (males)	26.3 ± 3.7	No Information	trained	Across an entire Portuguese football season	44 weeks	Flow cytometry	Lymphoc–tes, CD3+, CD4+ counts
Ronsen et al. (2001)	Humans–(males)	21—29	7—82	trained	Nordic skiing season	8 weeks	Automate^{+} cell^{+}counter	Lymphocyte count
Sheyklouvand e (2018)	Humans (males)	24 ± 3	No Information	trained	Canoe paddling based HIIT 3d.wk, variable intensity (6 × 60 s at 100%, 110%, 120%, 130^{+}, 130^{+}, 130%, 120%,	3 weeks	Automated cell counter	Lymphocyte count

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TABLE 2 (*Continued*) Return of relevant studies from the systematic search. Studies in Bold Italics were eligible for inclusion from the systematic search, but were not suitable for inclusion into the meta-analysis.

Study	Participants	Age	$\dot{V}$ O_{2max}	Training status	Intensified training	Duration	Measurement method	Biomarker
					130%, 100% v $\dot{V}$ O_{2peak}) across 9 sessions			
Shing et al. (2007)	Humans (males)	27 ± 2	69.3 ± 1.3	trained	High intensity cycle training >VT. Day 1: 20 × 1 min at PPO, 2 min days covery at 50 W; Day 2: 60 min × at 100%VT; Day 3: 12 × 30 s sprints at 175% PPO, 4.5min at 50 W recovery; ay 4: 30 mins at 80%VT, 45 min at 100%VT; Day 5: 40 min TT	5 days	Flow cytometry	Lymphocyte, CD3+, CD4+, CD8+, NK cell counts, CD4/CD8 ratio, TNF-α , IFN- γ , I L-12p70
Shing et al. (2007a)	***Humans (males)***	***27 ± 2***	***69.3 ± 1.3***	***trained***	***High intensity cycle training >VT. Day 1: 20 × 1 min at PPO, 2 min recovery at 50 W; Day 2: 60 min at 100%VT; Day 3: 12 × 30 s sprints at 175% PPO, 4.5 min at 50 W recovery; Day 4: 30 mins at 80%VT, 45 min at 100%VT; Day 5: 40 min TT***	***5 days***	***Flow cytometry***	***NKCC %lysis***
Smith and Myburgh. (2006)	Humans (males)	22.6 ± 4.7	56.1 ± 4.7	trained	Cycling; 2d.wk-1 alternating sessions of (1) 80% PPO for 5 min, 1 min rest x 5-8 reps (increased by 1 bout.wk), and (2) 90% 5 kmTT speed for 5 km, 50% 5km speed for 20 min, 90% 5 km TT speed 5 km.	4 weeks	Flow cytometry	CD3+, CD4+, CD8+, NK cell counts, CD4/CD8 ratio
Sugiura et al. (2002)	***Rodents (males)***	***6 week***	***Not Applicable***	***Not Applicable***	***Wheel running 3 d.wk-1, 12 h.d-1. Distance increased from 7 km.d-1 to 8km.d-1, peaking at 10 km.d-1 at 5 wk.***	***8 weeks***	***CON A and PHA induced thymidine incorporation via liquid scintillation***	***Lymphocyte proliferation***
Tanimura et al. (2009)	Humans (males)	19.6 ± 0.9	46.8 ± 3.4	trained	Kendo training; 310 $min.d^{-1}$	6 days	Flow cytometry	Lymphocyte, CD4+, CD8+ cou nts
Verde T . J et al. (1992)	***Humans (males)***	***288 ± 1.7***	***>60***	***trained***	***Increased running training load by via***	***3 weeks***	***Flow cytometer, PHA and CON A induced thymidine incorporation via liquid scintillation***	***CD4/CD8 ratio, lymphocyte proliferatio , resting CD3+ count***
Verde T et al. (1992)	Humans (males)	28.8 ± 1.7	65.3 ± 4.9	trained	Increased running training load by 38%	3 weeks	Flow cytometry	CD4+, CD8+ counts
Wang et al. (2011)	Humans (males)	23.1 ± 0.8	43.9 ± 2.3	untrained	Cycling at 50%Wmax, 30 $min.d^{-1}$, 5 $d.wk^{-1}$	4 weeks	Flow cytometry	NK cell $count^{+}$ NK CD45RO/RA+ count
Wang et al. (2011)	Humans (males)	21.5 ± 0.7	44.1 ± 2.5	untrained	Cycling at 50% Wmax, 30 $min.d^{-1}$, 5 $d.wk^{-1}$	4 weeks	Flow $cyt^{+}metry$	Lymphocyte, CD4+, CD3+, CD8+ counts, CD4/CD8 ratio
Watson (1986)	Humans (males)	22.8 ± 4.7	54.0 ± 3. d	untrained	Running 40–50 $min.d^{-1}$, $5d.wk^{-1}$ at 70%–85% $\dot{V}$ O_{2max}	15 weeks	Haemocytometer, Flow cytometry, NK cell ^{51}Cr release	Lymphocyte and CD3+ counts, NKCC, T cell proliferation

(Continued on following page)

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TABLE 2 (*Continued*) Return of relevant studies from the systematic search. Studies in Bold Italics were eligible for inclusion from the systematic search, but were not suitable for inclusion into the meta-analysis.

Study	Participants	Age	$\dot{V}O_{2max}$	Training status	Intensified training	Duration	Measurement method	Biomarker
Weng et al. (2013)	Humans (males)	22.3 ⁺0.2	46.5 ± 1.7	untrained	Cycling 5d.wk^{-1}; 3 min intervals at 40 and 80% $\dot{V}O_{2max}$, 30 min.d^{-1}	5 weeks	Flow cytometry	Lymphocyte,–CD4+, CD8+ counts, CD4.CD8 ratio
Witard et al. (2012)	Humans (males)	27 ± 8	64.2 ± 6.5	trained	1 wk normal, 1 wk high intensity cycling (increase volume and intensity by 70% vs normal), 1-2 sessions.d^{-1}, days7d.wk. E d of each week; 120 min at 60% $\dot{V}O_{2ax}$ and 45 min TT at 85%–100% $\dot{V}O_{2max}$	2 weeks	Flow cytometry	Lymphocyte and CD8+ counts
Zhang et al. (2019)	Humans (males)	20.1 ± 2.4	No Information	trained	High intensity training, 8 h.d^{-1} at grade 5-6 intensity	4 weeks	Flow cytometry	L–mphocyte, CD4+, C days8+ and CD3+ counts, and CD /CD8 ratio.

et al., 2014; Córdova Martinez et al., 2015; Borges et al., 2018; Hasanli et al., 2021; Juirmae and Purge, 2021), dendritic cell co-stimulatory molecule and MHC II expression (Chiang et al., 2007; Mackenzie et al., 2016) and NK Cytolytic activity (Watson, 1986; Bury et al., 1998; Shing et al., 2007).

3.3 Meta-analysis

3.3.1 Immune cell counts

3.3.1.1 Total lymphocytes

Of the 57 included studies, 29 studies assessed lymphocyte count at rest. Overall, a period of intensified training significantly (Z = 4.07 ($p < 0.0001$)) reduced resting lymphocyte number with a moderate effect (d= −0.57, 95% CI [−0.85, −0.30]; Figure 2). However, there is substantial heterogeneity amongst the studies (Chi^2 = 79.50, df = 28 ($p < 0.00001$), I^2 = 65%). Subgroup analysis indicated significant decreases in resting lymphocyte count in all exercise durations of >7 days (8 days- 2 weeks (n = 4): Z = 2.29 (p = 0.02), d = −1.36, 95% CI [−2.53, −0.20]; 15 days- 4 weeks (n = 7): Z = 3.21 (p = 0.001), d = −0.65, 95% CI [−1.05, −0.26]; >4 weeks (n = 15): Z = 2.08 (p = 0.04), d = −0.38, 95% CI [−0.73, −0.02]). Exercise durations of ≤7 days (n = 3) did not alter lymphocyte counts at rest (Z = 1.04 (p = 0.30), d = −0.71, 95% CI [−2.04, −0.63]).

Of the 57 included studies, 8 studies assessed lymphocyte count in response to exercise. Overall, a period of intensified training did not change the total lymphocyte count immediately post exercise (Z = 1.47, (p = 0.14), d= −0.64, 95% CI [−1.50, 0.21]). There is substantial heterogeneity amongst the studies (Chi^2 = 34.43, df = 7 ($p < 0.0001$), I^2 = 80%).

3.3.1.2 T cells.

3.3.1.2.1 CD3⁺ T cells. Of the 57 included studies, 14 studies assessed CD3$^+$ count at rest. Overall, a period of intensified training did not change CD3$^+$ count at rest (Z = 1.67, (p = 0.10), d= −0.50, 95% CI [−1.08, 0.09]; Figure 3). However, there is considerable heterogeneity amongst the studies (Chi^2 = 77.41, df = 13 ($p < 0.00001$), I^2 = 83%). Subgroup analysis revealed that intensified training periods of 8 days- 2 weeks (n = 3) significantly decreased CD4$^+$ T cell count at rest (Z = 2.14 (p = 0.03), d = −0.80, 95% CI [−1.53, −0.07]).

Of the 57 included studies, 3 studies assessed CD3$^+$ count immediately post exercise. Overall, a period of intensified training did not change CD3$^+$ count immediately post exercise (Z = 0.82, (p = 0.41), d= −1.16, 95% CI [−3.93, 1.16]). However, there is considerable heterogeneity amongst the studies (Chi^2 = 35.40, df = 2 ($p < 0.00001$), I^2 = 94%).

3.3.1.2.2 CD4⁺ T cells. Of the 57 included studies, 19 studies assessed CD4$^+$ count at rest. Overall, a period of intensified training did not change CD4$^+$ count at rest (Z = 1.41, (p = 0.16), d= −0.30, 95% CI [−0.71, 0.12]; Figure 4). However, there is considerable heterogeneity amongst the studies (Chi^2 = 74.12,

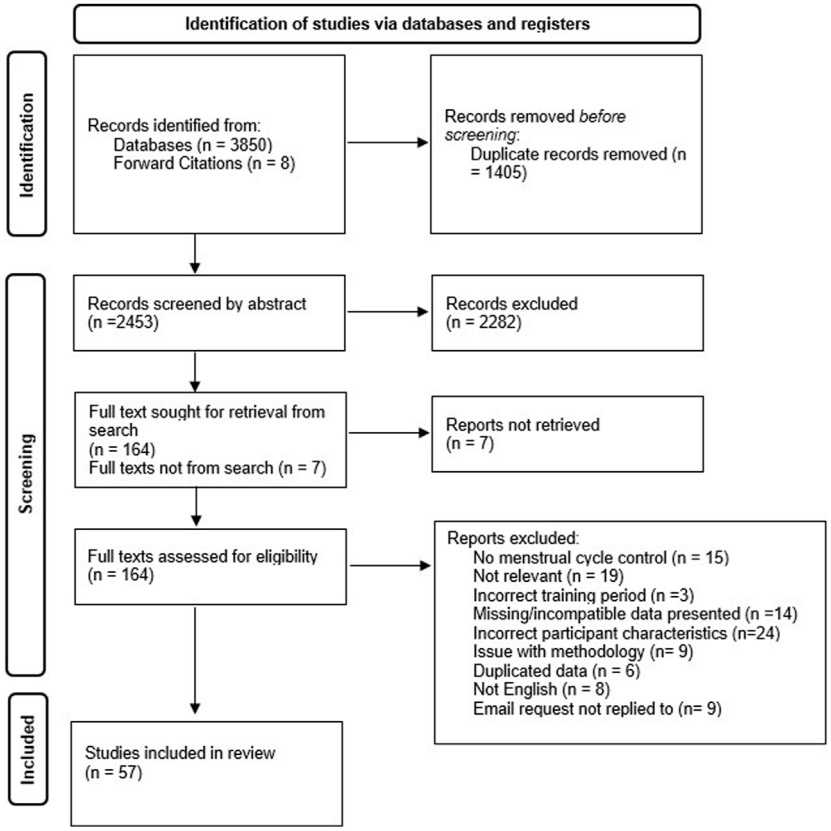

FIGURE 1
Systematic literature search PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow chart.

df = 18 ($p < 0.00001$), $I^2 = 76\%$). Subgroup analysis revealed that intensified training periods of 8 days- 2 weeks ($n = 3$) significantly decreased $CD4^+$ T cell count at rest (Z = 2.53 ($p = 0.01$), $d = -1.17$, 95% CI [−2.08, −0.26]).

Of the 57 included studies, 5 studies assessed $CD4^+$ count immediately post exercise. Overall, a period of intensified training did not change $CD4^+$ count immediately post exercise (Z = 0.80, ($p = 0.42$), $d = 0.35$, 95% CI [−0.50, 1.19]). However, there is substantial heterogeneity amongst the studies ($Chi^2 = 12.92$, df = 4 ($p = 0.01$), $I^2 = 69\%$).

3.3.1.2.3 $CD8^+$ T cells. Of the 57 included studies, 18 studies assessed $CD8^+$ count at rest. Overall, a period of intensified training significantly [Z = 2.18, ($p = 0.03$)] reduced $CD8^+$ count at rest with a small effect ($d = -0.37$, 95% CI [−0.7, −0.04]; Figure 5). However, there is substantial heterogeneity amongst the studies ($Chi^2 = 44.14$, df = 17 ($p = 0.0003$), $I^2 = 61\%$). Subgroup analysis revealed that intensified training periods of 8 days- 2 weeks ($n = 4$) were the only duration to significantly alter resting $CD8^+$ T cell count (Z = 2.98 ($p = 0.003$), $d = -0.79$, 95% CI [−1.30, −0.27]).

Of the 57 included studies, 6 studies assessed $CD8^+$ count immediately post exercise, before and after a period of overtraining. Overall, a period of intensified training did not change $CD8^+$ count immediately post exercise (Z = 0.54, ($p = 0.59$), $d = 0.37$, 95% CI [−0.97, 1.72]). However, there is considerable heterogeneity amongst the studies ($Chi^2 = 45.96$, df = 6 ($p < 0.00001$), $I^2 = 89\%$).

3.3.1.2.4 CD4/CD8 Ratio. Of the 57 included studies, 19 studies assessed CD+/CD8+ Ratio at rest. Overall, a period of intensified training did not change the resting CD4+/CD8+ ratio (Z = 0.91, ($p = 0.36$), $d = -0.15$, 95% CI [-0.49, 0.18]; Figure 6). However, there is substantial heterogeneity amongst the studies ($Chi^2 = 59.81$, df = 18 ($p < 0.00001$), $I^2 = 70\%$).

Of the 57 included studies, 7 studies assessed the CD4+/CD8+ ratio immediately post exercise. Overall, a period of intensified training did not change the CD4+/CD8+ ratio immediately post exercise (Z = 0.19, ($p = 0.85$), $d = -0.04$, 95% CI [−0.43, 0.35]). There is low heterogeneity amongst the studies ($Chi^2 = 7.15$, df = 6 ($p = 0.31$), $I^2 = 16\%$).

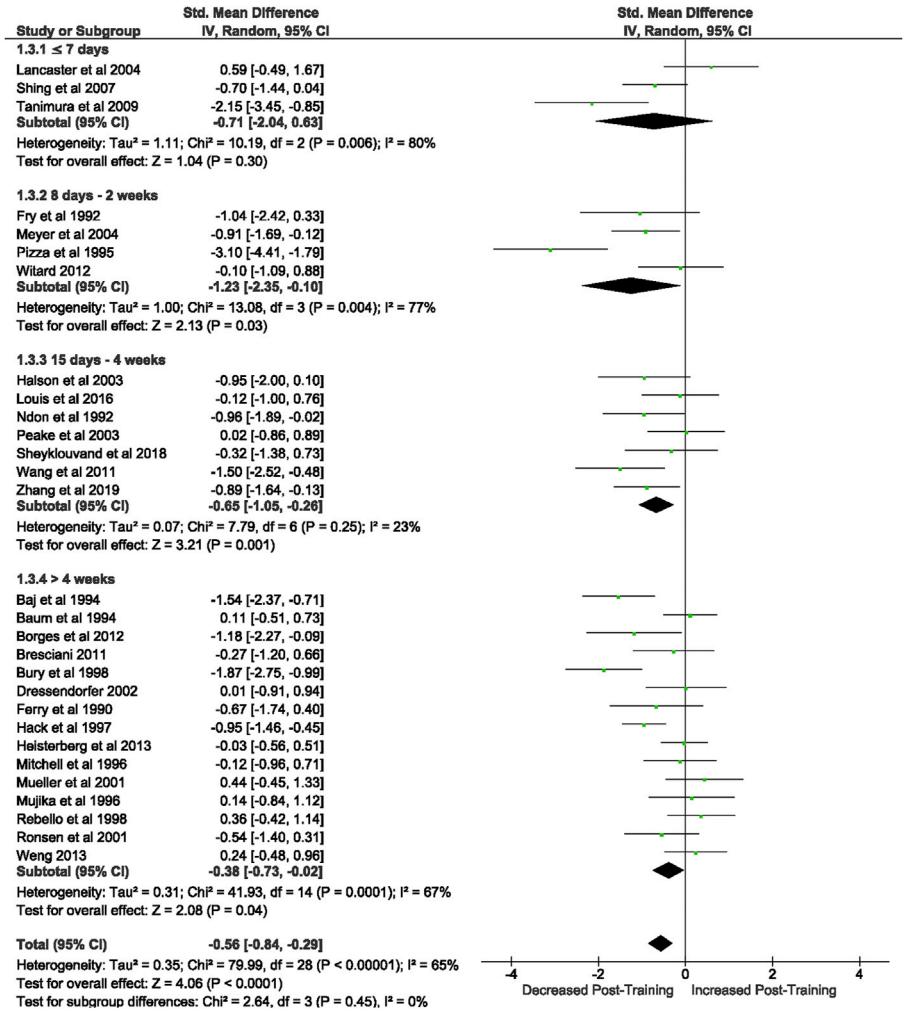

FIGURE 2
Effect of intensified training on resting lymphocyte counts, measured by FACS or Automated cell counter. Subgroup analysis is based on the duration of intensified training period; ≤ 7 days, 8 days—2 weeks, 15 days—4 weeks and > 4 weeks. All studies used human participants. *CI* Confidence interval.

3.3.1.2.5 Natural killer cells. Of the 57 included studies, 10 studies assessed NK cell count at rest based on $CD56^+$ expression. Overall, a period of intensified training did not change NK cell count at rest (Z = 1.18, (p = 0.24), d= −0.25, 95% CI [−0.67, 0.16]; Figure 7). However, there is substantial heterogeneity amongst the studies (Chi^2 = 24.78, df = 10 (p = 0.003), I^2 = 64%). Subgroup analysis revealed that resting NK cell count did not alter after a period of intensified training of any duration [i.e., ≤7 days (p = 0.36), 8 days-2 weeks (p = 0.18), 15 days-4 weeks (p = 0.62) or >4 weeks (p = 0.17)].

Of the 57 included studies, 3 studies assessed NK cell count based on $CD56^+$ expression immediately post exercise. Overall, a period of intensified training did not change NK cell count immediately post exercise (Z = 0.10, (p = 0.92), d= *0.04*, 95% CI [−0.74, 0.82]). However, there is substantial heterogeneity amongst the studies (Chi^2 = 4.35, df = 3 (p = 0.11), I^2 = 54%).

3.3.2 Immune function

3.3.2.1 Lymphocyte proliferation

3.3.2.1.1 Resting stimulated human peripheral blood (counts per minute). Of the 57 included studies, 5 different studies assessed human peripheral blood lymphocyte proliferation at rest, with 3 studies assessing lymphocyte proliferation to more than one stimulant, thus 8 results were entered into the meta-analysis. Overall, a period of intensified training did not change resting lymphocyte proliferation (Z = 0.04, (p = 0.97), d= −0.02, 95% CI [−1.10, 1.05]). However, there is considerable heterogeneity amongst the studies (Chi^2 = 75.72, df = 7 (p < 0.00001), I^2 = 91%).

3.3.2.2 Resting stimulated rodent spleen lymphocytes (counts per minute)

Of the 57 included studies, 6 studies assessed rodent spleen lymphocyte proliferation at rest with 2 studies assessing

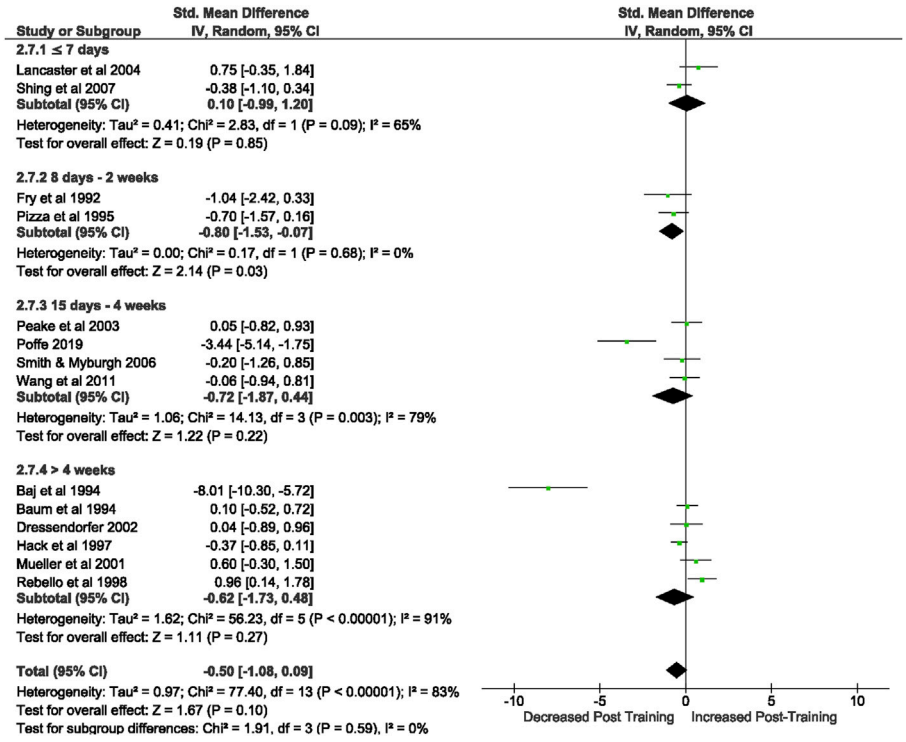

FIGURE 3
Effect of intensified training on resting CD3+ T cell counts, measured by FACS or Automated cell counter. Subgroup analysis is based on the duration of intensified training period; ≤7 days, 8 days—2 weeks, 1 days—4 weeks and > 4 weeks. All studies used human participants. *CI* Confidence interval.

lymphocyte proliferation to more than one stimulant, thus 8 results were entered into the meta-analysis. Overall, a period of intensified training did not change resting spleen lymphocyte proliferation (Z = 0.10, (p = 0.92), d = 0.09, 95% CI [−1.79, 1.97]). However, there is considerable heterogeneity amongst the studies (Chi^2 = 94.88, df = 7 (p < 0.00001), I^2 = 93%).

3.3.2.3 Exercise induced CON a stimulated rodent spleen lymphocytes (counts per minute)

Of the 57 included studies, 3 studies assessed spleen lymphocyte proliferation immediately post exercise. Overall, a period of intensified training did not change spleen lymphocyte proliferation immediately post exercise (Z = 0.77, (p = 0.44), d= −2.38, 95% CI [−8.44, 3.68]). However, there is considerable heterogeneity amongst the studies (Chi^2 = 44.46, df = 2 (p < 0.00001), I^2 = 96%).

3.3.3 NK cell cytolytic activity

Of the 57 included studies, 3 studies assessed NK cytolytic activity as %lysis of K-562 tumour cells. Overall, a period of intensified training did not change NK cell cytolytic activity (Z = 1.63, (p = 0.10), d = 1.13, 95% CI [−0.23, 2.49]). However, there is considerable heterogeneity amongst the studies (Chi^2 = 16.75, df = 2 (p < 0.0002), I^2 = 88%).

3.3.3.1 Cytokines

3.3.3.1.1 Unstimulated TNF- α. Of the 57 included studies, 10 studies assessed unstimulated TNF-α secretion at rest. Overall, a period of intensified training did not change resting TNF- α secretion (Z = 0.39, (p = 0.70), d= −0.13, 95% CI [−0.76, 0.51]; Figure 8). However, there is substantial heterogeneity amongst the studies (Chi^2 = 35.96, df = 9 (p < 0.00001), I^2 = 75%).

3.3.3.1.2 Unstimulated IFN-γ. Of the 57 included studies, 5 studies assessed unstimulated IFN-γ secretion at rest. Overall, a period of intensified training did not change resting IFN- γ secretion (Z = 1.33, (p = 0.18), d = 0.70, 95% CI [−0.33, 1.74]). However, there is considerable heterogeneity amongst the studies (Chi^2 = 19.42, df = 4 (p = 0.0006), I^2 = 79%).

3.3.3.1.3 Unstimulated IL-1β. Of the 57 included studies, 5 studies assessed unstimulated IL-1β secretion at rest. Overall, a period of intensified training significantly [Z = 2.69, (p = 0.007)] decreased resting IL-1 β secretion with a moderate effect (d= −0.63, 95% CI [−1.09, −0.17]; Figure 9).

There is very low heterogeneity amongst the studies [Chi^2 = 4.05, df = 4 (p = 0.40), I^2 = 1%].

3.3.3.1.4 Unstimulated IL-10. Of the 57 included studies, 3 studies assessed unstimulated IL-10 secretion at rest. Overall, a period of intensified training did not change resting IL-10 (Z = 1.62, (p = 0.11), d= *1.52*, 95% CI [−0.32, 3.37]). However, there is considerable heterogeneity amongst the studies [Chi^2 = 12.59, df = 2 (p = 0.002), I^2 = 84%].

3.3.3.1.5 Unstimulated IL-2. Of the 57 included studies, 2 studies assessed unstimulated IL-2 secretion at rest. Overall, a period of intensified training did not change resting IL-2 secretion (Z = 1.62, (p = 0.11), d= *1.41*, 95% CI [−0.30, 3.12]). However, there is considerable heterogeneity amongst the studies [Chi^2 = 4.26, df = 1 (p = 0.04), I^2 = 77%].

3.3.3.2 Dendritic cells (all rodent studies).

3.3.3.2.1 Dendritic cell CD80 expression. Of the 57 included studies, 2 studies assessed DC CD80 expression as a percentage of fluorescent intensity. Overall, a period of intensified training did not change rodent bone marrow derived DC CD80 expression (Z = 0.82, (p = 0.41), d = −0.33, 95% CI [−1.12, 0.46]; Figure 10). There is low heterogeneity amongst the studies [Chi^2 = 1.64, df = 1 (p = 0.20), I^2 = 39%].

3.3.3.2.2 Dendritic cell MHC II expression. Of the 57 included studies, 2 studies assessed DC MHC II expression. Overall, a period of intensified training did not change rodent DC MHC II expression (Z = 0.31, (p = 0.76), d= −2.30, 95% CI [−17.04, 12.43]; Figure 11). However, there is considerable heterogeneity amongst the studies [Chi^2 = 65.24, df = 1 (p < 0.00001), I^2 = 98%].

3.3.3.2.3 DC CD86 expression. Of the 57 included studies, 2 studies assessed rodent DC CD86 expression as a percentage of fluorescent intensity. Overall, a period of intensified training significantly [Z = 2.99, (p = 0.003)] increased DC CD86 expression with a large effect (d = 2.18, 95% CI [0.29, 4.07]; Figure 12). However, there is substantial heterogeneity amongst the studies [Chi^2 = 3.60, df = 1 (p = 0.06), I^2 = 72%].

4 Discussion

4.1 Overview

The purpose of this study was to bring together a body of research to characterise the T lymphocyte, NK cell and DC activity at rest and in response to exercise stress following periods of intensified training. To assess whether these immune biomarkers could provide insight into their use as diagnostic indicators of the negative states of overtraining, it was necessary to focus on research with appropriate intensity of training. Training protocols of >7 days are more likely to induce NFOR than shorter training periods of the same intensity (Halson et al., 2002). With 93% of included studies utilising a training protocol of >7 days in duration, we can be confident that NFOR was a possibility. In agreement with this, subgroup analysis conducted on resting immune cell counts within this review indicated that training durations of <7 days did not induce alterations in immune cell counts, whereas durations above this did. Understanding the relationship between resting and exercise induced immune cell markers and intensified training periods may be useful in the diagnosis of the negative states of overtraining e.g., NFOR or OTS. Currently no clear biomarker has been uncovered to support diagnosis of these states (Meeusen et al., 2013). Additionally, this review highlights further some gaps within the literature where further experimental research is required.

In total this meta-analysis examined 16 immune biomarkers; 7 were assessed at rest and in response to a bout of exercise, with the remaining 9 assessed at rest only. When comparing each variable from before to after an intensified training period, significant decreases in resting total lymphocyte and $CD8^+$ T cell counts, and unstimulated plasma IL-1β levels were found. In addition, resting DC CD86 expression was significantly increased in rodents only.

4.2 Total lymphocyte and $CD8^+$ T cell counts at rest

The meta-analysis found a significant decrease in resting total lymphocyte and $CD8^+$ T cell count after a period of intensified training. The magnitude, direction, and length of immune recovery after a period of intensified training is dependent upon the intensity, duration, and load of training (Simpson et al., 2020). Therefore, differences in exercise protocols used by studies utilised in this review may account for the inter-study variability in findings. To examine this further, we note that of the studies reporting intensity increases, the study displaying the largest effect size for a significant decrease in resting lymphocyte count elevated running training load by 200% across a 10-day period in trained participants (Pizza et al., 1995). Whereas the study with the lowest effect size increased cycling training load by 40% across a 13-day period in trained participants (Meyer et al., 2004). The use of sudden increases in workloads as large as 200% may not be reflective of practice within elite sport which could be suggested to limit the relevance of the findings to the wider athletic population. However, an overload of the immune system does provide insight into its response to training stress and helps highlight whether immune biomarkers could act as diagnostic indicators of NFOR or OTS. Although this may not be reflective of best practice in an athletes every-day regime, some individuals

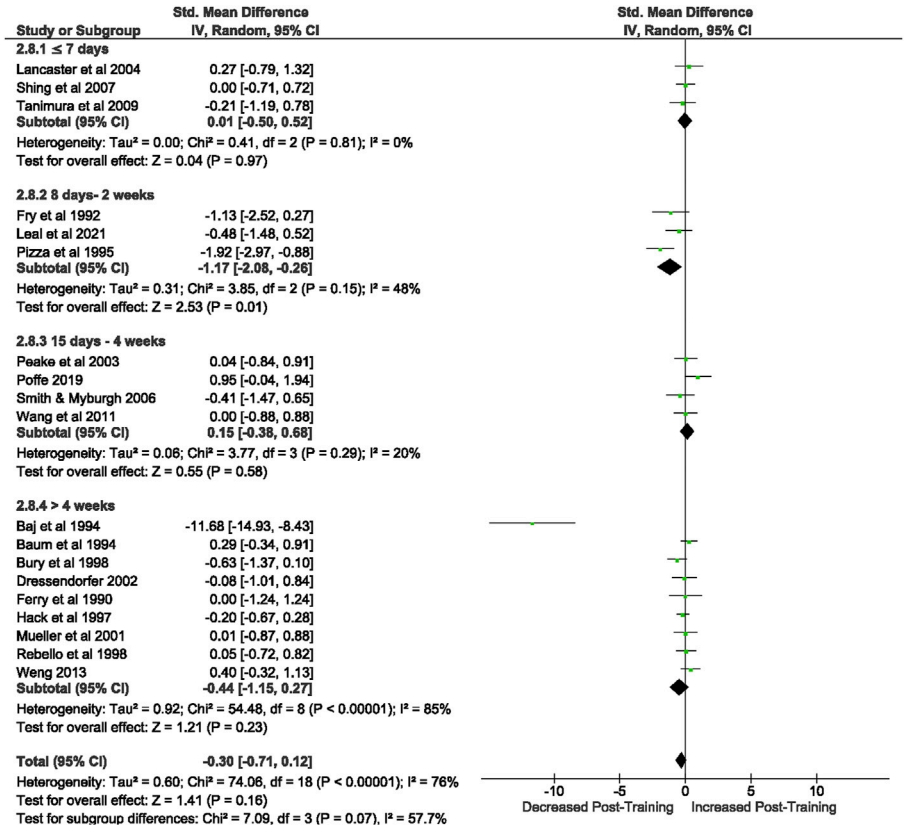

FIGURE 4
Effect of intensified training on resting CD4+ T cell counts, measured by FACS or Automated cell counter. Subgroup analysis is based on the duration of intensified training period; ≤ 7 days, 8 days—2 weeks, 15 day—4 weeks and > 4 weeks. All studies used human participants. *CI* Confidence interval.

may experience this overload. Additionally, the heart rate response and number of active muscles during running is known to be considerably higher than that of cycling exercise. In triathletes, it has been observed that the maximum heart rate achieved during cycling is often 6–10 bpm lower than obtained during running (Millet et al., 2009). Therefore, aside from the obvious intensity differences between Pizza et al. (1995) and Meyer et al. (2004), the use of running exercise in Pizza et al. (1995) induces larger internal stress and is thus more likely to push an athlete towards a state of NFOR than cycling protocols for the same given intensity and/or duration.

During exercise a transient period of lymphocytosis occurs followed by a period of lymphocytopenia after cessation of exercise (Shek et al., 1995). Traditionally it is thought that this lymphocytopenia creates a 3–72 h window of opportunity for infection (Pedersen and Ullum, 1994) which is prolonged and more severe if a second bout of exercise is performed within this time frame (Simpson et al., 2015). Exercise-induced lymphocyte apoptosis (Phaneuf & Leeuwenburgh, 2001) that can still be evident 24 h after a single bout of treadmill exercise to exhaustion (Mars et al., 1998) has been suggested as a possible cause for this. This mechanism is thought to be mediated by cortisol (Riccardi et al., 1999) by binding to glucocorticoid receptors within immune cells, leading to increased cell apoptosis (Cain and Cidlowski, 2017). A single bout of exercise (<1.5 h) has been shown to increase reactive oxygen species (ROS) and is further increased with higher intensities (Thirupathi et al., 2021), often peaking 2–3 days after exercise (Theodorou et al., 2011). This increase in ROS has also been attributed to initiating lymphocyte apoptosis *via* damaging the DNA of the immune cell (Mooren et al., 2004). Reactive oxygen species are oxygen containing molecules that are capable of independent existence, containing at least one or more unpaired electrons (Jakubczyk et al., 2020). At low levels, ROS may function in cell signalling processes, regulating cell growth and differentiation, inflammation, immune responses and immune survival (Romero and Agostinis, 2014). However, at higher levels, ROS may damage cellular DNA of immune cells and thus play a role in apoptosis (Mooren et al., 2004).

As the studies used in this review examined repeated exposures to exercise stress, exceeding the aforementioned duration and/or intensities, apoptosis could be a potential

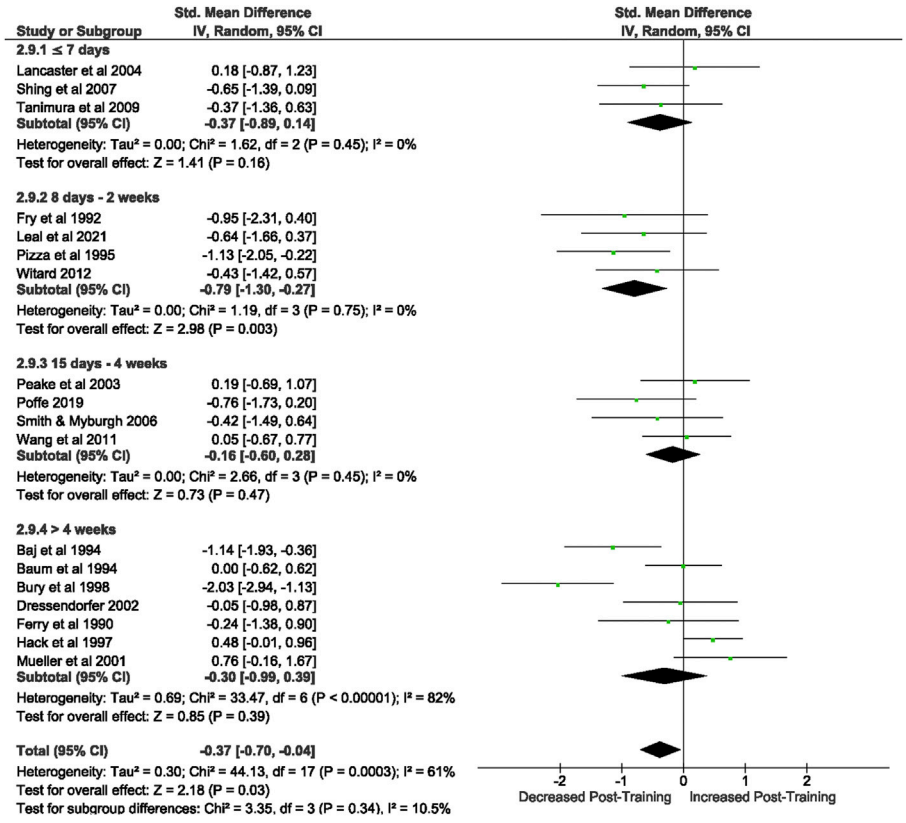

FIGURE 5
Effect of intensified training on resting CD8+ T cell counts, measured by FACS or Automated cell counter. Subgroup analysis is based on the duration of intensified training period; ≤ 7 days, 8 days—2 weeks, 15 day—4 weeks and > 4 weeks. All studies used human participants. *CI* Confidence interval.

reason for the decrease in resting lymphocyte numbers found after a period of intensified training. Despite earlier investigations suggesting that post-exercise lymphocytopenia is a result of apoptosis, Simpson et al. (2007) reported limited lymphocyte markers of apoptosis (Annexin-V (+) or HSPA60) 1 h after treadmill exercise to fatigue completed at 80% $\dot{V}\,O_{2max.}$, yet lymphocytopenia was evident. Moreover, the levels of apoptosis reported in studies indicating an increase in cell death are usually very small i.e., <5% (Mooren et al., 2002; Simpson et al., 2007), and as such is unlikely to account for the 30%–40% reductions in blood lymphocyte count witnessed after exercise (Peake et al., 2016). Therefore, the reduced resting lymphocyte and $CD8^+$ T cell counts after a period of intensified training could instead be due to an exercise-driven redistribution of highly functional effector cells, such as $CD8^+$ T cells from the blood stream into the tissues and organs for heightened identification and eradication of tissue tumour cells (Simpson et al., 2020).

This redistribution has been demonstrated in rodents *via* fluorescent cell tracking following both running and swimming exercise (Kruger et al., 2007). In humans, cycling at 85% of maximum power output (Wmax) for 20 min prompted the preferential mobilisation of highly cytotoxic $CD8^+$ T cells possessing a high propensity to migrate into the peripheral tissues during exercise recovery (Campbell et al., 2009). The redistribution of highly functional effector cells is driven by increased haemodynamics and the release of catecholamines and glucocorticoids following the activation of the sympathetic nervous system and HPA axis (Simpson et al., 2015).

Catecholamines, for example adrenaline and noradrenaline, influence the mobilisation of $CD8^+$ T cells both directly, *via* the action of adrenaline on lymphocyte β_2-adrenergic receptors (Graff et al., 2018) and expression of adhesion molecules (Shephard, 2003), and indirectly, *via* increased cardiac output and shear stress mobilising lymphocytes from endothelial walls (Shephard, 2003). Both mechanisms result in the demargination of highly cytotoxic effector cells into the circulation (Dimitrov et al., 2010). $CD8^+$ cells are the T cell subset expressing the most adrenergic receptors (β_2 receptor) and are therefore more susceptible to change with increased exposure to catecholamines across the

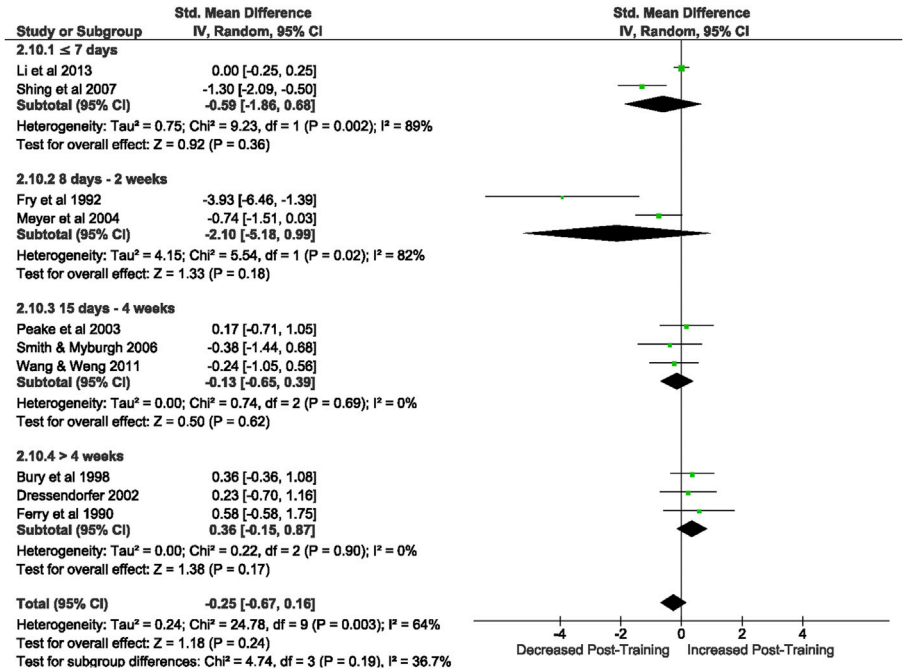

FIGURE 6
Effect of intensified training on resting CD4/CD8 ratio. Subgroup analysis is based on the duration of intensified training period; ≤ 7 days, 8 days—2 weeks, 15 days—4 weeks and > 4 weeks. Studies used a mixtureof human and rodent (Blank et al., 1994; Kaufman et al., 1994) participants. *CI* Confidence interval.

training period (Shephard, 2003). Whilst catecholamines drive the lymphocytosis of $CD8^+$ T cells during exercise, glucocorticoids such as cortisol are thought to influence the egress of $CD8^+$ T cells out of the peripheral blood and into the peripheral tissues and organs during exercise recovery. This is believed to be *via* heightened expressions of certain cell activation and adhesion molecules that facilitate migration, enabling them to pass through endothelial cells and into tissues (Simpson et al., 2006). During prolonged recovery from intensified exercise, substantial infiltration of certain subsets of T lymphocytes into damaged skeletal muscles also occur in order to enhance muscle repair (Jones et al., 1986). It is therefore possible that the reduction in resting lymphocytes, and more specifically $CD8^+$ T cells, is the result of a redistribution into damaged muscles, caused by repeated bouts of exercise over the training period (Pizza et al., 1995).

4.3 Exercise induced lymphocyte counts

Despite significant decreases in resting lymphocyte counts after a period of intensified training, there was no overall significant change in lymphocyte counts in response to an acute bout of exercise. This suggests that the lymphocyte response to exercise stress remains unchanged both before and after a period of intensified training. 8 studies investigated the exercise induced changes in lymphocyte count after a period of intensified training. Of those 8 studies, only 1 found a significant increase in exercise induced lymphocyte count (Wang et al., 2011). All other studies included either found no change (Ferry et al., 1990; Ndon et al., 1992; Ronsen et al., 2001; Witard et al., 2012) or a significant decrease (Lancaster et al., 2004; Shing et al., 2007; Hasanli et al., 2021).

On examination of the acute exercise bouts used before and after the intensified training period to assess the exercise induced lymphocyte changes in each study; all were all-out tests until volitional exhaustion. Differences in exercise intensities of these acute exercise bouts are therefore not the cause of the differences in lymphocyte response between Wang et al. (2011) and the rest of the studies. However, Wang et al. (2011) was the only study that used untrained, sedentary participants, with an average $\dot{V}O_{2max}$ of 44.1 ml/kg/min (classed as "Fair" (ACSM, 2017). All of the other studies utilised participants with a $\dot{V}O_{2max}$ > 60 ml/kg/min; classed as "Superior" (ACSM, 2017). It is known that trained and untrained individuals undergoing a period of intensified training show different cellular responses to exercise, thought to be related to differences in the elevated cortisol levels and alterations in the pro/anti-inflammatory

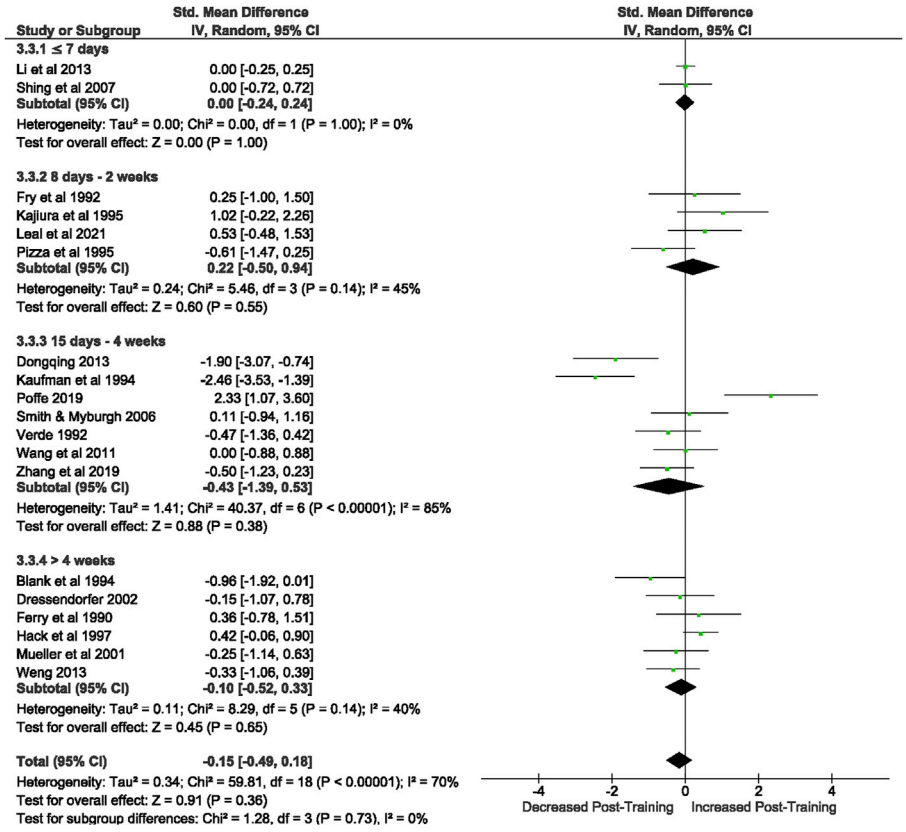

FIGURE 7
Effect of intensified training on resting Natural Killer cells, measured by FACS or Automated cell counter. Subgroup analysis is based on the duration of intensified training period; ≤ 7 days, 8 days—2 weeks, 15 days—4 weeks and > 4 weeks. All studies used human participants. *CI* Confidence interval.

balance in response to exercise (Walsh et al., 2011). Specifically, T cell counts appear to be sensitive to exercise load in well trained individuals undertaking a period of intensified training, but this sensitivity is reduced in sedentary individuals undertaking the same training (Walsh et al., 2011). If Wang et al. (2011) was to be removed, and the meta-analysis re-run using only trained participants, a significant reduction in total lymphocyte counts in response to an acute bout of exercise would be found.

Additionally, the intensity of the training intervention used by Wang et al. (2011) was lower than those used by the other studies assessing exercise induced lymphocyte counts. Wang et al. (2011) used a training intensity of 50% W_{max} which has previously been likened to ~55% $\dot{V}$ O_{2max} (Van Loon et al., 1999). According to Gore et al. (2013), any exercise <60% $\dot{V}$ O_{2max} is classed as light aerobic and represents the lowest training zone. The two studies displaying significant increases in exercise induced lymphocyte counts utilised intensities above the ventilatory threshold level (Shing et al., 2007) and 70–95% HRmax for most of the training duration (Lancaster et al., 2004). It is suggested that anaerobic exercise during maximal effort is the most powerful catecholamine and cortisol stimulator (Baj et al., 1994), therefore the differences in responses between these studies could be because the training protocol adopted by Wang et al. (2011) was not intense enough to elicit such hormonal changes that may blunt the exercise induced lymphocytosis post training that was seen in Lancaster et al. (2004) and Shing et al. (2007).

4.4 Resting unstimulated IL-1β

A significant decrease in resting unstimulated plasma IL-1β levels after a period of intensified training was found. High serum levels of IL-1β are thought to exacerbate damage during chronic disease and acute tissue injuries (Lopez-Castejon and Brough, 2011); commonly implicated in the pathogenesis of chronic diseases such as Rheumatoid Arthritis (Altomonte et al., 1992), Atherosclerosis (Kirii et al., 2003) and Chronic Obstructive Pulmonary Disease (Hammad et al., 2015). As

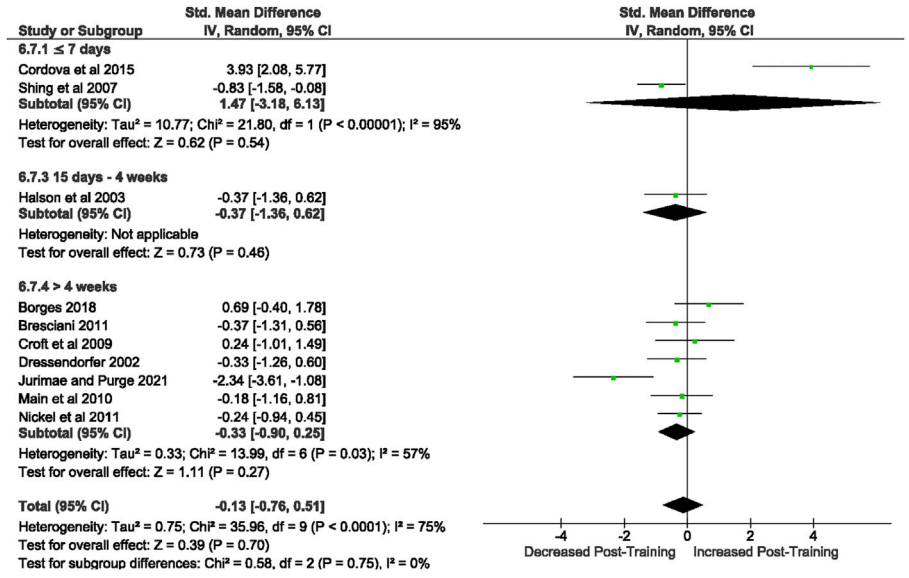

FIGURE 8
Effect of intensified training on resting unstimulated plasma TNF- α. Subgroup analysis is based on the duration of intensified training period; ≤ 7 day, 8 days—2 weeks, 15 days—4 weeks and > 4 weeks. All studies used human participants. *CI* Confidence interval.

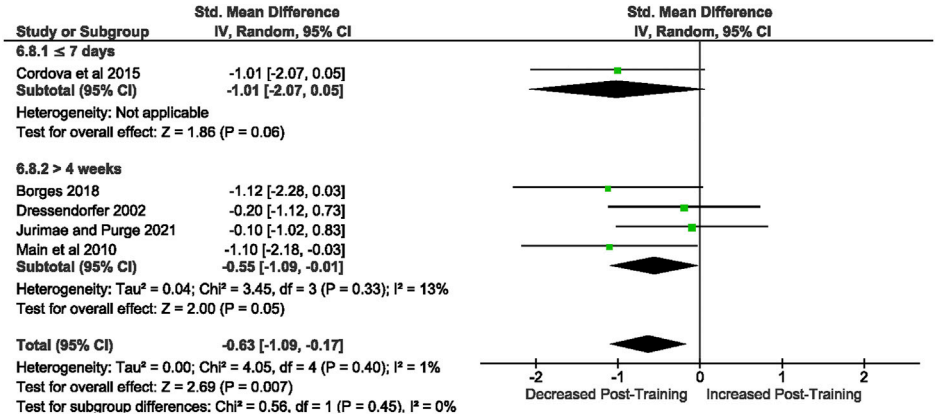

FIGURE 9
Effect of intensified training on resting unstimulated plasma IL-1 β. Subgroup analysis is based on the duration of intensified training period; ≤ 7 days, 8 days—2 weeks, 15 days—4 weeks and > 4 weeks. All studis used human participants. *CI* Confidence interval.

such, a reduction in resting serum IL-1β has been implicated in reducing low grade inflammation and is a target for many anti-inflammatory treatments (Dinarello et al., 2012). This indicates that reduced resting IL-1β levels after a period of training may be seen as a positive anti-inflammatory effect of exercise training. Conversely, IL-1β is essential for resistance to infections, and lower resting levels of IL-1β may reduce the ability to initiate a Type 1 immune response (Murray, 2013). However, in order to understand the true effects this reduction may have on immunity, stimulated cytokine release needs to be assessed.

On examination of the individual studies there needs to be a consideration of how resting IL-1β was defined. Córdova Martinez et al., 2015 took their post training, resting sample 3 h after a cycling race and Main et al. (2010) collected what they referred to as a resting sample 30 min after a water-based rowing session. A study investigating plasma and mononuclear mRNA IL-1β levels in response to a 3-h mixed cycling and running bout at 60%–65% $\dot{V}$ O_{2max} reported that plasma IL-1β levels were still elevated compared to resting pre-exercise levels at both 300 min and 24 h after cessation of exercise, but no change in mRNA was

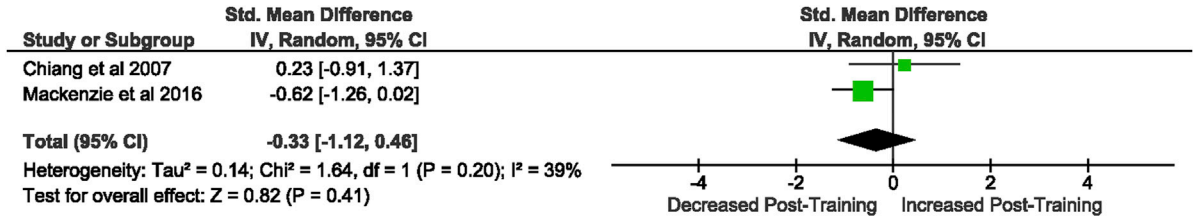

FIGURE 10
Effect of intensified training on resting dendritic cell CD80 expression. All studies used rodent participants. *CI* Confidence interval.

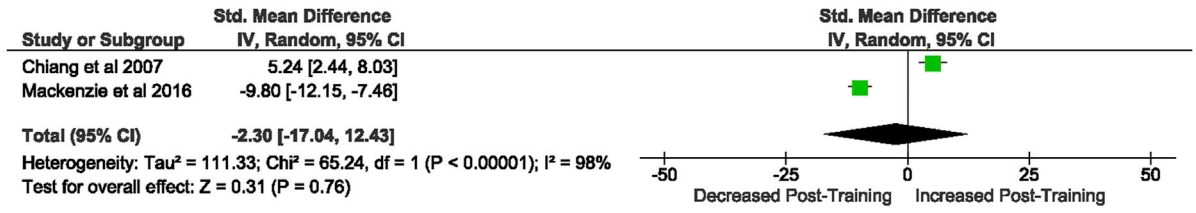

FIGURE 11
Effect of intensified training on resting dendritic cell MHC II expession. All studies used rodent participants. *CI* Confidence interval.

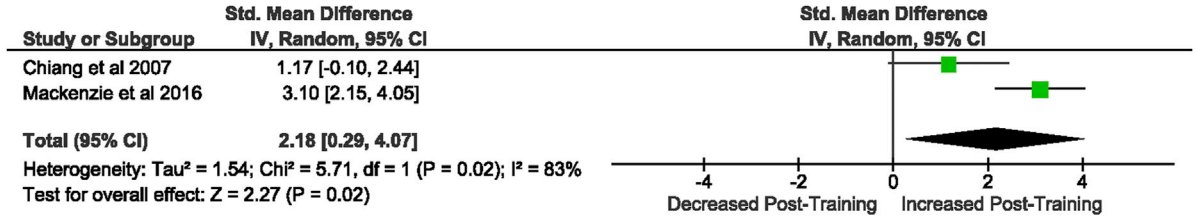

FIGURE 12
Effect of intensified training on resting dendritic cell CD86 expression. All studies used rodent participants. *CI* Confidence interval.

detected (Moldoveanu et al., 2000). Similarly, an acute bout of plyometric exercises consisting of 50 jumps and 50 drop jumps (Chatzinikolaou et al., 2010), and a marathon race (Ostrowski et al., 1999) have been shown to elevate plasma IL-1β levels immediately after exercise. It could therefore be argued that the true resting plasma IL-1β responses have not been shown and may account for the large differences in effect sizes between these studies and the two studies showing the smallest effect sizes. The two studies showing the smallest effect sizes, indicating little to no change in resting IL-1β levels, collected blood samples after at least 24 h of rest (Dressendorfer et al., 2002; Jurimae and Purge, 2021).

In both of the studies displaying little to no change in IL-1β, markers of performance were shown to increase. For example, in a group of competitive endurance cyclists, after 6.5 weeks of intensified training, average heart rate decreased at submaximal levels, cycling economy improved and no change in the testosterone:cortisol ratio was observed that would indicate any physiological stress that may evoke immune changes (Dressendorfer et al., 2002). Likewise, a group of elite rowers undergoing 6 months of volume extended training saw an improvement in performance in the form of increased aerobic power, indicating normal training adaptation (Jurimae and Purge, 2021). Therefore, regardless of sample timing, the results of these two studies may not be representative of the overtrained athlete, and insight into their use as a biomarker of overtraining may be limited.

Although acute bouts of exercise have been shown to elevate unstimulated plasma IL-1β, this review found that a period of intensified training led to significantly reduced levels. Pro-

inflammatory cytokines are mediated by both anti-inflammatory cytokines, such as IL-1ra and IL-6, and cytokine inhibitors, such as cortisol and adrenaline, which are known to increase markedly in the circulation following endurance exercise (Suzuki et al., 2002). Cortisol is known to possess anti-inflammatory effects (Blannin et al., 1996; Ortega et al., 1996) and adrenaline has been shown to downregulate the stimulated production of IL- 1β (Bergmann et al., 1999). Additionally, IL-6, the most notable cytokine secreted from contracting muscles, increases up to 100-fold during exercise, resulting in increased anti-inflammatory cytokine production, and decreased IL-1β production (Beavers et al., 2010). As such, the triggered anti-inflammatory effects of exercise could explain the significant decrease in resting IL-1β levels found after a period of intensified training in this review.

Whilst this review focused on unstimulated IL-1β concentrations, it is apparent that stimulated cytokine production from immune cells may be more informative of the overall immune state (Gleeson et al., 2013, P. 299). This is because IL-1β does not increase exponentially during exercise, which is different when compared to infections (Pedersen and Hoffman-Goetz, 2000). Therefore, stimulating the cytokine response from immune cells after exercise with stimulants such as LPS, mimics the initial innate immune response to bacterial infection. In line with this, Nielsen et al. (2016) found no significant changes in unstimulated IL-1β immediately after a half-marathon, but when measuring LPS-stimulated cytokines after the same bout of exercise, a significant decrease in plasma IL-1β was found. Despite this, only unstimulated cytokine responses were included in the meta-analysis because limited papers using the same stimulants were available for grouping.

4.5 Dendritic cell markers

The meta-analysis revealed a significant upregulation of stimulated CD86 expression after a period of intensified training in rodents, yet no significant changes were found for CD80 or MHC II expression. Only two papers satisfied the search criteria for this analysis, and both were in rodents. As such, it is reasonable to suggest that strong conclusions can not be drawn.

When looking at the trends across all papers that assessed DC markers, including those not suitable for meta-analyses, it is apparent that overall, there is a trend for increased CD86 (Chiang et al., 2007; Mackenzie et al., 2016; Fernandes et al., 2019) expression after a period of training, with conflicting results for MHC II (Chiang et al., 2007; Mackenzie et al., 2016) and CD80 (Liao et al., 2006; Chiang et al., 2007; Mackenzie et al., 2016; Fernandes et al., 2019). Chiang et al. (2007) found a significant increase in DC MHC II expression and IL-12 secretion in male sprawly rats in response to 5 weeks progressive endurance treadmill running, but no significant increase in CD80/86 expression. They suggested the upregulation in MHC II and IL-12 indicates enhanced DC differentiation and maturation, potentially implicating greater antigen presentation ability, and a greater Th1 response to elicit antitumor immunity (Chiang et al., 2007). Chiang et al. (2007) utilised a periodised endurance protocol; a well-designed training programme consisting of progressive intensity increases and sufficient active recovery periods. Periodised endurance training has been shown to modulate immunity in human models (Liao et al., 2006), allowing for sufficient recovery before the next training session. It could therefore be argued that the favourable outcomes seen in Chiang et al. (2007) may not represent the responses that would be seen in an overtrained athlete. Additionally, differences between these studies' findings could also be due to the use of different DC stimulants i.e., Chiang et al. (2007) used LPS, whereas Mackenzie et al. (2016) used OVA stimulation. It has been shown that the OVA-stimulated and LPS-stimulated DC cytokine responses are different in rodents, with LPS inducing a larger response (Huang et al., 2013). As such, these stimulants may also differently affect the expression of DC cell co-stimulatory molecules and MHC II on DCs upon stimulation.

Mackenzie et al. (2016) found a significant increase in DC CD86 expression, a significant decrease in MHC II expression and no significant differences in CD80 expression in mice who underwent 4 weeks of treadmill running at 6% max velocity for 1 $h.d^{-1}$, 5 $days.wk^{-1}$. DCs transmigrate between peripheral blood and the lymphatic system acting as immune sentinels (Brown et al., 2018). When infection occurs, DCs undergo maturation which involves the upregulation of co-stimulatory molecules CD80 and CD86, the MHC complex and IL-12 cytokine secretion (Wehr et al., 2019). All three of these signals are required for T cell activation, therefore, it is unlikely that an upregulation of one of these signals alone will significantly alter DC function, and ability to induce a T cell response. The discrepancies between findings of these studies (Chiang et al., 2007; Mackenzie et al., 2016), in addition to the lack of studies investigating these DC markers is a cause for expansion upon this preliminary work in rodents towards human models, which becomes increasingly important as exercise training may hold the potential to increase DC maturation, and thus antitumor immunity (Chiang et al., 2007). It is acknowledged however, that much is left to speculation, due to the limited numbers of studies assessing the DC response to intensified training, and those studies that have assessed the DC response, only examined rodent models. Therefore, we cannot make any conclusions in humans, but it does suggest some research in human models is required.

The immune system is a complex system. We cannot assume that periods of intensified training, or the cortisol alterations it may induce, affects all immune markers in the same way. Therefore it would not be unreasonable to see variation in the changes of different immune activation markers with intensified training. Furthermore, resilience is the ability of the body to resist, adapt to, recover or grow in response to stressors (Chow et al., 2022). For example, high levels of immune resilience can reduce illness episodes/

hospitalisations and accelerate immune recovery, as shown in COVID-19 patients (Justice et al., 2021). Differences in levels of immune resilience within the participants used in these studies could therefore lead to variations in immune activation and recovery to the same stress, and as such, we cannot assume that all immune systems will respond in the same way to the same level of stressor.

5 Conclusion

This review identified numerous immune biomarkers that have been investigated before and after a period of intensified training. Although this review focuses on the normal impact of high intensity training due to the difficulties surrounding confirmation of NFOR/OTS diagnosis, heavy training is a factor involved in the establishment of NFR/OTS, therefore, the results presented could provide evidence that these immune biomarkers are potentially indicative of NFOR/OTS. Results suggest that although some biomarkers indicated significant alterations after a period of intensified training (resting $CD8^+$ T Cell and total lymphocyte number, unstimulated IL-1β secretion, and DC CD86 expression), definitive immune biomarkers indicative of the negative states of overtraining are limited. Incompatibilities in methodologies and units of measurements between studies, as well as low study numbers contributed to the inability to identify more definitive immune biomarkers within the literature. This review highlights the need for further research into biomarkers specifically relating to dendritic cells, especially in human models. Additionally, although this review aimed to include females, no study returned from the systematic search controlled for menstrual cycle meaning only male data could be included. Therefore, future research should aim to conduct a controlled study of immune biomarkers in female subjects in response to a period of intensified training in order to widen the applicability of findings. The inclusion of humoral immunity, such as the measure of salivary immunoglobulin A was not considered for this review, however, future reviews should include this as a possible immune biomarker to provide a more holistic overview of the immune state after a period of intensified training. Overall, a period of intensified training has been shown to significantly decrease resting total lymphocyte counts, resting $CD8^+$ T cell counts and unstimulated IL-1β levels, and significantly increase DC CD86 expression.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

CB and JH carried out the literature search and screened abstracts and full texts for inclusion. CB and JH independently assessed risk of bias for articles included once screened by full text. CB wrote the manuscript with support from JH, JH, and JP. JH was the main supervisor of this project.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The role of exercise-and high fat diet-induced bone marrow extracellular vesicles in stress hematopoiesis

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Exercise and obesity regulate hematopoiesis, in part through alterations in cellular and soluble components of the bone marrow niche. Extracellular vesicles (EVs) are components of the bone marrow niche that regulate hematopoiesis; however, the role of exercise training or obesity induced EVs in regulating hematopoiesis remains unknown. To address this gap, donor EVs were isolated from control diet-fed, sedentary mice (CON-SED), control diet-fed exercise trained mice (CON-EX), high fat diet-fed, sedentary mice (HFD-SED), and high fat diet-fed, exercise trained mice (HFD-EX) and injected into recipient mice undergoing stress hematopoiesis. Hematopoietic and niche cell populations were quantified, and EV miRNA cargo was evaluated. EV content did not differ between the four groups. Mice receiving HFD-EX EVs had fewer hematopoietic stem cells (HSCs) ($p < 0.01$), long-term HSC ($p < 0.05$), multipotent progenitors ($p < 0.01$), common myeloid progenitors ($p<0.01$), common lymphoid progenitors ($p < 0.01$), and granulocyte-macrophage progenitors ($p < 0.05$), compared to mice receiving HFD-SED EVs. Similarly, mice receiving EX EVs had fewer osteoprogenitor cells compared to SED ($p < 0.05$) but enhanced mesenchymal stromal cell (MSC) osteogenic differentiation in vitro ($p < 0.05$) compared to SED EVs. HFD EVs enhanced mesenchymal stromal cell (MSC) adipogenesis in vitro ($p < 0.01$) compared to CON EVs. HFD-EX EVs had lower microRNA-193 and microRNA-331-5p content, microRNAs implicated in inhibiting osteogenesis and leukemic cell expansion respectively, compared to HFD-SED EVs. The results identify alterations in EV cargo as a novel mechanism by which exercise training alters stress hematopoiesis and the bone marrow niche.

KEYWORDS

physical activity, obesity, microRNA, myelopoiesis, adipogenesis

Introduction

Hematopoiesis, the process of blood cell formation from hematopoietic stem cells (HSCs), occurs in the bone marrow and is regulated by complex interactions between HSCs and their niche. Cellular components of the HSC niche include mesenchymal stem/stromal cells (MSCs), endothelial cells, osteoblasts, adipocytes, neurons, and as well as HSC progeny at various stages of differentiation. MSCs are particularly relevant due to their role in regulating hematopoiesis through their secretome and forming other cellular constituents of the HSC niche through their differentiation. Bone marrow cells communicate *via* paracrine mechanisms that have not been completely described. A greater appreciation for the role in lifestyle factors in regulating the HSC niche and hematopoiesis has recently developed. Preclinical models of diet-induced obesity results in an accelerated accumulation of bone marrow adipose tissue *via* promoting the adipogenic differentiation of MSCs, that is, related to the overproduction of inflammatory myeloid cells from HSCs (Emmons et al., 2019). This aberrant myelopoiesis is believed to contribute to systemic inflammation in obesity. Conversely, data from our lab and others have shown that participation in increased levels of physical activity and exercise training reduces marrow adipose tissue due to increased osteogenic MSC differentiation, reverses aberrant myelopoiesis, and decreases HSC turnover under obesogenic conditions (Styner et al., 2014; Emmons et al., 2019). The paracrine mechanisms responsible for the effects of obesity and physical activity/exercise training on hematopoiesis and the bone marrow niche have not been completely described.

Systemically, obesity is characterized by chronic low-grade inflammation, that is, accompanied by elevated levels of active immune cells in peripheral tissues that secrete pro-inflammatory cytokines (Benova and Tencerova, 2020). In bone marrow, inflammation induces HSC proliferation that promotes pre-emptive HSC pool exhaustion and skews HSC differentiation along the myeloid lineage to form common myeloid progenitor cells (CMPs), granulocytes, and monocytes, thus further exacerbating systemic inflammation (Trottier et al., 2012; Singer et al., 2014). This myeloid cell skewing from HSCs is due, in part, to increased marrow adipose tissue content formation from MSCs (Ambrosi et al., 2017). Styner and colleagues previously linked diet-induced obesity to marrow adipose tissue content formation in mice through high-fat diet (HFD) feeding, finding that exercise was capable of mitigating adipocyte production (Styner et al., 2014). Furthermore, other reports have shown that bone marrow adipocytes are capable of promoting HSC proliferation and differentiation (Poloni et al., 2013; Zhang et al., 2019). Recent work from our lab and others has shown that exercise influences hematopoiesis through alterations to the HSC niche. For example, we have previously shown that acute exercise stimulates HSC mobilization from the bone marrow and alters MSC gene expression (Emmons et al., 2016). Other work from our lab investigating exercise training surrounding a single exposure to sublethal whole-body irradiation found lower adipose tissue accumulation and differentially expressed soluble factors in the marrow of exercise trained mice compared to sedentary groups (Frodermann et al., 2019). Furthermore, Frodermann and colleagues identified leptin signaling from adipocytes and C-X-C motif chemokine ligand 12 (CXCL12) from MSCs as key mediators of HSC expansion (Frodermann et al., 2019). The current literature suggests that paracrine signaling between bone marrow cells regulate obesity- and exercise-induced alterations in hematopoiesis (Poloni et al., 2013; Emmons et al., 2016, 2019; Zhang et al., 2019).

EVs are membrane-bound particles that are secreted by all cells and act as intercellular mediators of communication (Yáñez-Mó et al., 2015; Doyle and Wang, 2019). EVs transfer miRNA and protein to alter recipient cell activity and fate (Ratajczak et al., 2006; Valadi et al., 2007; Skog et al., 2008). Szatmári and colleagues found that injecting EVs from irradiated mice into non-irradiated mice reduced HSC content comparable to direct radiation treatment (Szatmári et al., 2017). Conversely, Wen and colleagues showed non-irradiated MSC-derived EV injections into irradiated mice improves circulating white blood cell concentration recovery, thereby suggesting a role for EVs in promoting stress hematopoiesis (Wen et al., 2016). These findings demonstrate that EVs influence HSC activity and fate. To date, there has not been a full characterization of the paracrine factors regulating hematopoiesis with exercise and HFD. However, previous reports have shown exercise impacts EV production. In skeletal muscle, miR-486 is downregulated following a bout of acute exercise (Aoi et al., 2013); miR-486 has also been linked to erythrocyte formation, thereby indicating a systemic role for EVs in affecting hematopoiesis (Shi et al., 2017). Other reports have shown exercise training increases total circulating EV and circulating endothelial cell-derived EV concentrations (Bei et al., 2017; Ma et al., 2018).

Although prior studies have examined the role of EVs in regulating stress hematopoiesis (Wen et al., 2016; Szatmári et al., 2017), no study has investigated the alterations to bone marrow-derived EV cargo by exercise or HFD, or their impact on HSC and HSC niche cell content. Therefore, the purpose of the current study was to examine the effects of EVs derived from exercise trained and sedentary mice given a HFD or control (CON) diet on stress hematopoiesis in naïve mice. We hypothesized that EVs from mice fed a HFD would increase HSC and adipocyte progenitor cell concentrations in the bone marrow, and that EVs from exercise-trained mice would attenuate these effects.

Materials and methods

Experimental design

All protocols were approved by the University of Ottawa Animal Care Committee in accordance with the *Animals for*

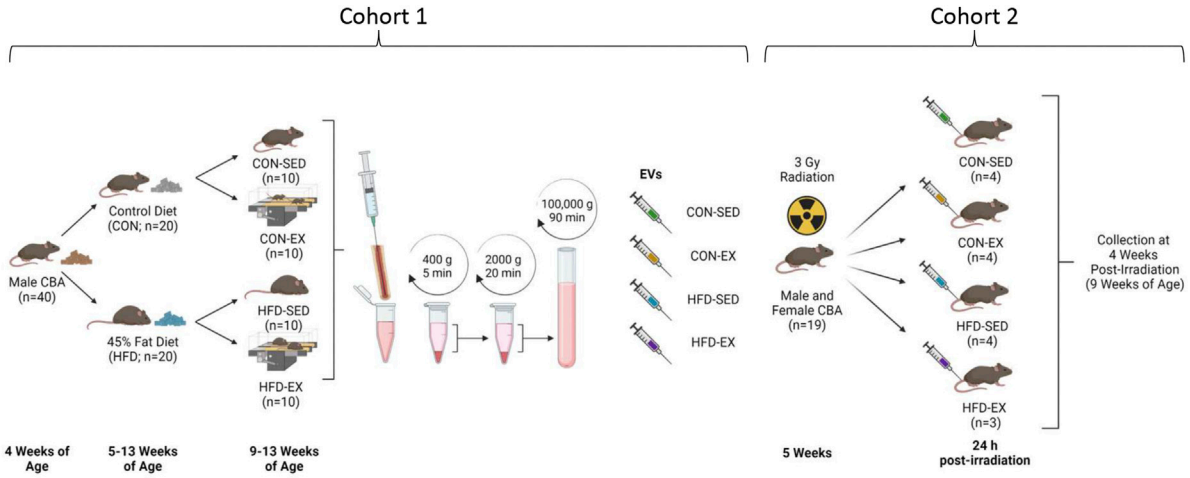

FIGURE 1
Study outline for EV isolation and injection. Male CBA mice (n = 40) were placed on high-fat (HFD) or control (CON) diets for 8 weeks with the latter 4 weeks including exercise (EX) or sedentary (SED) conditions. Bone marrow was extracted, and extracellular vesicles (EVs) isolated by differential centrifugation. Collected EVs were combined by group and injected into new male and female CBA mice by tail vein 24 h following ionising radiation injury. Bone marrow samples were collected at 4 weeks post-irradiation. Created with BioRender.com.

Research Act and by the Canadian Council on Animal Care. Mice were maintained on a 12:12 h light-dark schedule with food and water provided *ad libitum*. Cohort 1 was used to derive EVs. For the first cohort, male CBA (n = 40; Jackson Laboratories, United States) mice aged 5 weeks were randomly selected for 8 weeks of 45% fat (HFD; D12451, Research Diets, NJ, United States) or control (CON; D10012M, Research Diets, NJ, United States) diet feeding (Figure 1). After 4 weeks on their respective diets, half of the mice in each condition were randomly selected for exercise training (EX) while the rest remained sedentary (SED). Cohort 2 mice were EV recipients. For Cohort 2, male and female CBA mice (n = 3-4 per group) were irradiated with 3 Gy of whole-body gamma radiation using a X-Rad 320 biological irradiator (Precision X-Ray, Madison, Connecticut, United States) to induce stress hematopoiesis. At 24-h post-irradiation, Cohort 2 mice were injected with bone marrow-derived EVs isolated from non-irradiated Cohort 1 mice. Cohort 2 mice remained sedentary and were fed standard chow (Teklad 2018 Rodent Diet, Envigo, Indianapolis, IN, United States) *ad libitum* throughout the study. Four weeks after EV injection, bone marrow was collected for cell content analysis from Cohort 2 mice.

Endurance test and exercise training

Mice in Cohort 1 underwent an endurance test using a Exer 3/6 treadmill (Columbus Instruments, Columbus, OH, United States) angled upwards at 5°, as described previously, to evaluate training status (Baker et al., 2011; De Lisio et al., 2011, 2013; De Lisio and Parise, 2012; Emmons et al., 2019; Farber et al., 2021). All mice were acclimated for 10 min at 8 m min^{-1} the week prior to the endurance test. Cohort 1 mice ran at 10 m min^{-1} and increased speed once every 2 minutes by 1 m min^{-1} until the mice were resistant to running or were unable to keep their hind limbs on the treadmill for one full stage despite gentle encouragement. The mice were encouraged to run using a soft-bristle paint brush; electric shock was not used for any test. Following the endurance test, Cohort 1 mice underwent an exercise training program, as described previously (Baker et al., 2011; Emmons et al., 2019; Farber et al., 2021). The exercise training program involved three exercise sessions per week for 40–60 min, depending on the training week. The exercise protocol consisted of a warm-up period at 8 m min^{-1} for 10 min for the first 3 weeks (10 m min^{-1} for week 4) followed by an 8–10 m min^{-1} training speed for 25– (week 1), 35– (week 2), or 45– (weeks 3, 4) min and a cooldown period at 8 m min^{-1} for 5 min. Sedentary mice were placed in sham treadmills on top of the running treadmill to account for any stress associated with the treadmill vibrations, noise, and handling.

Extracellular vesicle characterization, injection, and miRNA quantification

EVs were isolated from the bone marrow supernatant *via* differential ultracentrifugation as described previously, with

minor modifications (Lobb et al., 2015). Briefly, bone marrow was extracted from Cohort 1 mice femurs and tibias using a 25-gauge needle and 1 ml of phosphate-buffered saline (PBS), followed by a 400 g centrifugation cycle at 4°C for 5 min. The supernatant was stored at −80°C for later analysis and injection. After thawing, the supernatant was centrifuged at 2000 g at 4°C for 20 min to remove any remaining cell fragments and apoptotic bodies. The supernatant was removed and ultracentrifuged thereafter at 100,000 g at 4°C for 90 min using an Optima MAX-micro-Ultracentrifuge (Beckman Coulter, Brea, CA, United States). EV pellets were resuspended with 110 µL sterile PBS, of which 10 µL was used for concentration and size distribution analysis at 1:100 dilution using the ZetaView PMX 110 Multiple Parameter Particle Tracking Analyzer (Particle Metrix, Meerbusch, Germany). Relative to *in vitro* isolation, bone marrow offers a small pool of EVs. Across all groups, we found that our bone marrow EV yield averaged 420 million particles per sample. Based on our quantification we were able to combine grouped samples to increase the EV injection counts to 650 million per mouse in Cohort 2, with some remaining samples available for follow-up miRNA content and *in vitro* C3H 10T1/2 MSC adipogenic and osteogenic analysis. Following EV quantification, EVs were combined by exercise and dietary condition and injected via tail vein. Mice were weighed prior to injection with sample volumes standardised across each mouse to 8 µL/g body weight. EV isolation was confirmed by Western blot using 30 µg of protein derived from six combined bone marrow EV samples and MSC lysate. Antibodies included anti-TSG101 (T5701, Sigma-Aldrich), anti-Alix (SAB4200476, Sigma-Aldrich), anti-Flotillin-2 (#3436S, Cell Signaling Technology) diluted 1:1000 in 5% bovine serum albumin-diluted tris-buffered saline with 1% Tween 20 (TBST). Horseradish peroxidase-linked secondary antibody (7074S, Cell Signaling Technology) was diluted 1:10,000 in 5% milk-TBST. The membranes were incubated for 5 min using SuperSignal West Pico PLUS enhanced chemiluminescent substrate (ThermoFisher Scientific, MA, United States) and visualized using a ChemiDoc Imaging System (Bio-Rad, CA, United States).

EV miRNA cargo from individual mice in Cohort 1 ($n = 3$ per group) was isolated using the Qiagen miRNEasy Micro kit (Hilden, Germany) according to the manufacturer's instructions. Extracted miRNA sample integrity and concentrations were measured using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, United States), with concentrations ranging from 47.7 to 166.6 ng/µL. Using 100 ng of isolated miRNA, specific miRNA content expression was identified using the Nanostring mouse v1.5 miRNA kit as per the manufacturer's instructions (Nanostring, Seattle, WA, United States). The prepared cartridges were read using a Nanostring nCounter station. One CON-SED and two CON-EX samples did not pass quality control measures on Nanostring's proprietary nSolver 4.0 software and were excluded from statistical analysis. The HFD-SED and HFD-EX samples were analyzed using the ROSALIND analysis program (Seattle, WA, United States). Briefly, the nCounter-derived RCC files for the HFD-SED and HFD-EX conditions ($n = 3$ per condition) were uploaded to the ROSALIND website. The samples were normalized by positive control and codeset normalization with fold-changes and significance calculated by the Student's t-test. Absolute-fold changes of ± 1.25 or greater (the default value by ROSALIND) and a p-value lower than 0.05 were considered significant. miRNAs that were differentially expressed underwent further investigation for potential gene targets using the miRWalk miRNA target mining feature (http://mirwalk.umm.uni-heidelberg.de/). All differentially expressed miRNAs were inserted to the search list and subsequent gene set enrichment analysis was selected for KEGG and GO:BP enrichment pathways. The top five results for the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology: Biological Processes (GO:BP) enrichment pathways were recorded for literature investigation.

Bone marrow collection and cell quantification

Mice were euthanized at 4 weeks post-EV injection by CO_2 asphyxiation followed by cervical dislocation. Femurs and tibiae were isolated for bone marrow extraction. Phosphate-buffered saline (PBS) was flushed at least 6 times through the bone marrow cavity to maximize bone marrow tissue collection. The cell suspension was centrifuged at 400 g at 4°C for 5 min and the EV-rich supernatant was frozen at −80°C immediately. The remaining cell pellet was gently resuspended with 5% fetal bovine serum (FBS) with PBS and stored on ice until further processing. To extract any remaining cells, the bones were crushed once with a pestle and mortar, cut into tiny fragments, and chemically digested for 45 min with 0.2% type II collagenase diluted in high glucose DMEM. The cells were triturated every 15 min to promote chemical digestion. Bone marrow content from flushing and bone digestion were combined, filtered through a 70 µm filter, and centrifuged at 400 g at 4°C for 5 min. The pellets were collected and resuspended in 5% FBS for flow cytometry staining.

Cohort 2 bone marrow bone marrow cells were quantified using an Attune NxT flow cytometer (Thermo Fisher Scientific), as described previously (De Lisio and Parise, 2012; Emmons et al., 2016, 2019). All cell identification strategies are summarized in Table 1, and all antibodies, fluorophores, and associated catalog numbers are summarized in Table 2. Live cells were used for the Zombie Yellow viability dye compensation. The flow cytometry gating was based on unstained and single-stained bone marrow cells (Supplemental Figures S1–S3).

TABLE 1 Flow cytometry gating strategies for HSPC and niche cell populations.

Cell population	Gating strategy
Hematopoietic Stem Cells (HSCs)	Lineage$^-$, Sca-1$^+$, c-Kit$^+$ (LSK)
Long-Term HSCs	LSK, CD48$^-$, CD150$^+$
Short-Term HSCs	LSK, CD48$^-$, CD150$^-$
Multipotent Progenitors	LSK, CD48$^+$, CD150$^-$
Common Myeloid Progenitors	LSK, CD16/32^{int}, CD34^{int}
Common Lymphoid Progenitors	Lineage$^-$, Sca-1^{int}, c-Kit$^-$
Granulocyte-Monocyte Progenitors	LSK, CD16/32^{hi}, CD34$^+$
Megakaryocyte-Erythroid Progenitors	LSK, CD16/32$^-$, CD34$^-$
Mesenchymal Stromal Cells	Ter119$^-$, CD45$^-$, CD31$^-$, CD51$^+$, CD140α$^+$
Endothelial Cells	Ter119$^-$, CD45$^-$, CD31$^-$, CD51$^-$
Adipocyte Progenitors	Ter119$^-$, Sca-1$^+$, CD45$^-$, CD31$^-$, CD51$^-$
Osteoblasts	Ter119$^-$, Sca-1$^-$, CD45$^-$, CD31$^-$, CD51$^+$
Osteoprogenitors	Ter119$^-$, Sca-1$^+$, CD45$^-$, CD31$^-$, CD51$^-$

C3H 10T1/2 proliferation and differentiation assays

C3H 10T1/2 cells are a model of bone marrow stromal cells that are capable of induced osteogenic and adipogenic differentiation (Shea et al., 2003; Huang et al., 2009). To observe the effects of HFD- and EX-induced EVs on MSC proliferation and differentiation directly, C3H 10T1/2 cells were plated on 96-well plates (1250 cells per well) with 100 µL of growth media (10% FBS, 1% penicillin-streptomycin, in high-glucose Dulbecco's Modified Eagle Medium; DMEM) and incubated at 37°C for 72 h to reach 90% confluence. Cells were then given either adipogenic or osteogenic differentiation media. For the initial plating and each concurrent media change, cells were supplemented with 5.1 million EVs. This value represented approximately twice the EV concentration injected into the mice and was selected to directly measure the effects of HFD- and EX-EVs on MSC differentiation and/or proliferation. Adipocyte differentiation was induced using the Mesencult adipogenic differentiation kit according to the manufacturer's instructions (Stemcell Technologies, Vancouver, BC, Canada). Two days after adding differentiation media, cells were supplemented with 1 µg/ml of insulin to induce adipocyte cell maturation for another 3 days. After 5 days of differentiation media supplementation, cells were fixed in 10% formalin on a rocker for 30 min at room temperature, washed twice with 100 µL of distilled water, and stained with 100 µL of 0.5% Oil Red O dye on a rocker for 30 min at room temperature while protected from light. Unbound dye was washed away by washing the wells 3 times with distilled water. The remaining bound dye was removed by adding 200 µL of isopropanol and was incubated on a rocker at room temperature for 10 min while protected from light. 100 µL of the isopropanol-Oil Red O dye solution was transferred to a new 96-well plate and measured by spectrophotometry at 492 nm. Osteogenesis was induced using the Mesencult osteogenic differentiation kit according to the manufacturer's instructions (Stemcell Technologies). Osteogenic differentiation media was replaced every 3 days for 14 days. After 14 days of differentiation, cells were fixed in 10% formalin on a rocker for 30 min at room temperature, washed twice with 100 µL of distilled water, and stained with 100 µL of 0.2% Alizarin Red S dye on a rocker for 20 min at room temperature while protected from light. Alizarin Red S preparation and extraction was conducted as outlined by Gregory and colleagues using 10% of the listed volumes to account for the 96-well plate volume capacity (Gregory et al., 2004). Absorbance was read on a spectrophotometer at 405 nm.

The effects of HFD- and EX-EVs on C3H 10T1/2 cell proliferation were measured using the Alexa Fluor 488 Click-iT EdU cell proliferation kit according to the manufacturer's

TABLE 2 Antibodies used for flow cytometry.

Antibody	Conjugate	Dilution	Product number
CD16/32	BV711	1:200	101337
CD31	BV510	1:200	563089
CD34	Pe-Cyanine5	1:200	119312
CD45	PE-Cyanine7	1:200	552848
CD48	BV510	1:200	563536
CD51	BV421	1:200	740062
Sca1 (Ly6A/E)	PE	1:200	553336
cKit (CD117)	PE-Cyanine7	1:200	558163
CD140a	PE	1:200	562776
CD150	BV421	1:200	562811
Lineage Panel (5)	Biotin	1:200 (5:200 total)	559971
Streptavidin	FITC	1:800	554060
Viability	Zombie Yellow	1:300	423104

instructions (C10337, Thermo Fisher Scientific). Briefly, 1250 cells were added to each well in a 96-well plate with 100 µL of growth media, as described above, and were incubated for 2 h at 37°C to allow cell adhesion to the plate. After 2 h, 50 µL of the cell culture media was removed and replaced with 50 µL of 20 µM EdU labeling solution (for a final concentration of 10 µM) containing 5.1 million EVs per well. Cells were fixed with 100 µL 4% formaldehyde at 6-, 12-, 24-, and 48-h post-media change, washed twice with 100 µL of 3% BSA in PBS, and permeabilized with 100 µL 0.5% Triton X-100 in PBS. To detect EdU-incorporated cells 50 µL of the Click-iT Plus reaction cocktail (85.76% 1X Click-iT reaction buffer, 4% Copper protectant, 0.24% Alexa Fluor picolyl azide, 10% 1X Click-iT EdU buffer additive) was added to each well and incubated for 30 min at room temperature while protected from light. Wells were then washed with 100 µL 3% BSA in PBS and again with 100 µL PBS. To stain all DNA, 1X Hoeschst 33342 (5 µg/ml) was added to each well and incubated for 30 min at room temperature while protected from light. The wells were then washed twice with 100 µL PBS. Plates were imaged and analyzed with a ZEISS CellDiscoverer7 microscope.

Statistical analysis

Flow cytometry and C3H 10T1/2 *in vitro* proliferation and differentiation data were analyzed using a two-factor (diet and exercise) ANOVA followed by a Sidak post-hoc analysis for interaction effects. The two-factor ANOVAs were carried out using GraphPad Prism 9 (GraphPad Software). A Student's t-test was conducted by ROSALIND software to compare HFD-SED and HFD-EX miRNA data. All data are presented as mean ± SEM with $p < 0.05$ being considered statistically significant. Investigators were blinded to all files and experimental groups for all analyses.

Results

Exercise increases extracellular vesicle concentration at specific sizes

EV isolation was confirmed through Western blot analysis using the positive markers; ALIX, Flotillin-2, and TSG 101, according to recommended guidelines (Willms et al., 2016; Théry et al., 2018), with MSC lysate included as a positive control (Figure 2A). NTA revealed no difference in average particle concentration between any groups ($n = 10$ per condition) (Figure 2B). Most EV particles were smaller than 200 nm in size, an expected observation as a large proportion of extracellular vesicles are small EVs that are typically 30–200 nm in size (Maumus et al., 2020). However, EV concentration was higher in CON-EX *vs.* CON-SED at 225 ± 15 nm ($p < 0.05$), and there was a main effect of exercise having higher EV concentrations at 315 ± 15 and 345 ± 15 nm ($p < 0.05$) (Figure 2C).

Extracellular vesicles from exercise training mice reverse HSC expansion and myeloid progenitor cell skewing with HFD during stress hematopoiesis

Mice injected with HFD-EX EVs had lower concentrations of HSC (Figure 3A), long-term HSC (LT-HSC) (Figure 3B), multipotent progenitor (MPP) (Figure 3D), CMP (Figure 3E), common lymphoid progenitor (CLP) (Figure 3F), and granulocyte-macrophage progenitor cells (Figure 3G) compared to mice injected with HFD-SED EVs (all $p < 0.05$). There was also a trend for fewer short-term HSCs (ST-HSCs) following injection of EX EVs compared to SED (Figure 3C, $p = 0.056$). There were no effects of HFD or EX EVs on megakaryocyte-erythroid progenitor (MEP) cells (Figure 4H) or on total bone marrow cellularity (Figure 3I).

Extracellular vesicles from exercise training mice reduce osteoprogenitor cell concentration during stress hematopoiesis

Mice injected with neither EX nor HFD EVs impacted MSC (Figure 4A), adipocyte progenitor (HFD-EX vs. HFD-SED interaction $p = 0.14$) (Figure 4B), endothelial (Figure 4C), osteoblast (Figure 4E) during stress hematopoiesis. Mice injected with EX EVs; however, had lower osteoprogenitor cell concentrations ($p < 0.05$) (Figure 4D).

HFD Extracellular vesicles increase MSC adipogenic differentiation and EX EVs increase osteogenic differentiation

Since EX EVs decreased osteoprogenitor cell concentrations, we investigated whether HFD and EX EVs directly impact MSC fate. There was no effect of HFD EVs or EX EVs on MSC proliferation, although a trend ($p = 0.098$) was seen for higher proliferation at 6 h post-Edu addition with CON-SED EVs compared to CON-EX EVs (Figure 5A). MSCs treated with HFD EVs *in vitro* had higher adipogenic differentiation ($p < 0.01$) compared to MSCs treated with CON EVs (Figure 5B). Conversely, MSCs treated with EX EVs had higher osteogenic differentiation ($p < 0.05$) compared to MSCs treated with SED EVs (Figure 5C).

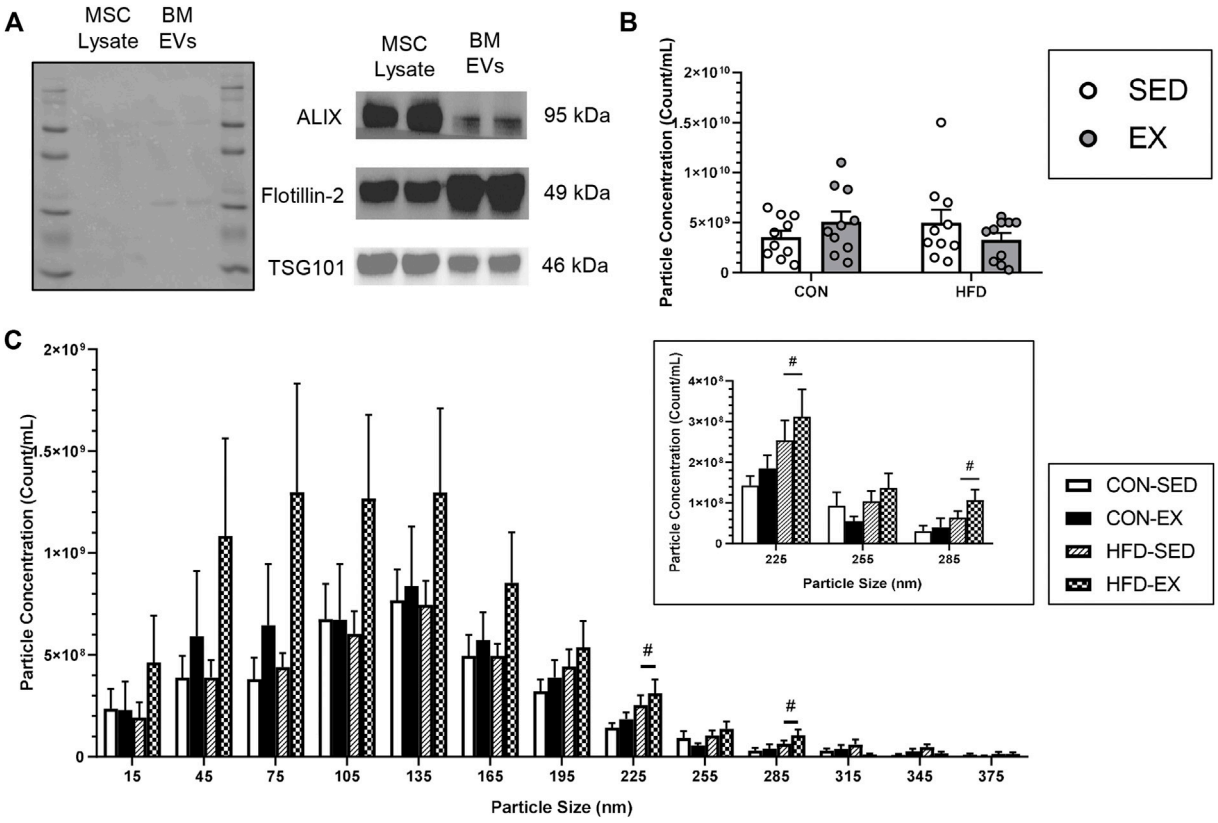

FIGURE 2
Characterization of extracellular vesicle (EVs) isolated from bone marrow supernatant of high-fat (HFD) or control (CON) diet-fed mice placed on exercise (EX) or sedentary (SED) conditions. **(A)** EV presence confirmation by mesenchymal stromal cell (MSC) lysate positive control measures of ALIX, Flotillin-2, and TSG101 proteins, **(B)** Total extracted particle concentration measured by nanoparticle tracking analysis (n = 10 per group), and **(C)** EV size distribution of injected EVs. $^{\#}p < 0.05$, high-fat diet difference *versus* control diet (n = 10 per group).

HFD-EX EVs have lower miR-193 and miR-331-5p content compared to HFD-SED Extracellular vesicles

We conducted a high-throughput miRNA screen from EVs in the current study to determine if alterations in miRNA cargo may explain our observed alterations to the various bone marrow cell populations. We focused this screen on HFD-SED and HFD-EX based on changes observed in our flow cytometric results and to determine the extent to which exercise training could reverse the effects of HFD. Our analysis of 577 distinct mouse miRNAs revealed lower miR-193 (2.00 fold, $p < 0.01$) and miR-331-5p (2.63 fold, $p < 0.05$) content in HFD-EX (n = 3) compared to HFD-SED (n = 3) (Figure 6A).

Using miRWalk, we identified the top five predicted target pathways of both miR-193 and miR-331-5p combined by KEGG and GO:BP analysis (Figure 6B). KEGG analysis predicts miRNA control of genes and the affected general metabolic pathways while GO:BP predicts more detailed molecular functions. KEGG analysis showed predictive activity in the MAPK, ErbB, and Rap1 signaling pathways alongside pathways in cancer and axon guidance, while GO:BP analysis showed predictive interactions with the canonical Wnt signaling pathway, transcription regulation, apoptosis, endocytosis, and intracellular signal transduction.

Discussion

Exercise and obesity induce significant alterations to bone marrow architecture and HSC activity (Styner et al., 2014; Emmons et al., 2016). Given that EVs regulate hematopoiesis (Wen et al., 2016; Szatmári et al., 2017), and exercise training increases circulating EV concentrations (Bei et al., 2017), we sought to understand the role of exercise- and high fat diet-induced EVs as molecular mediators of stress hematopoiesis and bone marrow remodeling. In diet-induced obesity, exercise-induced EVs reversed the effects of diet on HSC concentrations and induced MSC differentiation along the osteogenic lineage during stress hematopoiesis. Interestingly,

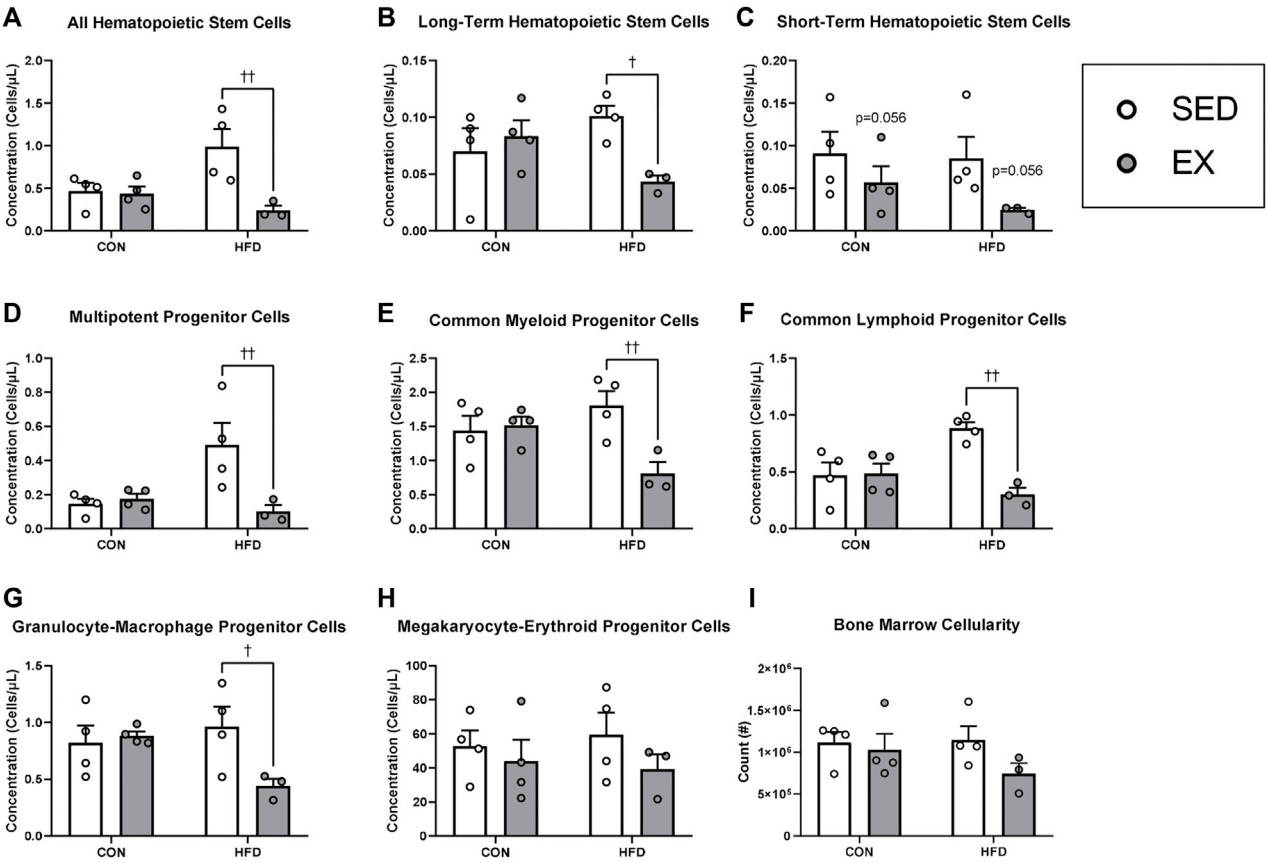

FIGURE 3
Extracellular vesicles from exercise-trained (EX) mice given a high-fat diet (HFD) return hematopoietic stem (HSC) and progenitor cell concentrations to control diet levels. **(A)** All HSCs, **(B)** long-term HSCs, **(C)** short-term HSCs, **(D)** multipotent progenitor cells, **(E)** common myeloid progenitor cells), **(F)** common lymphoid progenitor cells, **(G)** granulocyte-macrophage progenitor cells, **(H)** megakaryocyte-erythroid progenitor cells **(I)** total bone marrow cellularity. $^{\dagger}p < 0.05$ interaction effect; $^{\dagger\dagger}p < 0.01$ interaction effect ($n = 4$ per group, HFD-EX $n = 3$).

HFD-induced obesity resulted in bone marrow EVs that promoted adipogenesis *in vitro*. We further identified down-regulation of miR-193 and miR-331-5p content in exercise-induced EVs from high fat diet-fed mice as a potential molecular target responsible for these effects. Together, our findings suggest that bone-marrow derived EVs from EX mice partially reverse the effects of HFD EVs on hematopoiesis and the HSC niche providing a novel mechanism for the effects of exercise on hematopoiesis.

Previous studies that have examined EVs in response to exercise have focused mostly on the effects of acute exercise-induce and circulating EVs on skeletal muscle adaptations to exercise (Nederveen et al., 2021). The present study provides the first description of exercise-induced EVs specifically from the bone marrow, and also the first investigation of the effects of exercise-induced EVs on hematopoiesis. Acute exercise studies have shown elevated circulating EV concentrations immediately following a stepwise cycling test to exhaustion that subsided at 90-min post-exercise (Frühbeis et al., 2015). However, similar to our findings in bone marrow, previous work has indicated that chronic exercise training does not result in any changes to total EV concentration but found an altered miRNA expression profile (Hou et al., 2019). As such, it appears that changes in total EV concentrations may be part of the normal stress response to unaccustomed exercise, while training may have a larger impact on EV cargo. Despite no alterations to total EV concentration with HFD or EX, we found a redistribution of EV size in response to EX with EX mice having higher EV concentration at 315 ± 15 and 345 ± 15 nm, and specifically in CON diet condition compared to HFD at 225 ± 15 nm. This size range corresponds to large EVs (>100 nm) which predominantly originate as microvesicles that are formed directly from plasma membrane budding (Ståhl et al., 2019). This origin disparity leads to varying cargo profiles (Bruschi et al., 2019) that differentially impact cell function (Wen et al., 2016). These data suggest EX alters EV release in a way that partially reverses the consequences of diet-induced obesity.

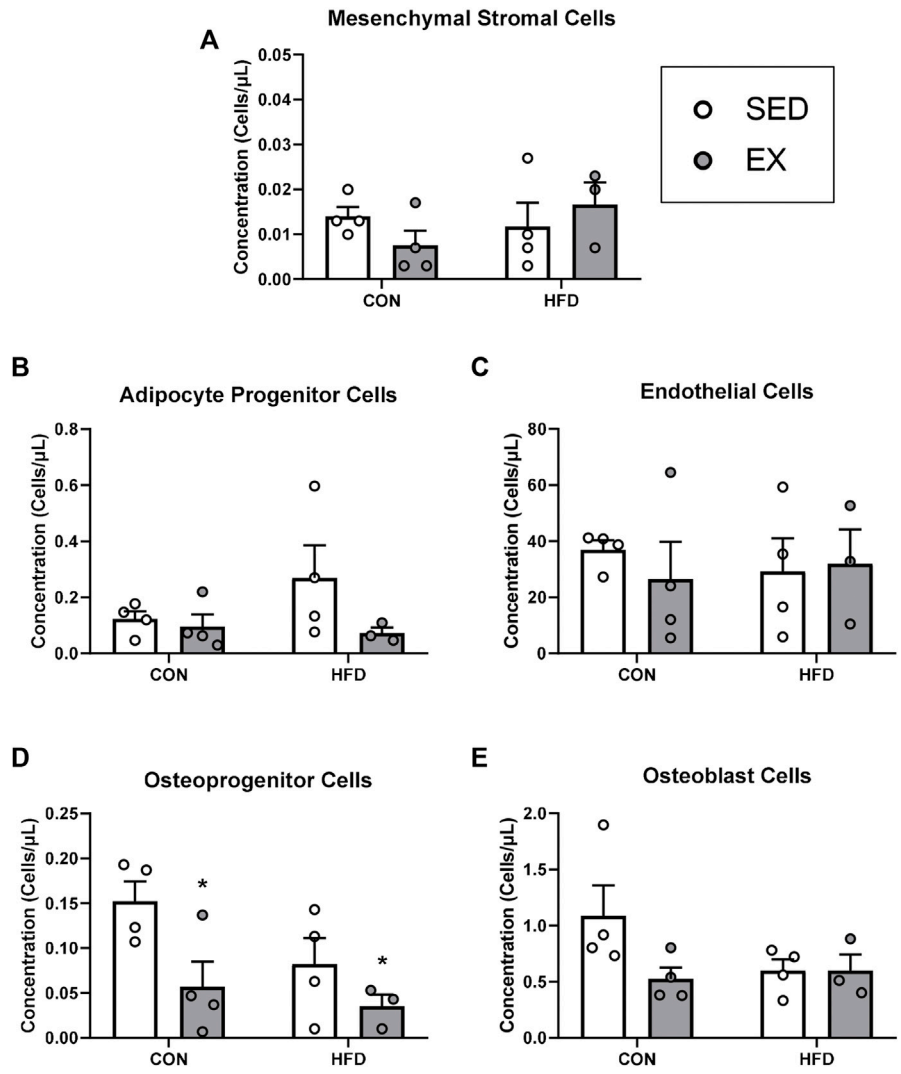

FIGURE 4
Extracellular vesicles from exercise-trained mice decrease osteoprogenitor cell concentrations. **(A)** Mesenchymal stromal cells, **(B)** adipocyte progenitor cells, **(C)** endothelial cells, **(D)** osteoprogenitor cells, and **(E)** osteoblast cells. *$p < 0.05$ main effect of exercise ($n = 4$ per group, HFD-EX $n = 3$).

Previous reports from our lab and others indicate exercise impacts hematopoiesis *via* paracrine actions in the HSC niche (Emmons et al., 2016; Wen et al., 2016; Szatmári et al., 2017; Frodermann et al., 2019). Therefore, to determine the role of EVs in these effects, we injected the isolated EVs from HFD and EX mice into naïve mice undergoing radiation-induced stress hematopoiesis. We injected EVs 24 h post-irradiation because Wen and colleagues had previously shown that injecting EVs at this timepoint following radiation altered hematopoiesis (Wen et al., 2016). Similar to previous work examining the effects of exercise in obesity on hematopoiesis (Frodermann et al., 2019), we found that exercise-induced EVs attenuated hematopoiesis in the HFD condition as evidenced by reduced concentrations of total HSC, LT-HSC, MPP, and CLP concentrations. Previous work from our lab used a similar model to the current study, where mice instead were given 3 Gy irradiation after 8 weeks of HFD or CON diet interventions and 4 weeks of EX or SED, while continuing the EX and dietary interventions for another 4 weeks after radiation (Emmons et al., 2019). Interestingly, in that study we found that EX increased LT-HSC, ST-HSC, MPP, and CLP cell counts without an effect of HFD (Emmons et al., 2019). Discrepancies in these studies could be due to different durations of exercise training, the direct effects of exercise *versus* indirect effects of exercise-induced EVs, or the age when the mice were irradiated. In the current study mice were irradiated at 5 weeks of age while the mice in the previous study were irradiated at

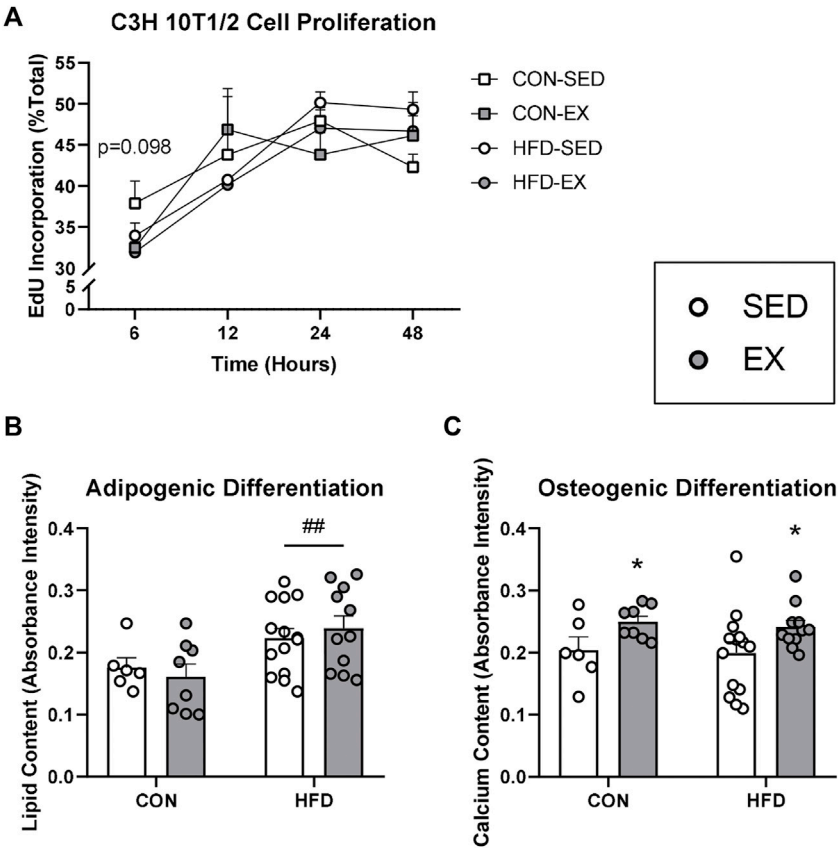

FIGURE 5
Extracellular vesicles (EVs) impact mesenchymal stromal cell (MSC) differentiation activity *in vitro*. C3H/10T1/2 MSCs were differentiated into either adipocytes and stained with Oil Red O or osteoblasts and stained with Alizarin Red S dye. **(A)** Proliferation by EdU stain at 6, 12, 24, and 48 h **(B)** EVs from high-fat diet-fed mice increased adipogenic differentiation and **(C)** EVs form exercise-trained mice increased osteogenic differentiation. $^{*}p < 0.05$ main effect of exercise; $^{\#\#}p < 0.01$ main effect of high-fat diet (n = 6–14 per group).

13 weeks of age. These ages represent mice pre- and post-sexual maturity (Dutta and Sengupta, 2016), which may impart differing hematopoietic and MSC differentiation responses to irradiation, exercise, and high-fat diets.

Our lab and others have previously determined that the effects of exercise on hematopoiesis are mediated by alterations in cellular communication in the bone marrow (Yamazaki et al., 2011; Kunisaki et al., 2013; Bruns et al., 2014; Hérault et al., 2017; Emmons et al., 2019). As a first step towards understanding if EVs were influencing hematopoiesis indirectly through HSC niche cells, we quantified MSCs, their progeny (i.e., osteoprogenitor, adipocyte progenitor, and osteoblast cells), and endothelial cells. These analyses revealed that EX EVs decreased osteoprogenitor cell concentrations with no effects of EX- or HFD-induced EVs on other bone marrow cell populations. HFD is known to increase bone marrow adipose tissue content which can be prevented (Styner et al., 2014; Emmons et al., 2019) or reversed (Emmons et al., 2018) by exercise training. Conversely, exercise training is known to increase osteogenesis from MSCs (Baker et al., 2011; Zhang et al., 2020), thus we hypothesized that the lower concentration of osteoprogenitors in the mice that received exercise-induced EVs may be due to enhanced differentiation. Our *in vitro* findings support this hypothesis showing that treating MSCs with exercise-induced EVs enhanced their osteogenic differentiation. Previous work has suggested that enhanced osteogenic differentiation in response to exercise training is due to mechanotransduction signaling in MSCs (Sen et al., 2011), thus our findings provide a novel paracrine mechanism whereby exercise may be enhancing osteogenesis. Interestingly, our *in vitro* analyses also revealed a role for HFD-induced EVs in enhancing adipogenesis which provides an additional mechanism explaining the enhanced adipogenic differentiation of MSCs and accumulation of marrow adipose tissue in HFD-induced obesity (Emmons et al., 2019).

As previous studies have shown alterations to cell function through variations to EV cargo (Hou et al., 2019), we reasoned that the exercise-induced effects were partially due to alterations in EV cargo. Previous studies have examined muscle-derived EV cargo in the context of exercise, yet the EV cargo of the bone marrow

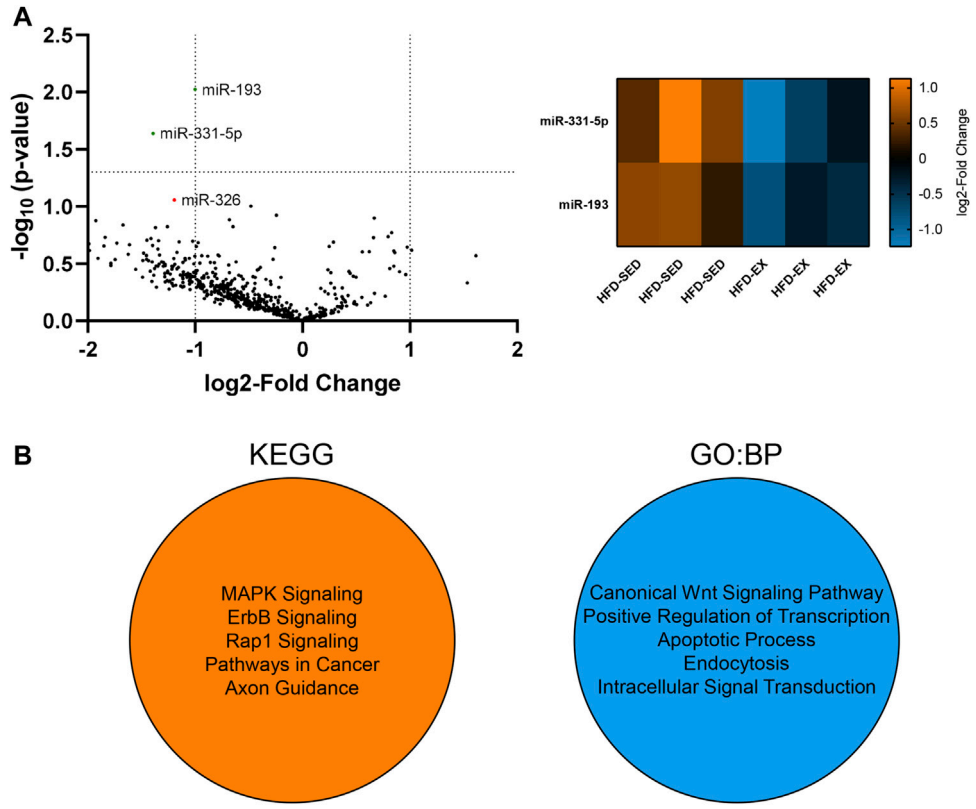

FIGURE 6
Extracellular vesicles (EVs) from high-fat diet exercise-trained (HFD-EX) mice have lower miR-331-5p ($p < 0.05$) and miR-193 ($p < 0.01$) expression compared to HFD-sedentary (HFD-SED) mice. **(A)** Volcano plot of all measured miRNAs and heatmap of differentially expressed miRNAs. miR-326 approached lower expression in HFD-EX EVs compared to HFD-SED ($p = 0.0877$). The vertical dotted lines represent the margin for physiologically meaningful expression changes as per default ROSALIND analysis. The horizontal dotted line represents statistical significance ($p = 0.05$). ($n = 3$ per group). **(B)** Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology: Biological Processes (BP) predicted target pathways by miRWalk combined analysis of miR-193 and miR-331-5p.

environment remains unexplored. Here, we examined the miRNA cargo of the isolated EVs due to their established role in cell signaling (Avraham and Yarden, 2012; Vu et al., 2019). We found miR-331-5p ($p < 0.05$) and miR-193 ($p < 0.01$) downregulation in HFD-EX EVs compared to HFD-SED EVs. Correlational findings from clinical studies suggest lower miR-331-5p expression is related to leukemia relapse (Feng et al., 2011) and worsened responses to therapies for acute myeloid leukemia (Butrym et al., 2015). Previous data from our lab has shown that acute myeloid leukemia incidence is increased with HFD, that is, attenuated by exercise throughout the lifespan (Farber et al., 2021), thus downregulated miR-331-5p expression in HFD-EX EVs may be implicated in this observation. Relatively more information is available regarding miR-193 and hematopoiesis. Haetscher and colleagues found that miR-193b is upregulated in mouse hematopoietic stem cells while miR-193a expression is found primarily in committed myeloid cells, such as monocytes and granulocytes (Haetscher et al., 2015). Along these lines, overexpression of miR193a in HSCs impaired their regenerative capacity in transplantation assays, but had augmented granulocytic differentiation *in vitro* (Krowiorz et al., 2018), suggesting mirR-193b may be involved in maintaining HSC stemness, while mir-193a may be involved in myeloid differentiation.

miR-193a also plays a role in regulating MSC fate by suppressing osteogenic differentiation in human MSCs (Wang S.-N. et al., 2018). Further, miR-193a downregulation induced osteoblast differentiation (Wang W. et al., 2018). These findings align nicely with our data as miR-193 was lower in exercise-induced EVs, and exercise-induced EVs promoted osteogenesis. miR-193a-3p has been shown to downregulate leucine-rich repeat-containing G-protein coupled receptor 4 (LGR4) and activating transcription factor 4 (ATF4) (Wang W. et al., 2018). LGR4 nonsense mutations have previously been implicated decreased bone mineral density and elevated risk of osteoporotic fractures (Styrkarsdottir et al., 2013), and ATF4 has been reported to positively regulate HSC expansion in mouse fetal liver (Zhao et al., 2015). miR-193a-3p downregulation

may be promoting *lgr4* and *atf4* gene transcription to increase osteogenesis as we observed *in vitro*. Our KEGG analysis predicted the MAPK signaling pathway as a potential target for miR-331-5p and miR-193 which is corroborated by work from Lv and colleagues showing that miR-193a-3p downregulation increases MAPK signaling (Lv et al., 2020). The MAPK signaling cascade is important for cell proliferation and differentiation. The pathway is composed of multiple components, including JNK and ERK. Previous reports showed elevated JNK signaling promotes early MSC osteogenic differentiation (Brito et al., 2019; Mizerska-Kowalska et al., 2019), and others have shown that ERK inactivation in osteoprogenitors leads to decreased bone mass (Kim et al., 2019). miR-193 and 331-5p downregulation may be partially responsible for the upregulated osteogenesis seen *in vitro* in the current study.

In conclusion, our results indicate that exercise training-induced EVs restore normal hematopoiesis and enhance osteogenic differentiation in the context of HFD-induced obesity. Mechanistically, these findings may be explained by elevated large EV release alongside downregulated miR-193 and miR-331-5p content in HFD-EX EVs compared to HFD-SED EVs. These results provide a novel mechanism the regulation of hematopoiesis and MSC fate by exercise. Future studies should identify the cellular source of these exercise-induced EVs to better-understand how intercellular crosstalk occurs between HSCs and their niche cells in the context of exercise training.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by University of Ottawa Institutional Animal Care and Use Committee.

Author contributions

JV designed and performed the experiments and drafted the manuscript and figures. WK assisted in all sample collections and assisted in drafting the methods and results sections. LE completed the C3H 10T1/2 proliferation assay, drafted the C3H 10T1/2 proliferation assay methods section, and assisted in the Nanostring assay. MN optimized the Western Blot assay and EV isolation methods. NC assisted with flow cytometry data collection. MD supervised the project, provided funding, designed the studies, contributed to the analysis, and edited the manuscript. All authors reviewed the manuscript and approved its submission.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.1054463/full#supplementary-material

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The effects of physical activity on glutamate neurotransmission in neuropsychiatric disorders

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Physical activity (PA) is an effective way of increasing cognitive and emotional health and counteracting many psychiatric conditions. Numerous neurobiological models for depression have emerged in the past 30 years but many struggle to incorporate the effects of exercise. The hippocampus and pre-frontal cortex (PFC) containing predominantly glutamate neurotransmission, are the centres of changes seen in depression. There is therefore increasing interest in glutamatergic systems which offers new paradigms of understanding mechanisms connecting physical activity, stress, inflammation and depression, not explained by the serotonin theories of depression. Similar hippocampal glutamate dysfunction is observed in many other neuropsychiatric conditions. Excitatory glutamate neurones have high functionality, but also high ATP requirements and are therefore vulnerable to glucocorticoid or pro-inflammatory stress that causes mitochondrial dysfunction, with synaptic loss, culminating in depressed mood and cognition. Exercise improves mitochondrial function, angiogenesis and synaptogenesis. Within the glutamate hypothesis of depression, the mechanisms of stress and inflammation have been extensively researched, but PA as a mitigator is less understood. This review examines the glutamatergic mechanisms underlying depression and the evidence of physical activity interventions within this framework. A dynamic glutamate-based homeostatic model is suggested whereby stress, neuroinflammation and PA form counterbalancing influences on hippocampal cell functionality, which manifests as depression and other neuropsychiatric conditions when homeostasis is disrupted.

KEYWORDS

exercise, neuroimmunology, depression, glutamate, inflammation, stress

Introduction

Depression is defined by diagnostic criteria from the Diagnostic and Statistical Manual 5 (DSM5). including depressed mood or anhedonia (loss of interest or pleasure), with additional symptoms including appetite or weight changes, difficulty sleeping, diminished ability to think or concentrate, fatigue or loss of energy, feelings of worthlessness, or excessive guilt and suicidality (1). The heterogeneity of clinical presentations, presence of comorbid conditions and psychosocial confounding variables is challenging for the reproducibility of studies of the underlying mechanisms.

Major depressive disorder (MDD) is a leading cause of global disease burden that affects over 300 million persons worldwide (2). This burden is high across the entire lifespan, genders, global distributions and the incidence is rising. The total estimated number of people living with depression worldwide increased by 18.4% between 2005 and 2015 to 322 million equating to 4.4% of the world's population, with a lifetime risk of 15%–18%.

In terms of disability, depression ranked third in the causes of years lived with disabilities (3). Importantly, no reduction in the global prevalence or burden has been detected for depression or anxiety since 1990, despite compelling evidence of interventions that reduce their impact (4). The high costs to individuals and society demand efficacious treatments, yet 30% of patients fail to respond to current serotoninergic-based pharmacotherapeutics, while 70% do not achieve complete remission (5). Outcomes for Major Depressive Disorder (MDD) are associated with a reduced life expectancy of 10–20 years, despite active treatment. This is mostly due to cardiometabolic disease related to chronic treatments and physical inactivity (6). The high levels of treatment resistance, undesirable side effects, and the high economic and social cost to society have prompted a diversification of the search for effective treatment options (7).

Physical activity (PA) in its many forms has, since antiquity, been associated with antidepressant effects although the neurobiological mechanisms have remained elusive due to the heterogeneity of clinical depression and difficulty of studying neurochemistry *in vivo*, large differences in individual responses and poor uptake of sustained exercise interventions (8).

Volumetric magnetic resonance (MR) imaging of depressed patients has established for some time that, more than other brain areas, the hippocampus shows volume reduction in chronic psychiatric conditions which is partially restored by regular exercise (9, 10). However, previous models of depression, such as the monoamine theory of serotonin and noradrenaline, fail to provide a mechanistic framework for these effects, leading to a historical lack of research into exercise as a treatment.

There have been many neurobiological theories of depression, with many overlapping themes. The theories of monoamines, hypothalamic pituitary adrenal (HPA) axis hyperactivity, reduced neuroplasticity, hypoGABA-ergic theory and neuroinflammation are all validated hypotheses (11–15). Glutamate neurotransmission and associated mitochondrial function within the hippocampus, underpin and therefore unify many of these related concepts. However advances since the 2000s in knowledge of glutamate neurotransmission in the limbic and prefrontal cortex, have generated an overarching glutamate hypothesis of depression, which has a wide evidence base and increasing clinical acceptance (16). Importantly, a glutamate-based model of depression provides mechanistic explanations for how physical activity impacts on mental health. Therefore, the aim of this review is to provide an integrative view of neuroplasticity, glutamatergic and inflammatory theories of depression, centred on mitochondrial dysfunction in the hippocampus and to describe the integral role of PA within this schema. As depression is by far the most researched field for the effects of PA, we discuss the mechanisms for a model in relation to depression and then extend to other disorders. Given the rising societal prevalences of mental health disorders, social isolation, stress and sedentariness, the need to research the precise effects of exercise is more important than ever. Psychiatric conditions where PA has an evidence base and areas where PA remains under-researched are also discussed.

Glutamatergic model of depression

The glutamate hypothesis of depression emerged in the 1990s, when antagonists of the N-methyl-D-aspartate (NMDA) receptor, an ionotropic glutamate receptor, produced antidepressant-like effects in mice (17). In a seminal human trial by Berman in 2000, ketamine (a long-standing anaesthetic agent and glutamate NMDA receptor antagonist) demonstrated a rapid-onset and prolonged mood improvement in MDD, including in cases of treatment-resistant depression (TRD) (18). Further proof of concept emerged when Magnetic Resonance Spectroscopy (MRS) scans revealed decreased levels of glutamatergic metabolites in the medial frontal cortex in patients with depression (19) and MR imaging showed volume reductions in MDD patients in the hippocampus which is a glutamate neurotransmission predominant area (20).

This prompted research into glutamate receptor subtypes and ligands as possible therapeutic targets and agents respectively. There are two classes of receptors that exhibit functional differences (21). Inotropic GluRs (iGluRs) are ligand-gated ion channels for fast signal transmission. Metabotropic receptors, GluRs (mGluRs) are G protein-coupled receptors (GPCRs) which control cellular processes *via* G protein signalling cascades. The first iGluR and mGluR members were cloned in 1989, and 1991 respectively and intense research into receptor subtypes and ligands has continued ever since in the search for possible new paradigms of treatment (22, 23).

Specific iGluR agonists have revealed three main subfamilies of receptors, named after the agonist: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPARs), kainic acid (KARs), and N-methyl-D-aspartate (NMDARs) (24). NMDARs, the target site of ketamine, were first cloned in 1991 (25). Within each receptor group genetic mechanisms generates further structural variations within the receptor subunits, which creates functional diversity (26). This receptor-encoded complexity sets glutamate apart from other neurotransmitters and enables the functional capacity required for complex processing in pattern and memory encoding.

Experimentally depression and anxiety like behaviours are reproduced by stimulating or blocking the subunits of these receptors with pharmacological ligands. Where specific receptor ligands are unknown, gene targeting models in mice can investigate the contribution of subunit subtypes. In a knockout mouse model of the GluN2A subunit of NMDARs, mice exhibited reduced anxiety and depression behaviour during experimental conditions such as the forced swim test and tail suspension tests (27). Similarly effects were seen with deletions of GluN1 GluN2B NMDA subunits, or the administration of the GluN2B antagonist Ro 25-6981 (28).

The precise mechanisms of NMDAR antagonism reducing depression are still unclear as pure NMDAR antagonists alone do not reduce depressive symptoms (29). The antidepressant effects of NMDAR antagonism in preclinical experiments require simultaneous activation of AMPARs (30). Although the reasons are unclear, the GluA1 subunit of AMPAR seems to be related to dysfunctional synaptic plasticity during depression. The selective

deletion of this GluA1 AMPA subunit in the hippocampus, reduces experience dependent behavioural despair (31).

Glutamate is the predominant excitatory neurotransmitter in the central nervous system (CNS) accounting for over 90% of excitatory function (32). Glutamatergic synapses are found throughout the CNS but are especially concentrated in the hippocampus, amygdala, caudate nucleus, pre frontal cortex (PFC) and the cerebellum (33).

Glutamate neurone distribution therefore neatly overlays the main anatomical areas implicated in depression, namely the hippocampus and PFC. The excitatory nature of glutamate neurotransmission and vast array of receptor combinations, enables the complex synaptic activity required by the hippocampus for rapid pattern recognition. Additional functions include: spatial reference and working memory, pattern discernment and mapping, emotional activation and decision making, all in response to new environmental stimuli. Impairment of these functions leads to low mood and anhedonia. Prefrontal inhibition leads to inflexible negative biases in cognition, predisposing to rigidly held negative beliefs and poor motivation, leading to negative cognition arising the low mood (34).

It is the unique properties of glutamatergic neurotransmission, enabling memory and cognition, that create a vulnerability to dysfunction, leading to depression and other conditions. Unlike other neurotransmitters, glutamate causes synaptic excitation at micromolar concentrations and cannot then be enzymically deactivated in the synaptic space. Therefore, to avoid the lethal effects of synaptic overstimulation and excitotoxcity, adjacent astrocytes have developed a receptor-based system of reuptake transporters: GLT-1, GLAST, and EAAT (35). After uptake, glutamate is metabolised within the astrocyte, to non-excitatory glutamine and returned to the neurone *via* the glutamate–glutamine cycle, or oxidised and entered into the citric acid cycle as a fuel substrate. This active system of glutamate uptake against steep concentration gradients, requires significant amounts of ATP energy and therefore mitochondrial involvement. Glutamate neurotransmission therefore accounts for 80% of entire brain energy expenditure, and 20% of the total body expenditure. Hence, prolonged mental concentration, involving widespread glutamatergic activity, causes sensations of fatigue (36, 37).

The link of the glutamine-glutamate cycle with the tricarboxylic acid (TCA) cycle in astrocyte mitochondria, enables glutamate neurotransmission to recycle some of the cellular energy loss, thus avoiding potential local energy mismatch issues. Despite this, the net demands on astrocytic mitochondria remain very high and still render the hippocampus very vulnerable to metabolic overload or inflammatory stress. This dependency of hippocampal function and therefore mood, on mitochondrial function, is demonstrated by resveratrol, a drug whose action is purely to increase mitochondrial function. In animal models of depression, significant increases in mood are observed, without a direct effect on neurotransmitters (38).

Mitochondria, as providers of ATP energy for the cell, therefore also have a role in sensing and regulating cellular ATP requirements and oxidative stress. Mitochondria are therefore sensitive to numerous signalling pathways involving oxidative stress and inflammation. As such, the glutamate-based model of depression, through glutamatergic astrocytic mitochondria, demonstrates direct links between depression symptoms and physiological systems such as HPA stress responses, physical activity and less intuitively, the immune system.

Decades of depression mechanistic research have converged on three main, but interrelated, systems associated with depression; stress *via* the HPA axis, inflammation and neuroplasticity. This review discusses these aspects in relation to glutamate function, the hippocampus and physical activity.

Glutamate mediates the relationship of stress with depression

The hippocampus, within the limbic system, is the primary centre of receiving and interpreting external stimuli and determining appropriate and rapid emotional, cognitive, neurological and endocrine responses. The primary endocrine response, when threats are perceived, is *via* the HPA axis, culminating in glucocorticoid release from the adrenal glands. The process starts when impulses from the hippocampus excite corticotrophin release factor (CRF) cells in the paraventricular nucleus (PVN) of the hypothalamus. The excitation is *via* glutamate receptors, demonstrating one of many relationships between glutamatergic activity and the endocrine stress response (39). Common to many glutaminergic microcircuits, GABA interneurones control the effect of excitation by inhibiting CRF cell secretion. GABA inhibition is itself deactivated when GABA ion channels open, causing depolarisation. Depolarisation is regulated by chloride cotransporters, which determine the transmembrane electrochemical gradient (40). In animal models, psychological stress and the inflammatory mediator IL-6 affect the number and function of these cotransporters and therefore act to deactivate GABA inhibition, hence increasing CRF and cortisol secretion (41). Cortisol acts *via* glucocorticoid (GR) receptors to cause GABA inhibition of CRF secretion and hippocampal deactivation to complete the negative feedback loop. Such close hippocampal-hypothalamic interactions explain the tight association of stress and mood.

In the short term, stress-induced deactivation of GABA, enabling cortisol-mediated protective mechanisms, is an appropriate physiological response to a perceived threat. Cortisol, the primary adrenal glucocorticoid, has anti-inflammatory and catabolic effects, necessary to contain infection, trauma and other defined periodic immune stresses.

Medium term stress, over weeks or months, such as bereavement, divorce, financial or criminal issues, and illness, produce perpetuation of the HPA axis activation. This occurs as a result of a self-amplification effect, whereby HPA glucocorticoids provide feedback to the CNS and activate GR receptors on the original glutamate presynaptic neurones, provoking further CRF cell activation. Although the underlying precipitants and perpetuating causes are not fully established, external agents that promote glutamatergic excitation and

suppress GABA inhibition are clearly involved. This is sometimes referred to as "the GABA-ergic deficit hypothesis of major depressive disorder" (42). The high levels of circulating cortisol causes persistent GR receptor activation and a positive feedback loop.

GR receptor-stimulation also causes astrocytic release of ATP causing microglial proliferation, the release of pro-inflammatory mediators and the release of the growth factor Brain Derived Neurotrophic Factor (BDNF) (43). In animal models, the effects of persistent glucocorticoids on glutamate neurones are: increased glutaminergic excitability with astrocytic decline, reduced astroglial plasticity and reduced dendritic connectivity in hippocampal and frontal cortex regions (44, 45). Collectively this manifests as anxiety, depressed mood, disturbed or excessive sleep, fatigue, memory and cognitive problems, behavioural responses and, in extreme circumstances, suicide (46). Outside of the CN, cortisol causes depressed humoral and cellular immunity with dysmetabolic and vascular changes.

In short term stress, this cluster of cortisol-mediated, energy-conserving and self-protective symptoms, is seen in the "sickness behaviour" of people with severe infections. It also features behavioural changes, such as anorexia, fatigue, loss of interest in usual daily activities, social withdrawal, listlessness or malaise, hyperalgesia, sleep disturbances, and cognitive dysfunction (47). The "pathogen defence hypothesis of depression" is supported by the ancestral and continued proximity of alleles for depression and pro-inflammatory factors (48). This inflammatory depression of hippocampal function as an ancient protective mechanism becomes problematic in modern life when HPA activation is persistent.

The glutamate model explains why reduced physical activity, low motivation and depressed mood may in fact be inherent protective adaptations to stress and may explain the poor motivation and uptake of exercise in interventions for major depression.

From experimental simulations of stress, creating subtypes such as intermittent, restrained, or unpredictable forms, it is clear that high persistent cortisol levels also feature a pro-inflammatory state. This less understood aspect of the stress response is due to cortisol-mediated short term rises in plasma IL-6, TNF-alpha, and monocyte nuclear-factor kappa-B (NFkB), partially facilitated by the sympathetic nervous system (SNS) (49–51). As stress persists there is increasing SNS mediated B-adrenoceptor-activated monocyte migration into the CNS, causing pro-inflammatory cytokine secretion (52). Clearly, stress, anti-inflammatory HPA-stress responses, and raised pro-inflammatory mediators, have a complicated and sometimes seemingly contradictory relationship, although this may simply reflect different phases in the stress response and emphasises the dynamic nature of the hippocampal/HPA systems. This co-existent state of pro-inflammatory cytokines and raised CNS cortisol, causes double stimulation of astrocyte and neuronal receptors, leading to cellular exhaustion and apoptosis. This, in turn, leads to more chronic and structurally consolidated stress phenotypes (53).

Prolonged stress

Continual suppression of GABA inhibition leads to glutamate overstimulation and eventual exhaustion of CRF cells and the HPA axis, resulting in low levels of glucocorticoids despite ongoing stress. Low circulating glucocorticoids are seen in atypical depression, post-traumatic stress disorder (PTSD) and in suicide attempts (54). Reduced circulating glucocorticoids remove the suppressive influence the HPA axis exerts on pro-inflammatory mediators. The resulting pro-inflammatory state causes dendritic damage, reduced synaptic plasticity and the transformation of astrocytes into pro-inflammatory phenotypes, which then secrete cytokines in a "feed-forward" manner. Pro-inflammatory cytokines then act to reduce mitochondrial biogenesis leading to metabolic insufficiency with autophagy and apoptosis.

In severe or prolonged stress, cumulative cell loss eventually results in loss of volume of hippocampal and prefrontal areas on MRI, with reductions in glutamate neurotransmission on MRS brain imaging, as described above. These macroscopic changes represent significant neuronal loss and degeneration and are commensurate with the increasing evidence of chronic MDD or other major psychiatric conditions, converting to neurodegenerative conditions such as Dementia and Parkinson's Disease (55, 56). The transition from MDD to diseases featuring progressive neurological decline is unpredictable and poorly understood, but is consistent within the framework of the glutamatergic model.

Severe stress experienced in early life seems to amplify pro-inflammatory responses to stress in adulthood, possibly as a result of defective HPA/hippocampal integration during development, although the mechanism is not fully understood (57). The resulting chronic pro-inflammatory states may explain the connection between adverse life events in childhood and subsequent depression and chronic physical disease. Childhood Adverse Life Experiences (ALEs) are associated with a 2.4-times increase in mortality at the age of 65, compared to controls (58).

Glutamate, inflammation and depression

Patients with major depression have significantly higher plasma concentrations of the cytokines TNF-α and IL-6 than controls (59). Whilst CNS inflammatory mediators form part of the depression-associated stress response above, primary inflammatory conditions themselves can invoke depression (60). The relationship between inflammation and depression therefore is rather circular. Regardless of whether the origin is centrally derived or peripheral, inflammatory mediators produce similar effects on glutamate microcircuits to those seen with stress, namely increased oxidative and nitrosative stress, with reduced BDNF and other growth factors needed for maintenance of the glutamate astro-neuronal unit. Oxidative stress results from the imbalance of between mitochondrial reactive oxygen species ROS

production and removal and therefore excess superoxide and other ROS within the cell. This arises from overproduction and or deceased antioxidant capacity. Oxidative damage affects many cellular components, including lipids DNA and proteins and mitochondria themselves. Up to 2% of consumed oxygen is converted to superoxide through electron leakage within the mitochondrial ETC (61). In energetic cells, such as neurons, with raised mitochondrial activity, ROS production is increased (62). However ROS production is also increased when mitochondria become impaired and electron leakage increases (63).

In addition to the direct effects on cells, ROS also stimulates pro-inflammatory cytokines such as IL-6 and TNFa. This provokes a ligand-activated transcription factor: peroxisome-proliferator-activated receptor-γ (PPAR-γ). PPAR-γ suppresses pro-inflammatory transcription factors and activates mitochondrial biogenesis. This key metabolic controller also promotes anti-oxidant and angiogenesis pathways and raises glucose and lactate availability in astrocytes (64).

The high oxidative activity of neuronal mitochondria, makes the mitochondria ETC itself vulnerable to functional overload, producing excess ROS, ETC damage, then increased electron leakage and ROS in a vicious circle. Mitochondrial function declines and antioxidant defences are reduced. The increased oxidative state damages organelles and if prolonged risks progression to autophagy, *via* mTor signalling and apoptosis *via* activated Caspases (65, 66).

Effects of physical activity in increasing neuronal mitochondrial biogenesis

Counterbalancing these risks of functional overload are trophic mechanisms to promote cell enhancement and growth, which are strongly related to physical activity. PGC1a is a transcriptional co-activator stimulated by physical activity which, *via* PPAR-γ, facilitates mitochondrial biogenesis (67). This effect is not just confined to muscle. In the hippocampus, exercise induced biogenesis increases levels of oxidative phosphorylation giving neuroprotection to glutamate neurones, vulnerable to mitochondrial insufficiency (68). PGC1a is activated in muscle by a mitochondrial energy-sensing kinase, AMPK and stress inducible kinases including p38 mitogen-activated protein kinase (MAPK). Muscle activity in the form of exercise, particularly endurance exercise, creates energy demand and optimally stimulate production, with effects in the CNS (69).

In patients with treatment resistant depression (TRD), that is not responsive to serotoninergic drugs, it would be expected that exercise would stimulate increased PGC1a and hence hippocampal mitogenesis to reduce pro-inflammatory cytokines and improve mood. This was in fact observed in a study of 12 weeks of an aerobic exercise protocol in TRD patients (70). Increased mood correlated with reduction of IL-1β levels. Notably the higher the circulating basal TNF-α levels the greater the effects of PA on reducing the depression.

Physical activity also serves to lose weight, particularly visceral adiposity, which coupled with sedentariness are potent contributors of circulatory IL-6 and TNF-α, sometimes described as the "diseaseome of physical inactivity" (71).

Randomised trials show positive results for exercise improving mental health but due to the heterogeneity in responses, effect sizes are smaller than in large observational studies (72).

Most evidence indicates that exercise, especially aerobic exercise improves depression and anxiety outcome measures, with similar effect sizes to psychopharmacological treatments. Resistance exercise trials also show reduction in symptoms (73). Exercise benefits are seen in different forms of depression such as in the elderly and children and young people (74, 75). Notably the mental health effects continue during the intervention but decline rapidly after discontinuation (76). Sustained beneficial effects seem to be maintained only above a threshold of 150 mins per week, as reflected by WHO exercise guidelines (77). The considerable variation of exercise effects across patient populations and forms of activity indicates the need to understand the underlying mechanisms, although this has proved elusive. The discovery of the kynurenine pathway, linking muscle activity with neurophysiology was a major development in understanding.

Physical activity and the kynurenine pathway

A key inflammatory pathway in CNS glutamatergic systems, relevant to PA, is the kynurenine pathway. The kynurenines are bioactive metabolites of tryptophan (TRP), deriving from the gut and are therefore an important feature of traffic in the gut-muscle-brain axis (78). Much interest has focused on members of this pathway in terms of mediating inflammatory CNS conditions. In the brain kynurenine metabolites are generated locally by microglia or enter *via* the blood-brain barrier (BBB) from the circulation. Importantly, excess kynurenine substrate (KYN) is prevented from entering the brain, through conversion by exercising muscle into kynurenic acid (KA), which cannot pass the BBB. In the brain, kynurenine catabolism divides into two pathways depending on local conditions: Either pro-inflammatory neurotoxic metabolites including Quinolinic acid, (QA) (a glutamate NMDA receptor agonist), or neuroprotective metabolites including kynurenic acid, KynA (an NMDAr antagonist). Initially, the kynurenine pathway was thought to cause depression by depleting serotonin, but the principal site of action is now known to be glutamatergic transmission (79). The ratio of KynA to QuinA has been demonstrated to be low (i.e., neurotoxic), due to the excess QuinA released from microglial cells, in the anterior cingulate cortex in depression (80). Moreover, the kynurenine pathway itself is modulated peripherally in adverse conditions such as infection and stress. However, in exercise, beneficial effects occur, with muscle enzyme increases or decreases, causing changes in the KynA: QuinA ratio (81). In addition to reducing CNS entry of kynurenine, exercise promotes enzymic switches towards an increased KynA/QuinA ratio, which promotes PGC1a mediated mitochondrial biogenesis. This is responsible for the beneficial

effects of exercise such as improved energy homeostasis, the promotion of an anti-inflammatory environment, and neuroprotection, and is thought to be one of the mechanisms underlying the beneficial effects of exercise on depression (82).

Neuroplasia and depression

From a structural point of view, the hippocampus is a central processor of incoming information, rapidly sorting, evaluating and storing sensory input and connecting with the PFC, amygdala and HPA axis for cognitive, emotional and endocrine responses. The speed and complexity of this most challenging of brain activities requires high levels of neurogenesis and neuroplasticity in these exclusively glutamate neurones. Neurogenesis, the formation of new neurone cells, continues throughout adult life, only in two areas of the adult brain; a zone of the lateral ventricles and the dentate gyrus of the hippocampus (83). Neurogenesis seems a prerequisite for memory formation. Neuroplasticity involving the growth, consolidation and pruning of hippocampal dendrites is a vital part of memory consolidation and memory removal, when modelled by activity-dependent changes in synaptic transmission such as long-term potentiation (LTP) and long-term depression (LTD) (84). Stress acts through many mechanisms to impair synaptic plasticity and neurogenesis, by direct decrease of dendritic branching or activating corticosteroids to downregulate neurogenesis (85). Severe and continued impairment is reflected in overall loss of hippocampal volume.

As described above, the link between impairment in the memory forming regions of the hippocampus and depression is strong but not self-evident. However evidence is incontrovertible that reduced neuroplasticity in areas of memory function correlates to symptoms of depression (86). Trophic factors that promote synaptogenesis or improve mitochondrial function to fuel the process therefore also improve mood. Growth factors, or neurotrophins, are produced locally or enter *via* the BBB from muscle and other tissues. The mammalian neurotrophins include: Brain derived neurotrophic factor (BDNF), insulin like growth factor (IGF-1) vascular endothelial growth factor (VEGF) fibroblast growth factor (FGF-2) epidermal growth factor (EGF) nerve growth factor (NGF) (87). Irisin, produced in muscle, is involved in the regulation of neural differentiation, neuroplasticity and energy expenditure (88).

BDNF is a vital growth factor for sustaining neurones and promoting growth and repair and is synthesised in neurones in physiological circumstances and astrocytes during inflammation or injury (89). BDNF expression is highest in hippocampal and frontal areas corresponding to emotional and cognitive function. It is well established that serum BDNF levels are reduced in depression and are normalised with treatment by antidepressants or PA (90). PA increases plasma levels threefold, partially due to muscle derived BDNF and contributes to brain plasticity and neurogenesis. Aerobic and anaerobic exercises promote neurogenesis *via* BDNF, lactate and Vascular Endothelial Growth Factors (VEGF). In contrast, for resistance exercise, neuroplasticity and neurogenesis is mediated *via* muscle derived irisin and IGF-1 (91).

As BDNF expression features highly in those hippocampal circuits responsible for interpreting external sensory information, then environmental enrichment appears to increase BDNF further and be the primary source of the ability of environmental enrichments to enhance cognitive processes (92).

Summary of neurological effects of PA

Physical activity significantly improves brain function as measured by neuronal survival, resilience to brain physical insults, cognitive function, brain vascularisation, neuroplasticity and neurogenesis, neuroinflammatory stability and resistance to the effects of ageing. Physical activity mechanisms operate not in isolation but form part of the network of the gut-muscle-brain axes, in addition to environmental stressors as depicted in **Figure 1**.

The primary focus of these effects centres on the hippocampus and involves glutamatergic neurotransmission. Key to maintaining homeostasis and promoting growth are the neurotrophic factors produced locally or peripherally in muscle, which emphasises the crosstalk between brain and the musculoskeletal system. In terms of neuropsychiatric conditions, these changes have the effect of stabilising mood, reducing negative cognitive bias, increasing resilience to psychosocial stress and increasing motivation (93). There is also evidence of exercise directly stimulating hippocampal circuitry from other parts of the CNS. Glutamate neurotransmission frequencies are seen to increase almost instantaneously to the start of exercise. The mechanisms and neural pathways are unclear but the neuroplastic promoting effects on hippocampal neuronal health are similar to the other effects of PA (94).

In addition to the direct neurohumoral benefits of exercise, PA also provides numerous indirect benefits due to the nature of different sports and the external environment. Complex tasks, such as strategizing and decision making, risk management and social interaction, all stress and stimulate hippocampal and PFC circuits in beneficial ways (95). Exercise tends to behaviourally influence other positive activities such as diet quality and social interactions, all with individual beneficial effects on neuroimmunology (96).

In a negative context, the importance of environment is illustrated by the increasing incidence of mental health disorders in professional athletes, illustrating the power of environmental stress factors to override the antidepressant effects of even large quantities of physical activity (97).

PA in other neuropsychiatric conditions

The physiological effects of exercise impact positively on the vast majority of psychiatric conditions from affective disorders,

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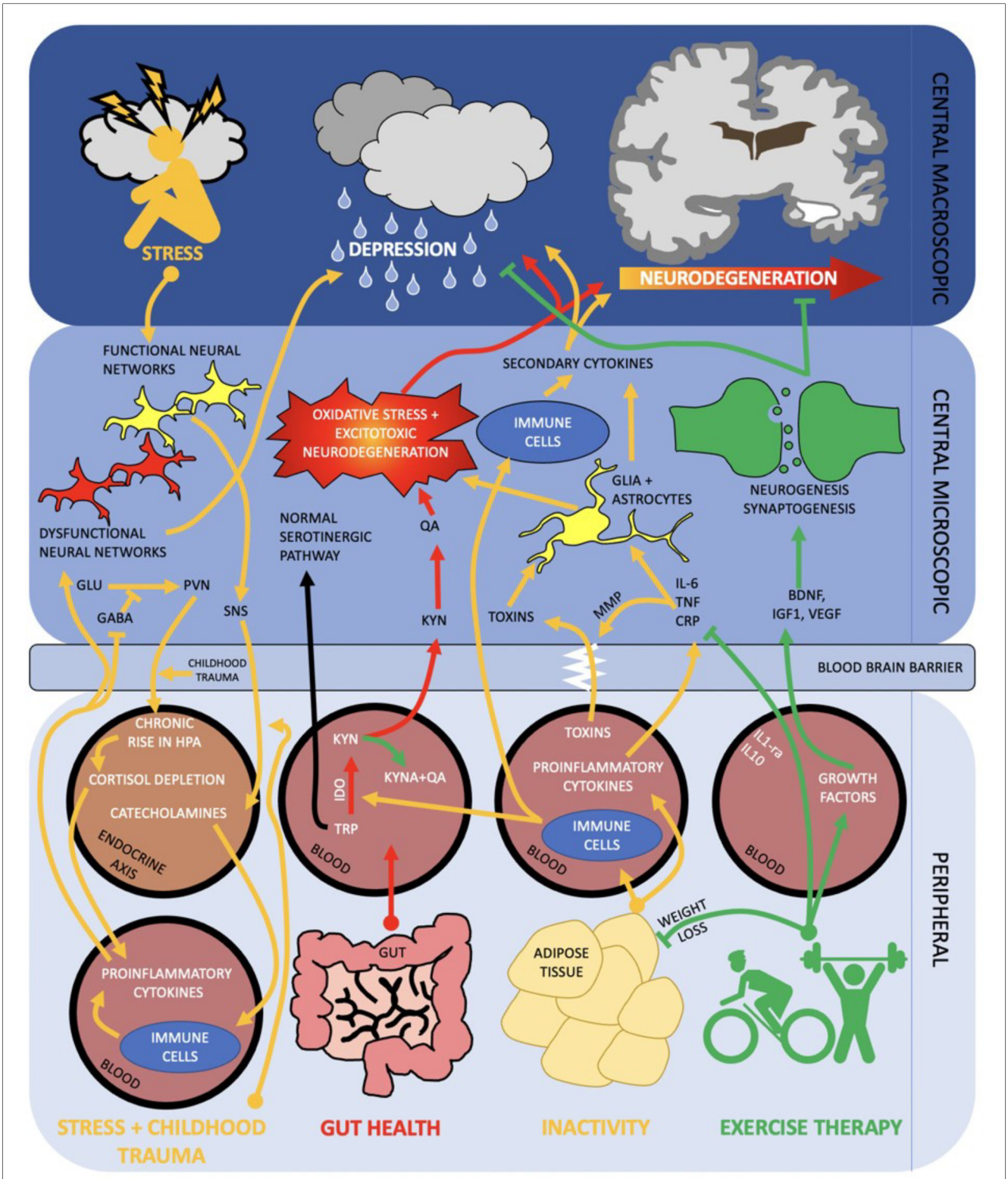

FIGURE 1
Diagram of the brain and peripheral neuroimmune interactions in terms of the gut—muscle-brain axes. RED activation of kynurenine pathway *via* poor gut health propagates oxidative stress and excitotoxic neurodegeneration. ORANGE inactivity and subsequent raised adiposity elicits an inflammatory cascade which traverses the blood brain barrier and contributes to neurodegeneration and depression. Stress and negative early life events may activate a parallel pathway, in which peripheral inflammation and HPA axis dysregulation contribute to dysfunctional neural networks and depression. GREEN exercise therapy both acutely (arrow) and chronically (inhibition arrow) inhibit inflammatory and kynurenine pathways whilst promoting neurogenesis and synaptogenesis. Combined, this can help stave off neurodegeneration and onset of depression. SNS, sympathetic nervous system; MMP, mixed metalloproteases; PV, paraventricular nucleus; IDO, indoleamine 2,3-dioxygenase; Other abbreviations described above.

schizophrenia disorders to trauma-based and developmental disorders.

Unlike the glutamate model, many of the previous depression models of pathogenesis do not transfer across diagnostic boundaries. In a previous review we described the validity of the glutamatergic model across diverse neuropsychiatric categories, involving common themes such as excitotoxicity, and suggest possible novel treatment avenues (98). Through cytokines and neurotrophins the glutamate model is also able to provide a theoretical basis for physical activity impacting on depression and other psychiatric disorders. Many disorders that feature depression and hippocampal pathology, respond to PA as described. However, other disorders may improve along different channels and await further research. A full description of the clinical research on the benefits of physical activity on the spectrum of neuropsychiatric conditions is outside the scope of this mechanism-based review. However, **Table 1** summarises the evidence of effect, taken from systematic reviews, where available, in the main DSM-5 disease categories.

It is interesting to note the contemporaneous nature of much of this research, which demonstrates how psychopharmacology has dominated psychiatric treatment research in previous decades. Also, the inconsistency in the volume of research for each disorder, across the spectrum, is evident in **Table 1**. While there is a substantial research base into PA for depression and neurodegenerative disorders, research into bipolar disorder and schizophrenia is a more recent phenomenon and much smaller in volume. Research into clinical benefits in the remaining categories is at an embryonic stage, despite strong evidence of underlying glutamatergic dysfunction and therefore plausible responses to PA. At the time of writing there are no identifiable studies of PA in Personality Disorders. The reasons for this are not clear.

TABLE 1 Quantity of evidence for physical activity influencing neuropsychiatric conditions (Symbols "+", "++", "+++" and "++++" refer to approximate proportional quantities, relative to zero).

DSM V classification	Evidence of PA preventing disease	Evidence of PA treating symptoms	Volume of evidence
Depressive disorders	+++	+++	++++
Anxiety	+++	+++	++++
Schizophrenia	0	++	(99, 100)
Bipolar disorder	0	++	Paucity (101)
Neurodevelopmental disorders			
Autism	0	+	+ (102)
ADHD	0	+	+ (103)
Intellectual disorders	0	+	+ (104)
Trauma and stressor related disorders			
PTSD	0	++	+ (105)
OCD	0	+	+ (106, 107)
Personality disorders	0	0	No studies
Neurodegenerative disorders			
Alzheimers	+++ (108)	++ (109)	++
Parkinsons	+++ (110)	+++ (111)	+++

The seesaw model of hippocampal glutamatergic function

Multiple previous models of depression (inflammatory, neuroplastic) have been discussed in relation to increasing evidence supporting glutamate neurotransmission as the key player. Many previous theories consider systems in isolation and without temporal features. As a result most theories, although partially correct, struggle to incorporate or explain the complex effects of exercise on depression neurobiology.

The conclusion from this review is that physical activity, HPA stress, and neuroinflammation are three distinct, necessary and constant influences forming a dynamical balance or "seesaw", as depicted in **Figure 2**, that controls glutamate neurotransmission in the hippocampus and PFC, to maintain mood and cognitive function. Numerous other factors such as diet and genetic influences are relevant but for simplification only the best known influencers of glutamatergic stability are included.

It is the hope that providing a robust simple model will enable researchers to identity gaps and conduct much needed research in this field.

The implication of this model is that physical activity is not an adjunctive treatment for depression, but an essential component of basic functioning, without which harm is caused. This absence of PA, (sedentariness), is predicted this model to tip the balance towards a pro-inflammatory state. Indeed chronic sedentariness is seen to correlate with chronic low-grade pro-inflammatory states leading to depression and cardiometabolic disease.

Too much of a good thing

A second implication is that moderate amounts of hippocampal stimulation from the HPA axis and neuroinflammation systems are required for system stability, despite often being considered as pathological forces. With dynamic systems the quantity and temporal behaviour sometimes determine the beneficial or harmful nature of the agent. Taking this to an extreme, to demonstrate proof of model concept, it can be seen that even beneficial physical activity, in excessive amounts, can cause a pro-inflammatory state and be harmful. Excessive exercise training schedules with insufficient recovery time and extreme ultra-distance running events causing muscle injury and pro-inflammatory states are common examples of this (112–114).

Too little of a bad thing

The converse scenario of the seesaw model shows too little stimulation from HPA or inflammatory systems. This leads to reduced levels of BDNF, reduced synaptoplasticity and a slow decline in glutamate functionality. Clinically there is evidence of anxiety and depression resulting from lack of stress and stimulation (115). Social isolation is related to this. Small amounts of "healthy" acute stress such as public speaking,

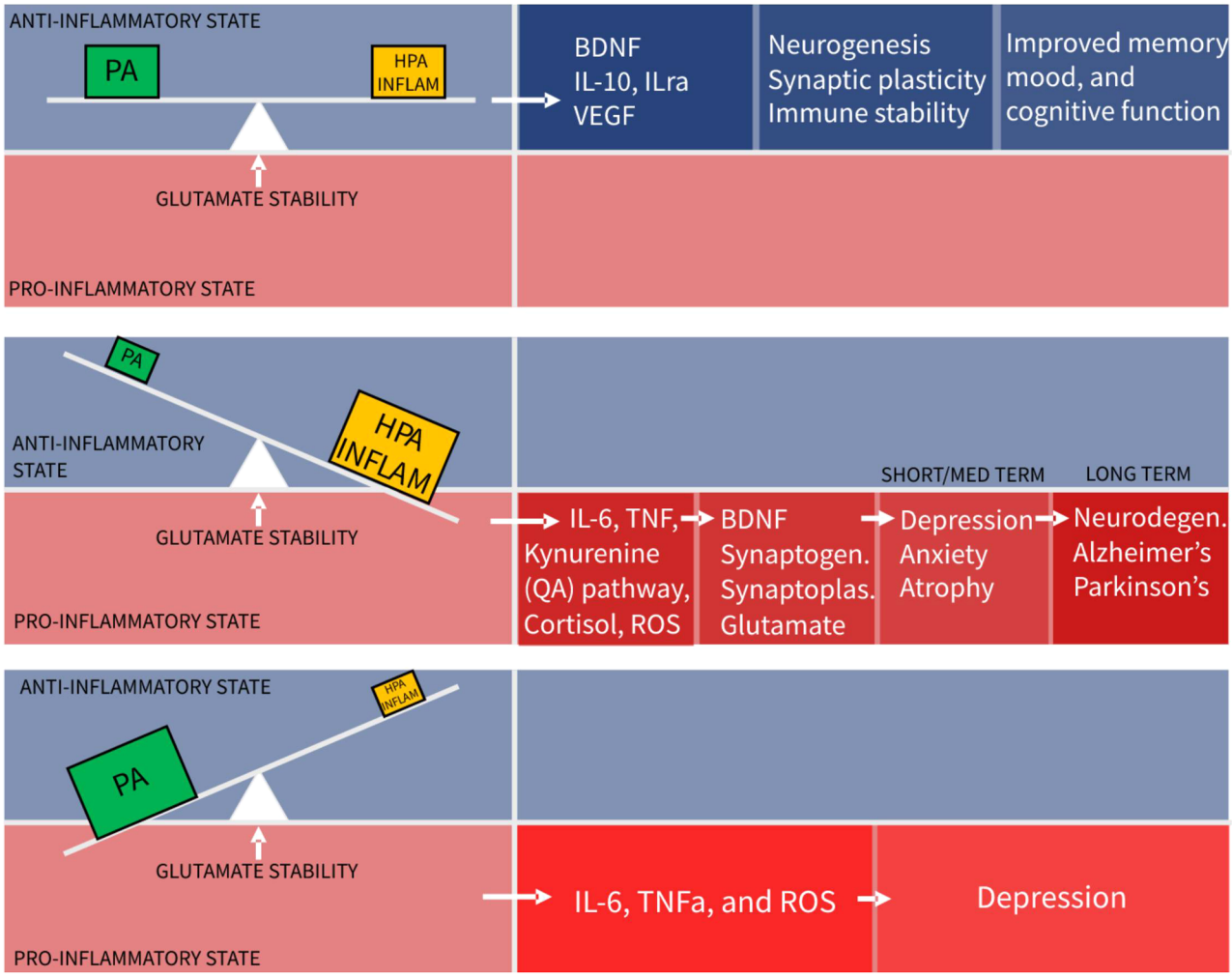

FIGURE 2
The seesaw principle of glutamatergic homeostasis (PA, physical activity; HPA, hypothalamo-pituitary-adrenal axis).

produce cortisol mediated dentate gyrus neurogenesis leading to improvements in memory and performance 2 weeks after stress exposure (116).

How much physical activity is required to maintain health or homeostatic balance, is seen in this model to be a varying and dynamic quantity, depending on the counterbalancing stress and inflammation within the individual. Hence, increased stress, tipping glutamate neurones towards dysfunction can be rebalanced by increased amounts of physical activity and this is borne out in clinical research. The underlying state of individual balances may explain some of the variation in responses of individuals to mass exercise interventions for mental health and the difficulties in prescribing optimal exercise interventions for individuals.

Another application of this model is in the depression associated with the chronic metabolic diseases, such as diabetes, characterised by low grade pro-inflammatory states. In this case, systemic cytokines enter the brain causing neuroinflammation with reduced mood and cognition. This condition has only relatively recently been recognised despite affecting up to 20% of patients (117).

As a result there is very little research available to date, on PA interventions to improve this form of depression in chronic disease groups. Limited studies have shown improvement in mood and motivation in chronic lung disease, diabetes and rheumatoid patients after exercise, independent of the state of the underlying condition (118).

The important implication of the glutamate model is that treatment of chronic disease-associated depression, by physical activity, not only reduces depression symptoms in current disease, but is neuroprotective against future chronic metabolic disease-associated-neurodegeneration, such as diabetes associated dementia and Parkinson's disease. This is also a recently recognised and significant clinical burden, which is not currently addressed by conventional disease management (119, 120).

In a parallel process, the glutamate balance model also indicates that chronic psychiatric conditions, such as Major Depressive Disorder, could theoretically progress on to later neurodegenerative disease. Conditions where PA is less studied, such as Borderline Personality Disorder, theoretically risk future neurodegenerative disease, because they already demonstrate the pre-requisites of chronic inflammation and reduced hippocampal

volume as a result of developmental in HPA responses (121). This emphasises the urgent need for neuroprotective exercise research in these poorly studied groups and other psychiatric conditions that stem from Adverse Childhood Experiences (ACEs).

Lastly it is important to distinguish this dynamic glutamate "seesaw" model based on the non-linear laws of cellular energetics and energy balance, from the psychological associations observed in depression. Hence stress and depression are often associated with reduced physical activity levels, despite the fact that this model clearly indicates the need to increase. The psychology of why human behaviour does not necessarily follow what the underlying balance state is indicating, is complex and outside the subject of this review.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Astaxanthin supplementation counters exercise-induced decreases in immune-related plasma proteins

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Objectives: Astaxanthin is a dark red keto-carotenoid found in aquatic animals such as salmon and shrimp, and algae (Haematococcus pluvialis). Astaxanthin has a unique molecular structure that may facilitate anti-oxidative, immunomodulatory, and anti-inflammatory effects during physiological stress. The primary objective of this study was to examine the efficacy of 4-week ingestion of astaxanthin in moderating exercise-induced inflammation and immune dysfunction using a multi-omics approach.

Methods: This study employed a randomized, double blind, placebo controlled, crossover design with two 4-week supplementation periods and a 2-week washout period. Study participants were randomized to astaxanthin and placebo trials, with supplements ingested daily for 4weeks prior to running 2.25h at close to 70%VO_{2max} (including 30min of 10% downhill running). After the washout period, participants repeated all procedures using the counterbalanced supplement. The astaxanthin capsule contained 8mg of algae astaxanthin. Six blood samples were collected before and after supplementation (overnight fasted state), immediately post-exercise, and at 1.5, 3, and 24h-post-exercise. Plasma aliquots were assayed using untargeted proteomics, and targeted oxylipin and cytokine panels.

Results: The 2.25h running bout induced significant muscle soreness, muscle damage, and inflammation. Astaxanthin supplementation had no effect on exercise-induced muscle soreness, muscle damage, and increases in six plasma cytokines and 42 oxylipins. Notably, astaxanthin supplementation countered exercise-induced decreases in 82 plasma proteins (during 24h recovery). Biological process analysis revealed that most of these proteins were involved in immune-related functions such as defense responses, complement activation, and humoral immune system responses. Twenty plasma immunoglobulins were identified that differed significantly between the astaxanthin and placebo trials. Plasma levels of IgM decreased significantly post-exercise but recovered after the 24h post-exercise recovery period in the astaxanthin but not the placebo trial.

Discussion: These data support that 4-week astaxanthin versus placebo supplementation did not counter exercise-induced increases in plasma cytokines and oxylipins but was linked to normalization of post-exercise plasma levels of numerous immune-related proteins including immunoglobulins within 24h. Short-term astaxanthin supplementation (8mg/day during a 4-week period)

provided immune support for runners engaging in a vigorous 2.25h running bout and uniquely countered decreases in plasma immunoglobulin levels.

KEYWORDS

astaxanthin, exercise, proteomics, oxylipins, inflammation, cytokines

Introduction

Athletes experience recurrent training and competitive increases in inflammation, oxidative stress, and immune dysfunction (1). Nutrition-based strategies including the recent emphasis on increased intake of plant phytochemicals are being explored as countermeasures to exercise-induced physiological stress (2). A recent focus in our research group has been the use of metabolomics, lipidomics, and proteomics to capture the complex responses from nutrition interventions within an exercise context (3–7).

The primary objective of this study was to examine the efficacy of 4-week ingestion of the keto-carotenoid astaxanthin in moderating exercise-induced inflammation and immune dysfunction. Astaxanthin is a dark red carotenoid found in aquatic animals such as salmon and shrimp. Humans cannot synthesize astaxanthin and can only acquire it through their diet. In the dietary supplement industry, natural astaxanthin is extracted from algae (Haematococcus pluvialis). Astaxanthin lacks pro-vitamin A activity but is more bioactive than other carotenoids such as zeaxanthin, lutein, and carotene in exerting anti-oxidative, immunomodulatory, and anti-inflammatory effects (8, 9).

Astaxanthin has a unique molecular structure with a high capacity to scavenge reactive oxygen nitrogen species (RONS) and other reactive species (sulfur and carbon) directly by donating electrons and bonding with the free radical to form a non-reactive product (8, 9). Astaxanthin may protect muscle cell membranes in salmon during their long migrations, and this finding prompted rodent-based studies that showed attenuation of exercise-induced damage in skeletal and heart muscle (10, 11). Astaxanthin plays a regulatory role with transcription factors involved in cellular redox homeostasis and inflammation including nuclear-factor erythroid 2-related factor 2 (Nrf2) and nuclear factor κB (NF-κB), respectively (12). In addition, astaxanthin may exert immune-regulatory effects by augmenting immunoglobulin production and enhancing natural killer and T lymphocyte responses (12–14).

Taken together, these data suggest that astaxanthin has the potential to mitigate post-exercise oxinflammation and immune dysfunction (2, 7). Few high quality randomized controlled trials utilizing prolonged and intensive exercise bouts have been published, and these studies used disparate dosing regimens, varied research designs, and limited outcome measures that provided inconclusive findings (15–20). To better assess the potential influence of astaxanthin supplementation on exercise-induced physiological stress, this study emphasized a multi-omics approach (4, 7). Oxylipins are bioactive oxidation products generated during stressful exercise from the metabolism of *n*-6 and *n*-3 polyunsaturated fatty acids (PUFAs) by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) enzyme systems. Oxylipin generation during exercise can be moderated through nutritional interventions (1, 2, 5–7, 21). Untargeted proteomics is the large-scale study of the proteome or the entire set of proteins produced in response to a wide variety of stresses (7). The utilization of untargeted proteomics in human sports nutrition studies is an emerging science and has high potential to improve scientific understanding regarding the complex interplay between exercise-and nutrition-related influences on immune and metabolic responses (7, 22, 23).

Methods

Study participants

Healthy, non-smoking male and female runners were invited to take part in this study if they met the inclusion criteria including 18–57 years of age, capable of running 2.25 h on laboratory treadmills at 70% maximal oxygen consumption rate (VO_{2max}), and a willingness to avoid supplements and medications such as non-steroidal anti-inflammatory drugs (NSAIDs) with a potential to influence inflammation and immune function. Participants also agreed to avoid foods and supplements with astaxanthin during the 10-week study (other than what was provided) including algae, yeast, salmon, trout, krill, shrimp, and crayfish. After 42 participants were assessed for eligibility, 21 were entered into the study, with 18 completing the protocol (Figure 1). The study participant number provided more than 84% power to detect a difference with an effect size 0.7 at alpha 0.05 using two-sided *t*-tests. Participants voluntarily signed the informed consent, and procedures were approved by the university's Institutional Review Board. Trial Registration: ClinicalTrials.gov, U.S. National Institutes of Health, identifier: NCT05409092.

Study design

This study employed a randomized, double blind, placebo controlled, crossover design with two 4-week supplementation periods and a 2-week washout period (Figure 2). The study included seven lab visits at the Appalachian State University Human Performance Laboratory (HPL) at the North Carolina Research Campus, Kannapolis, NC.

Study participants were randomized to astaxanthin and placebo trials, with supplements ingested daily (with the first meal) for 4-week prior to participation in the first 2.25 h running session. After a 2-week washout period, participants repeated all procedures using the counterbalanced supplement. The astaxanthin and placebo supplements were supplied by the sponsor (Lycored, Be′er Sheva, Israel). The astaxanthin capsule contained 8 mg of

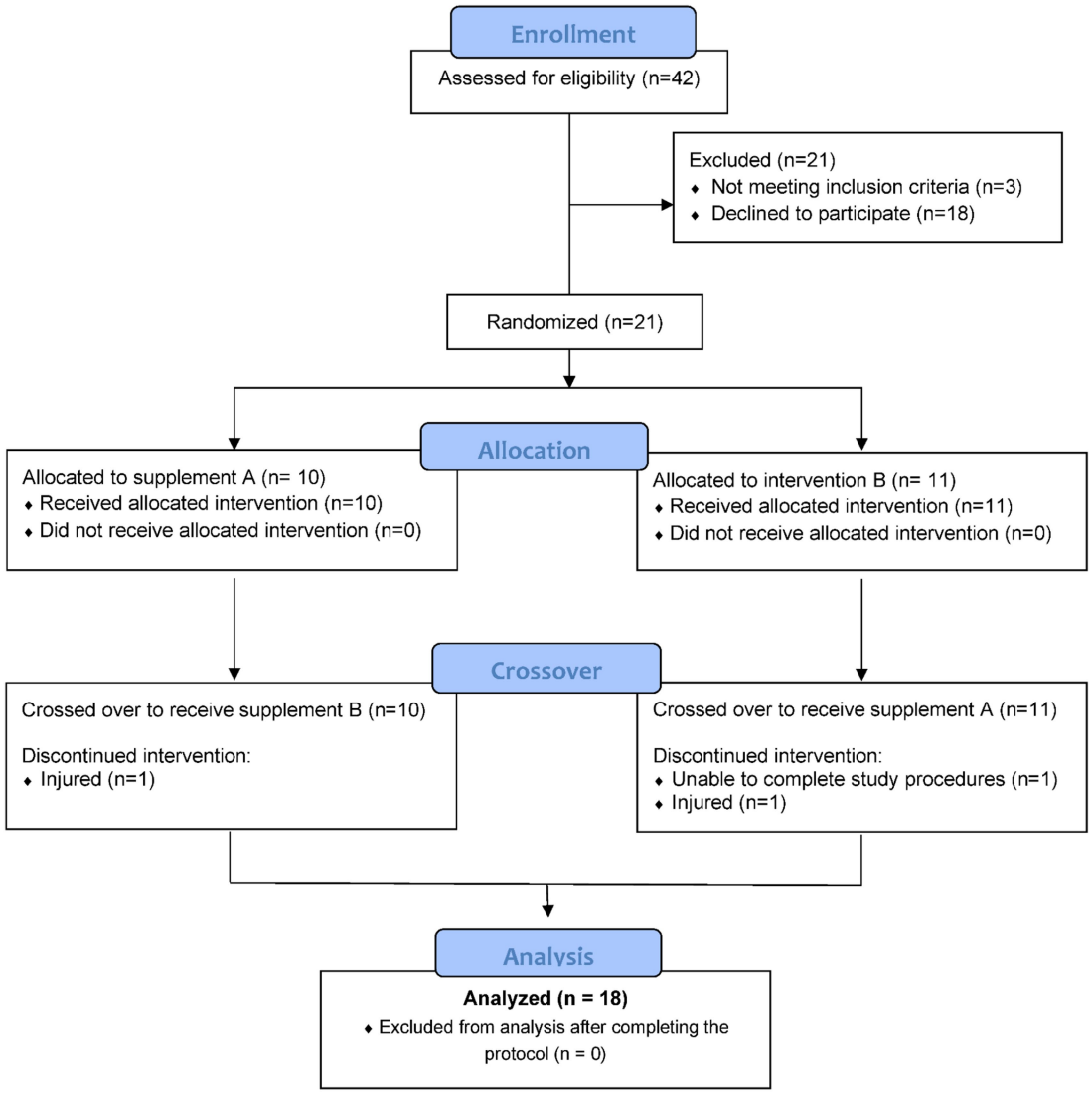

FIGURE 1
Study participant flow diagram.

astaxanthin from algae in starch beadlets, and the placebo capsules contained the starch beadlets without astaxanthin. The astaxanthin and placebo capsules were identical in appearance and color. Four ounces (113 g) of sockeye salmon contains about 4.5 mg of astaxanthin, with much higher levels in arctic shrimp and krill. Natural astaxanthin is sold around the world as an antioxidant supplement with a recommended dosage of 4–12 mg a day. A 4-week supplementation period with 8 mg/day astaxanthin has been shown to significantly increase plasma astaxanthin in human subjects (13). Lycored uses astaxanthin from Haematococcus pluvialis and the family Haematococcaceae.

Six blood samples were collected before and after 4-week supplementation (overnight fasted state, before 8:00 am in the morning), immediately post-exercise, and at 1.5, 3, and 24 h-post-exercise. Blood samples were aliquoted and stored at −80°C prior to analysis for the outcome measures. After each of the blood draws, participants provided a muscle soreness rating using a 1–10 scale (DOMS) (24).

Study participants signed the consent form and were given a complete orientation to the study protocol during the first lab visit. Study participants provided an overnight fasted blood sample and recorded responses to the DOMS questionnaire. Height and body weight were assessed, with body composition measured using the BodPod system (Cosmed, Rome, Italy). Study participants were tested for VO_{2max} during a graded, treadmill test with the Cosmed CPET metabolic cart (Cosmed, Rome, Italy). Supplements for the first and second 4-week supplementation periods were supplied in coded bottles. To facilitate compliance to the supplementation protocol, study participants were contacted *via* email on a regular basis and returned the coded bottles. Participants reported 100% compliance with the supplementation regimen and no adverse events were recorded.

During the 3-day period prior to the 2.25 h running session, subjects tapered exercise training and ingested a moderate-carbohydrate diet using a food list restricting high fat foods, visible fats, and astaxanthin. Participants recorded all food and beverage

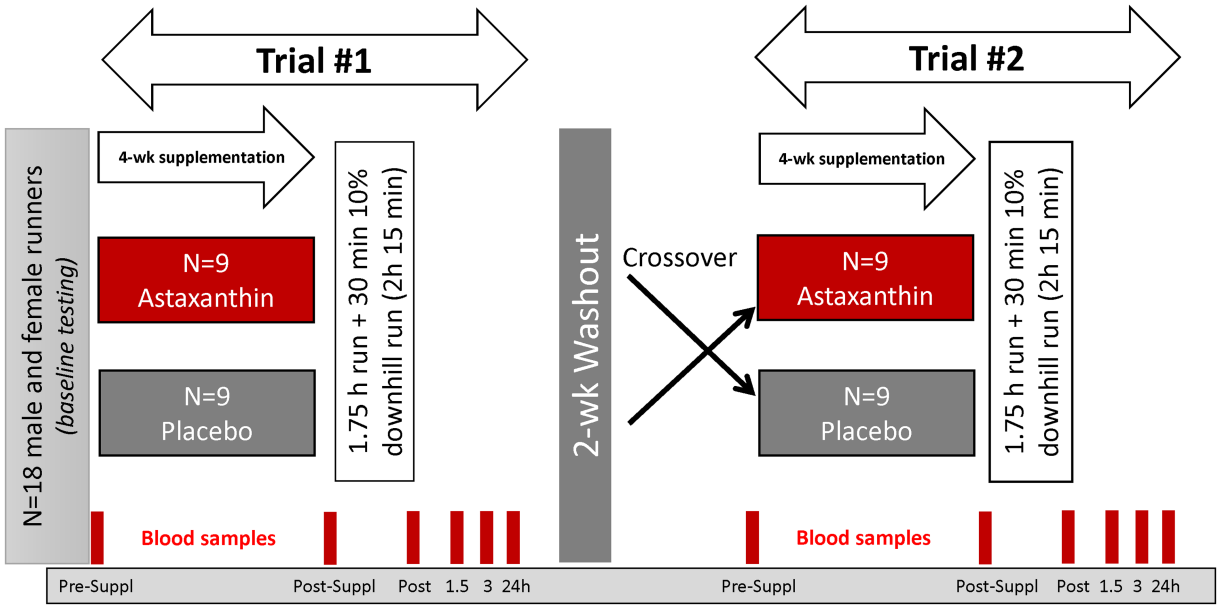

FIGURE 2
Study design.

intake for 3 days at the end of the 4-week supplementation periods to assess the background diet. Macro-and micro-nutrient intake was assessed using the Food Processor dietary analysis software system (Version 11.11, ESHA Research, Salem, OR, United States).

Study participants reported to the Human Performance Lab in an overnight fasted state, provided a blood sample, ingested the astaxanthin or the placebo supplement with water, and then ran 2.25 h at high intensity (70% VO_{2max}) while ingesting water alone (3 mL/kg every 15 min). Oxygen consumption carbon dioxide production, respiratory exchange ratio, and ventilation were measured using the Cosmed Quark CPET metabolic cart after 15 min and then every 30 min. Subjects ran 1.75 h followed by 30 min of downhill running (10%) at the same intensity. Blood samples were collected at 0, 1.5, 3, and 24 h post-exercise. Immediately after the 1.5 h post-exercise blood sample, all subjects consumed 8 kcal per kilogram of body weight of a fortified nutrient beverage (Boost, Nestlé S.A., Vevey, Switzerland).

Sample analysis

Serum creatine kinase and myoglobin, plasma cortisol, and complete blood counts with a white blood cell differential count were analyzed each day samples were collected using Labcorp services (Burlington, NC). Plasma aliquots were prepared and stored in a −80°C freezer until analysis for cytokines, proteomics, and oxylipins after the study was completed.

Plasma cytokines

IL-6, IL-8, IL-10, IL-1ra, monocyte chemotactic protein (MCP-1), and granulocyte colony-stimulating factor (GCSF) from plasma aliquots were measured with the multiplexed immunoassay platform –Ella™ (Protein Simple, CA) (25). Briefly, each individual sample was diluted 2-fold and 50 µL of the diluted sample was loaded to each well on the 32-sample cartridge or 72-sample cartridge, and the concentration of each cytokine was determined with built-in calibration curves. For quality control purposes and measurement reproducibility, aliquots of pooled plasma samples were processed the same as each individual sample to control the variation between cartridges.

Plasma oxylipins

Plasma arachidonic acid (ARA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and oxylipins were analyzed using a liquid chromatography-multiple reaction monitoring mass spectrometry (LC-MRM-MS) method as fully described elsewhere (26). Resultant data files were processed with Skyline and the auto-integrated peaks were inspected manually. Concentrations of each oxylipin were determined from calibration curves of each analyte, which were constructed by normalizing to the selected deuterated internal standards followed by linear regression with 1/x weighting (Supplementary Data Sheet 1). The coefficient of variation for the quality control standards was <15% as reported in the method development paper (26).

Plasma proteome and statistical procedures

Plasma samples were centrifuged at 10,000 × *g* for 10 min to filter out particles. 5 µl of the supernatants were transferred to 96-well plates containing 55 µl of lysis buffer (8 M urea and 10 mM dithiothreitol in 50 mM triethylammonium bicarbonate). The plasma proteins were denatured and reduced by incubating at 30°C for 60 min followed by alkylation with 10 mM iodoacetamide. After dilution to reduce urea concentration to under 1 M, samples were digested with trypsin/lysine-C. The resulted peptides were acidified by adding 10 µL of 5% formic acid. After peptide concentration measurement using the standard bicinchoninic acid (BCA) assay,

200 ng peptides were loaded onto disposable EvoTip trap-columns (EV-2003, EvoSep, Denmark) and separated on an EvoSep One ™ LC system (EV-1000, EvoSep, Denmark) using a 21 min gradient with 1 µL/min flow rate. Effluents were analyzed on a high resolution Orbitrap Exploris 240 (Thermo) mass spectrometer using the data independent acquisition (DIA) method. The plasma protein library was generated using the gas-phase fractionation DIA method from peptide samples with and without depletion of the top 14 high-abundance plasma proteins (14,120 precursors, 960 proteins). For analysis of the individual samples, the range of MS1 scan was 400–1,010 m/z with 60 k orbitrap resolution and the maximum injection time was set to 50 ms with AGC target of 3×10^6. The peptides were fragmented every 23 m/z scan window from 400 to 950 m/z with 1 m/z overlapping and scanned with 30 k orbitrap resolution. For the MS2 scan, maximum injection time was set to 120 ms with the automatic gain control (AGC) target of 1×10^6. Pooled plasma samples were used as quality controls, injecting one per every two subject samples. The obtained LC–MS/MS dataset was searched for protein identification and quantitation using DIA-NN(27). Data were normalized by referencing to the protein levels of the first time point from the same individual subject to effectively correct for inter-individual variations (28) (Supplementary Data Sheet 2). The normalized values were statistically analyzed using the ANOVA test with two trials and six timepoints. To consider the protein as significantly changing between or within effects, the positive false discovery rate (FDR) was set to less than 0.05. Pearson-correlated hierarchical clustering analysis was used to cluster proteins with similar level patterns, and the results were visualized as a heatmap with the averaged value of each time-point after normalization by z-score. The list of significantly changed proteins in the enriched clusters were functionally enriched using STRING (Ver.11.5, https://string-db.org/). The top 10 enriched biological processes from STRING analysis were selected to represent the functions of the proteins. The STRING database does not include immunoglobulins in their analysis of protein–protein interactions. Thus, the functional enrichment analysis did not include immunoglobulins. Immunoglobulins were included in the heat map hierarchical clustering analysis, and IgM and IgG were quantified by summing all peptides belonging to the specific immunoglobulin heavy constant mu and gamma 2, respectively. Paired *t*-tests were used to compare astaxanthin and placebo trial values for IgM and IgG at each time point immediately before and after the running bout.

Additional statistical procedures

The data are expressed as mean ± SE and were analyzed using the generalized linear model (GLM), repeated measures ANOVA module in SPSS (IBM SPSS Statistics, Version 28.0, IBM Corp, Armonk, NY, United States). The statistical model utilized the within-subjects approach: 2 (trials) × 6 (time points) repeated measures ANOVA and provided time (i.e., the collective effect of the running exercise bout) and interaction effects (i.e., whether the data pattern over time differed between trials). If the interaction effect was significant ($p \leq 0.05$), then *post hoc* analyses were conducted using paired *t*-tests comparing time point contrasts between trials. An alpha level of $p \leq 0.01$ was used after Bonferroni correction for five multiple tests. The positive false discovery rate (FDR or "*q* value") was calculated for multiple testing correction of the plasma oxylipin and plasma proteomics data.

Results

Characteristics for the $n = 18$ study participants ($n = 11$ males, $n = 7$ females) completing all aspects of the study protocol are summarized in Table 1. Male and female runners had similar ages, training histories, body compositions, and maximal oxygen consumption rates (VO_{2max}). This study was not powered to compare outcome measures for the male and female runners, and outcome measures for this randomized, crossover study are presented for all participants combined.

Three-day food records collected at the end of the 4-week supplementation period to assess the background diet revealed no significant differences in energy, carbohydrate, and micronutrient intake between trials (data not shown). For the entire group, energy intake of the background diet averaged 2,486 ± 173 kcal/day (7.24 ± 0.33 MJ/day), with carbohydrate, protein, fat, and alcohol representing 45.9 ± 1.6, 17.7 ± 1.1, 35.4 ± 1.6, and 3.0 ± 0.7%, respectively of total energy.

Performance data for each trial are summarized in Table 2. As designed, the two trials were similar in all performance measures during the first 1.75 h (level grade) including treadmill speed, total distance covered, and percent of maximal heart rates and oxygen

TABLE 1 Subject characteristics (n=18) for male (n=11) and female (n=7) runners.

	Sex	Mean	SE	*p* value
Age (years)	1 = male	40.7	2.7	0.477
	2 = female	43.7	2.9	
Body mass (kg)	1	75.4	3.8	0.008
	2	58.7	3.3	
Height (cm)	1	175.8	1.3	0.001
	2	162.8	3.7	
Body mass index (BMI) (kg/m^2)	1	24.3	1.0	0.149
	2	22.1	0.9	
Body fat (%)	1	17.4	2.2	0.151
	2	22.9	2.9	
Maximum oxygen consumption (VO_{2max}) ($ml \cdot kg^{-1} min^{-1}$)	1	52.7	2.9	0.162
	2	46.3	2.8	
Maximum heart rate (beats/min)	1	175.2	2.3	0.062
	2	168.0	2.6	
Maximum ventilation (L/min)	1	138.7	6.6	<0.001
	2	94.3	6.9	
Maximum respiratory exchange ratio (RER_{max}) (VCO_2/VO_2)	1	1.13	0.03	0.496
	2	1.09	0.03	
Maximum respiratory rate (breaths/min)	1	51.0	4.2	0.277
	2	44.4	3.4	
Run training distance (km/wk)	1	44.7	6.4	0.591
	2	38.8	8.9	

consumption rates. During the final 30 min of the 2.25 h run, participants in the astaxanthin and placebo trials ran on a 10% downhill grade at increased speeds (11.3 ± 0.4 and 11.3 ± 0.4 km/h, respectively), similar heart rates (146 ± 2.9 and 145 ± 3.4 bpm, respectively), increased ratings of perceived exertion (RPE; 14.0 ± 0.5 and 14.3 ± 0.5, respectively), and decreased oxygen consumption rates (28.6 ± 1.3 and 28.6 ± 1.1 ml·kg^{-1} min^{-1}, respectively) compared to the 1.75 h level grade phase of the running bouts.

Inflammation related data are summarized in Table 3. The 2.25 h running trials induced significant increases in delayed onset of muscle soreness (DOMS), serum concentrations for the stress hormone cortisol and the muscle damage biomarker creatine kinase, and plasma concentrations for six cytokines (all time effects, $p \leq 0.012$). Significant post-exercise increases were also measured for serum myoglobin and the blood neutrophil-to-lymphocyte ratio (time effects, $p < 0.001$, data not shown). Interaction effects revealed no differences in the patterns of change in these biomarkers between trials.

Of 81 oxylipins detected in study samples, a total of 42 oxylipins exhibited significant time effects during GLM statistical analysis (Supplementary Data Sheet 1). These 42 oxylipins were summed for a composite variable (Figure 3). GLM analysis showed a significant time effect ($p < 0.001$) of 2.25 h running on this composite variable of 42 oxylipins, but without trial differences (interaction effect, $p = 0.412$) or differences between male and female runners (supplement × time × sex interaction effect, value of $p = 0.800$). Two other composite variables were calculated including nine oxylipins generated from arachidonic acid and cytochrome P-450 (ARA-CYP) and four abundant oxylipins generated from linoleic acid and CYP (9,10-DiHOME, 12,13-DiHOME) and lipoxygenase (LOX; 9-HODE, 13-HODE; LA-DiHOMES+HODES; Figure 3). Significant time effects were shown for ARA-CYP and LA-DiHOMES+HODES ($p = 0.007$ and $p < 0.001$, respectively), but without trial differences ($p = 0.432$ and $p = 0.505$, respectively). The nine oxylipins included with ARA-CYP are generally regarded as pro-inflammatory oxylipins and included 5,6-, 8,9-, 11,12-, and 14,15-diHETrEs, 5,15-diHETE, 16-, 17,- 18-HETEs, and the 20-HETE metabolite 20-coohAA.

A total of 608 plasma proteins were identified with an FDR <0.01, and 500 of them were quantified without any missing values across

TABLE 2 Average performance outcomes for n=18 runners during the first 1.75h of the astaxanthin and placebo trials.

	Astaxanthin		Placebo		
	Mean	SE	Mean	SE	p value
Treadmill speed (km/h)	10.4	0.32	10.4	0.32	0.84
Heart rate (beats/min)	145	2.09	144	2.37	0.29
Heart rate (% max HR)	84.4	1.05	83.5	1.47	0.31
Total distance (2.25 h; km)	24.3	0.74	23.9	0.74	0.26
Rating perceived exertion (at 1.75 h)	13.4	0.46	13.8	0.43	0.23
Oxygen consumption (ml·kg^{-1} min^{-1})	35.7	1.30	35.4	1.26	0.51
Oxygen consumption (% VO_{2max})	71.7	1.62	71.1	1.53	0.51
Ventilation (L/min)	69.9	2.46	68.0	2.76	0.10

TABLE 3 Trial comparisons of inflammation related outcomes.

Variable	Trial	Pre- study	4-week Suppl.	0h Post-Ex	1.5h Post-Ex	3h Post-Ex	24h Post-Ex	p value
DOMS (1–10 scale)	AS	1.4 ± 0.2	1.8 ± 0.2	5.6 ± 0.6	5.4 ± 0.5	4.9 ± 0.4	5.7 ± 0.5	<0.001; 0.818
	PL	1.5 ± 0.2	1.4 ± 0.1	5.4 ± 0.5	5.1 ± 0.5	4.5 ± 0.5	5.7 ± 0.5	
Creatine kinase (U/L)	AS	150 ± 14.9	164 ± 29.9	268 ± 35.5	265 ± 31.9	304 ± 34.2	535 ± 70.5	<0.001; 0.518
	PL	157 ± 18.6	162 ± 20.8	251 ± 29.6	262 ± 26.4	278 ± 27.8	462 ± 67.9	
Cortisol (μg/dL)	AS	15.2 ± 1.7	19.8 ± 1.4	19.9 ± 1.9	16.1 ± 2.4	15.8 ± 2.3	17.5 ± 1.0	<0.001; 0.114
	PL	16.7 ± 1.4	19.9 ± 1.2	18.3 ± 1.8	15.9 ± 1.5	13.3 ± 1.2	16.3 ± 1.4	
IL-6 (pg/mL)	AS	1.2 ± 0.1	1.7 ± 0.2	13.8 ± 2.2	9.9 ± 2.9	4.5 ± 0.5	1.8 ± 0.4	<0.001; 0.938
	PL	1.2 ± 0.2	1.9 ± 0.6	12.8 ± 2.7	8.3 ± 1.3	4.1 ± 0.4	1.8 ± 0.2	
IL-8 (pg/mL)	AS	3.3 ± 0.3	3.9 ± 0.3	8.5 ± 0.8	6.4 ± 0.6	4.6 ± 0.4	3.7 ± 0.3	<0.001; 0.123
	PL	3.6 ± 0.3	4.0 ± 0.4	7.7 ± 0.7	6.3 ± 0.5	4.4 ± 0.3	3.6 ± 0.3	
IL-10 (pg/mL)	AS	2.3 ± 0.5	3.7 ± 1.2	17.7 ± 5.4	9.1 ± 3.0	4.2 ± 0.9	2.9 ± 0.6	0.012; 0.079
	PL	2.4 ± 0.5	2.8 ± 0.6	12.4 ± 4.4	5.4 ± 1.5	2.7 ± 0.5	3.0 ± 0.5	
IL-1ra (pg/mL)	AS	119 ± 4.9	143 ± 12.8	244 ± 29.6	283 ± 38.3	291 ± 33.0	146 ± 11.4	<0.001; 0.225
	PL	122 ± 6.4	134 ± 6.9	182 ± 11.8	243 ± 28.0	244 ± 24.3	137 ± 7.6	
MCP-1 (pg/mL)	AS	149 ± 9.0	161 ± 11.9	292 ± 23.6	260 ± 20.6	216 ± 12.6	154 ± 9.3	<0.001; 0.123
	PL	153 ± 10.9	164 ± 9.2	261 ± 21.7	258 ± 16.8	213 ± 16.3	165 ± 8.3	
GCSF (pg/mL)	AS	12.7 ± 1.8	12.5 ± 1.9	20.4 ± 2.4	23.9 ± 5.5	18.3 ± 3.5	15.6 ± 3.4	<0.001; 0.565
	PL	11.4 ± 1.9	13.2 ± 2.0	19.3 ± 2.4	20.3 ± 2.4	15.9 ± 2.6	12.7 ± 2.0	

AS, astaxanthin; PL, placebo. p values represent time (first value) and trial × time interaction effects. All interaction effects were non-significant, so no post-hoc tests were conducted.

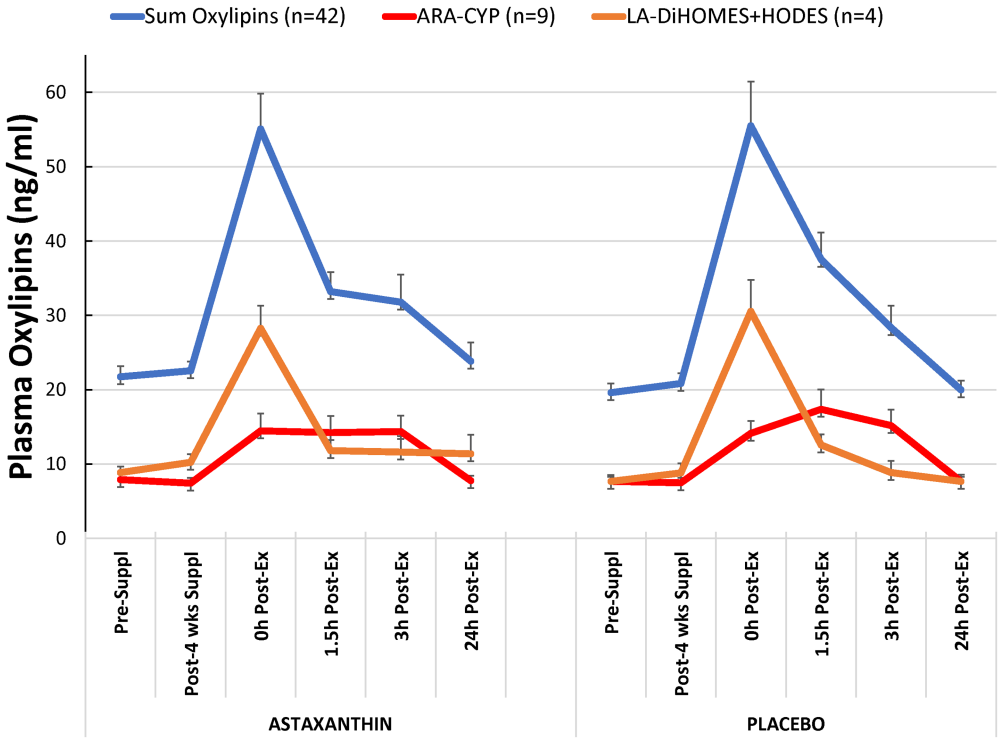

FIGURE 3
Plasma oxylipin concentrations for the astaxanthin and placebo trials. "Sum oxylipins" is the sum value for n=42 oxylipins with a significant exercise-induced time effect but no interaction effect (p=0.412). "ARA-CYP" is the sum value for n=9 arachidonic acid-cytochrome P-450 generated oxylipins (time effect, p<0.001, interaction effect, p=0.432). "LA-DiHOMES+HODES" is the sum value for n=4 linoleic acid CYP and LOX generated oxylipins (time effect, p<0.001, interaction effect, p=0.505).

214 samples analyzed (Supplementary Data Sheet 2). The plasma sample quality was assessed based on the plasma's potential main sources of protein contamination, indicating that the majority of samples (all but three) were not significantly contaminated with platelets, erythrocytes, or coagulations (Supplementary Figure S1A). Since the protein levels varied significantly between subjects as evidenced by the higher correlations within subjects than between subjects (Supplementary Figure S1B), the longitudinal dataset was normalized by calculating ratios to the protein levels at the first time point to increase the likelihood of discovering proteins dysregulated due to supplementation.

Of the 500 identified plasma proteins, 105 were significantly influenced by the running bouts (FDR < 0.05, Figure 4A). Cluster 1 proteins (23 total) were rapidly downregulated after exercise and then gradually recovered in both supplement trials within 24 h. A total of 82 proteins from clusters 2, 3, and 4 (Figure 4A) were immediately reduced post-exercise compared to pre-exercise levels and increased during the 24 h post-exercise period in the astaxanthin compared to the placebo trial. Biological process analysis revealed that most of the proteins were involved in immune-related functions such as defense responses, complement activation, and immune system responses (Figure 4B; Table 4). Two proteins in cluster 5, S100A8 and S100A9, were increased after exercise in both groups and then gradually returned to pre-exercise levels within the 24 h post-exercise recovery period. Their biological functions are related to neutrophil degranulation (FDR = 0.002) and the innate immune system (FDR = 0.0095). A total of 20 plasma immunoglobulins were identified that differed significantly between the astaxanthin and placebo trials (Figure 4C). Plasma levels of IgM were significantly downregulated post-exercise but recovered after the 24 h post-exercise recovery period in the astaxanthin but not the placebo trial (Figure 4D). The patterns of change in IgG did not differ between the astaxanthin and placebo trials.

Discussion

This study employed a strong research design and showed that 4-weeks astaxanthin supplementation had no effect in runners on 2.25-h running-induced muscle soreness, muscle damage, and elevations in six plasma cytokines and 42 oxylipins. The untargeted proteomics data, however, showed that astaxanthin supplementation did counter exercise-induced decreases in 82 plasma proteins involved in immune-related functions. Astaxanthin supplementation countered the post-exercise decrease in plasma immunoglobulins, especially IgM.

Other astaxanthin-based human clinical trials focused on limited and basic outcomes related to exercise performance, muscle damage (e.g., creatine kinase), oxidative stress (e.g., malondialdehyde, MDA, as a lipid peroxidation marker), and inflammation (e.g., C-reactive protein, CRP (15–20, 29). Most of these studies showed no effects of astaxanthin (6–20 mg/day during 1–13 weeks) on exercise performance (29), creatine kinase (17, 18), MDA (18, 29), or CRP (17, 18) after an exercise challenge. This is the first human clinical

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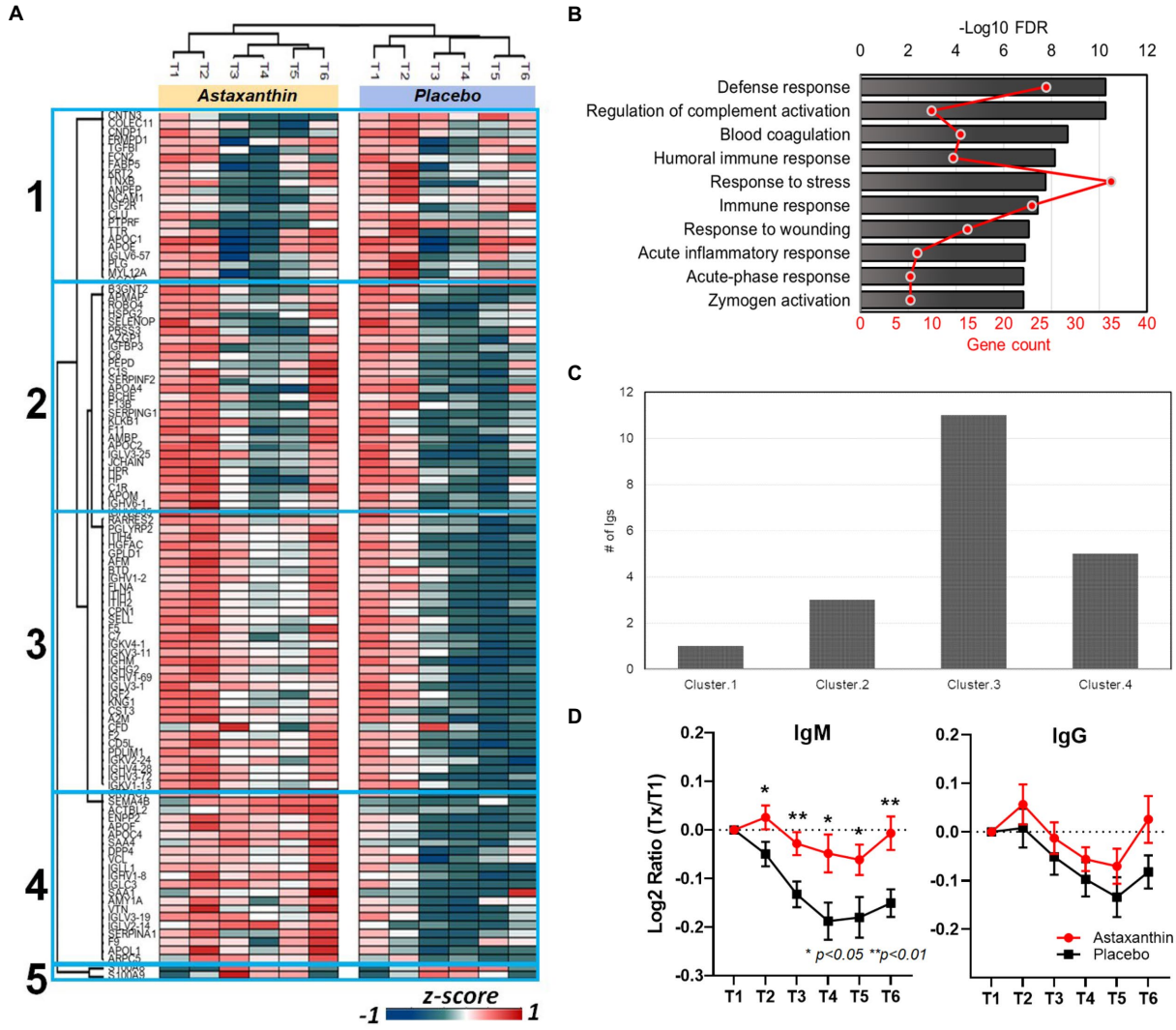

FIGURE 4
(A) Heatmap of clustered proteins in the astaxanthin and placebo trials. T1, pre-study; T2, 4-weeks supplementation, pre-exercise; T3, immediately post-exercise (2.25 h run bout); T4, 1.5 h post-exercise; T5, 3 h post-exercise; T6, 24 h post-exercise. **(B)** Associated biological processes for clusters 2–4, see Supplementary Table S1 for details. **(C)** Number of identified immunoglobulins in the clusters of **(A)**. **(D)** Changes of plasma IgM and IgG levels in plasma in subjects in response to the astaxanthin and placebo trials.

trial to measure physiological responses to astaxanthin supplementation after an intense exercise challenge using untargeted proteomics (500 proteins across all samples), a targeted and comprehensive panel of 81 oxylipins, and six cytokines.

The data indicate that 4-weeks astaxanthin supplementation had little effect on exercise-induced increases in most inflammation-related measures including six plasma cytokines, 42 plasma oxylipins, and plasma proteins in cluster 5 of this study. The running bout caused significant increases in plasma levels of IL-6, IL-8, IL-10, MCP-1, GCSF, IL1ra, and many different types of oxylipins as shown in previous studies (3, 5, 6, 21). The proteomics analysis showed that two proteins in cluster 5, S100A8 and S100A9 or calprotectin, were increased after exercise in both the astaxanthin and placebo trials, before gradually returning to pre-exercise levels within the 24 h post-exercise recovery period. Calprotectin is released during degranulation from activated neutrophils during the inflammatory process following intensive exercise. Calprotectin also promotes phagocyte migration, and functions as an alarmin and endogenous danger-associated molecular pattern (DAMP) (30). *In vivo* and *in vitro* data support a role for astaxanthin in decreasing inflammation, but the data from the present study indicate that these findings do not extend to mitigating transient exercise-induced inflammation (9, 17, 18).

Astaxanthin supplementation did have a strong effect in countering post-exercise decreases in many proteins related to immune function including 20 immunoglobulins. The major soluble proteins for humoral immunity are the immunoglobulins that can combine with specific antigens as a functional component of the host defense system. Previous studies have shown that serum immunoglobulin levels can be reduced for 1–2 days after prolonged and intensive exercise, as confirmed in the present study (31, 32). B lymphocyte suppression has been reported after sustained vigorous

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TABLE 4 Associated biological processes, gene counts, and matching proteins for clusters 2–4.

Biological processes	FDR *q* value	Gene count	Matching proteins, clusters 2,3,4
Defense response	5.51E-11	26	IGJ, C6, KLKB1, KNG1, ITIH4, SAA4, SERPING1, F2, APOL1, SERPINF2, C7, IGLL1, CFD, PGLYRP2, HP, APOA4, DPP4, CD5L, FLNA, HSPG2, CST3, SAA1, C1S, SERPINA1, RARRES2, and C1R
Regulation of complement activation	5.51E-11	10	VTN, C6, SERPING1, F2, C7, A2M, CD5L, CPN1, C1S, and C1R
Blood coagulation	2.08E-09	14	VCL, F9,KLKB1, KNG1, SERPING1, F2,A2M, F13B, F5, FLNA, SAA1,F11, SERPINA1, and HGFAC
Humoral immune response	7.39E-09	13	IGJ, C6, KNG1, SERPING1, F2, C7, IGLL1, CFD, PGLYRP2, PRSS3, C1S, RARRES2, and C1R
Response to stress	1.76E-08	35	VCL, F9, IGJ, C6,KLKB1, KNG1, ITIH4, SAA4, SERPING1, F2, APOL1, SERPINF2, C7, A2M, IGLL1, CFD, PGLYRP2, HP, APOA4, DPP4, F13B, F5, CD5L, FLNA, PDLIM1, HSPG2, CST3, SAA1, F11, C1S, SERPINA1, RARRES2, SEPP1, HGFAC, and C1R
Immune response	3.83E-08	24	VCL, VTN, SELL, IGJ, ENPP2, C6, KNG1, SERPING1, ARPC5, F2, APOL1, C7, IGLL1, CFD, PGLYRP2, HP, APOA4, PRSS3, CST3, SAA1, C1S, SERPINA1, RARRES2, and C1R
Response to wounding	8.98E-08	15	VCL, F9, KLKB1, KNG1, SERPING1, F2, A2M, F13B, F5, FLNA, CST3, SAA1, F11, SERPINA1, and HGFAC
Acute inflammatory response	1.29E-07	8	KLKB1, ITIH4, SAA4, F2, SERPINF2, HP, SAA1, and SERPINA1
Acute-phase response	1.51E-07	7	ITIH4, SAA4, F2, SERPINF2, HP, SAA1, and SERPINA1
Zymogen activation	1.51E-07	7	F9, KLKB1, PRSS3, CD5L, F11, HGFAC, and C1R

exercise and may in part be related to an inhibitory effect from activated monocytes (33). Several cell culture-based studies have shown that astaxanthin can increase immunoglobulin production under varying conditions (14, 27, 34–36). For example, astaxanthin enhanced IgM and IgG production by human lymphocytes in response to T cell-dependent stimuli (14). Animal studies support increases in plasma IgG and IgM and other biomarkers of immune function in astaxanthin-fed dogs and cats (37, 38). In the present study, astaxanthin supplementation countered the exercise-induced decrease in plasma IgM but not IgG levels. IgM represents about 10% of total blood immunoglobulins and is the predominant antibody produced early in an immune response. IgM is also the major immunoglobulin expressed on the surface of B cells (31).

Randomized clinical trials investigating the influence of astaxanthin supplementation on immune-related outcomes are limited. One study of young female adults ingesting 0, 2, or 8 mg/day astaxanthin daily for 8 weeks showing strong increases in plasma astaxanthin concentrations at 4 and 8 weeks with 2 or 8 mg/day, but inconsistent and modest improvements in T cell and natural killer cell function (13). Plasma immunoglobulins were not measured in this study. The data from the present study are the first human data to indicate that astaxanthin supplementation can counter exercise-induced decreases in plasma immunoglobulins and IgM in human subjects.

Conclusion

This study used a 2.25 h running bout with 30 min of downhill running to induce muscle soreness, damage, inflammation, and immune dysfunction. Astaxanthin supplementation (8 mg/day, 4 weeks) was tested under double blind procedures against placebo using a crossover design. The objective was to see if astaxanthin could serve as a nutrition-based strategy to mitigate exercise-induced physiological stress. A human systems biology approach was used to improve the ability to capture trial differences using untargeted proteomics, and comprehensive targeted oxylipin and cytokine panels. These data indicate that astaxanthin supplementation did not counter exercise-induced increases in plasma cytokines and oxylipins but was linked to normalization of post-exercise plasma levels of numerous immune-related proteins within 24 h. Thus, astaxanthin supplementation provided immune support for runners engaging in a vigorous running bout and uniquely countered decreases in 20 plasma immunoglobulins including IgM.

Data availability statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD040503.

Ethics statement

The studies involving human participants were reviewed and approved by Appalachian State University IRB. The patients/participants provided their written informed consent to participate in this study.

Author contributions

DN, QZ, AP, and GV designed the research project. DN, CS, KD, AP, and GV conducted the research project. JW, QZ, AO, YT, CS, and KD analyzed the samples, and DN, JW, and QZ conducted the data analysis. DN, JW, QZ, CS, KD, AO, AP, GV, and YT wrote and edited the paper. DN had primary responsibility for the final content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1143385/full#supplementary-material

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Human lymphocytes mobilized with exercise have an anti-tumor transcriptomic profile and exert enhanced graft-versus-leukemia effects in xenogeneic mice

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Background: Every bout of exercise mobilizes and redistributes large numbers of effector lymphocytes with a cytotoxic and tissue migration phenotype. The frequent redistribution of these cells is purported to increase immune surveillance and play a mechanistic role in reducing cancer risk and slowing tumor progression in physically active cancer survivors. Our aim was to provide the first detailed single cell transcriptomic analysis of exercise-mobilized lymphocytes and test their effectiveness as a donor lymphocyte infusion (DLI) in xenogeneic mice engrafted with human leukemia.

Methods: Peripheral blood mononuclear cells (PBMCs) were collected from healthy volunteers at rest and at the end of an acute bout of cycling exercise. Flow cytometry and single-cell RNA sequencing was performed to identify phenotypic and transcriptomic differences between resting and exercise-mobilized cells using a targeted gene expression panel curated for human immunology. PBMCs were injected into the tail vein of xenogeneic NSG-IL-15 mice and subsequently challenged with a luciferase tagged chronic myelogenous leukemia cell line (K562). Tumor growth (bioluminescence) and xenogeneic graft-versus-host disease (GvHD) were monitored bi-weekly for 40-days.

Results: Exercise preferentially mobilized NK-cell, CD8+ T-cell and monocyte subtypes with a differentiated and effector phenotype, without significantly mobilizing CD4+ regulatory T-cells. Mobilized effector lymphocytes, particularly effector-memory CD8+ T-cells and NK-cells, displayed differentially expressed genes and enriched gene sets associated with anti-tumor activity, including cytotoxicity, migration/chemotaxis, antigen binding,

cytokine responsiveness and alloreactivity (e.g. graft-versus-host/leukemia). Mice receiving exercise-mobilized PBMCs had lower tumor burden and higher overall survival (4.14E+08 photons/s and 47%, respectively) at day 40 compared to mice receiving resting PBMCs (12.1E+08 photons/s and 22%, respectively) from the same donors ($p<0.05$). Human immune cell engraftment was similar for resting and exercise-mobilized DLI. However, when compared to non-tumor bearing mice, K562 increased the expansion of NK-cell and CD3+/CD4-/CD8- T-cells in mice receiving exercise-mobilized but not resting lymphocytes, 1-2 weeks after DLI. No differences in GvHD or GvHD-free survival was observed between groups either with or without K562 challenge.

Conclusion: Exercise in humans mobilizes effector lymphocytes with an anti-tumor transcriptomic profile and their use as DLI extends survival and enhances the graft-versus-leukemia (GvL) effect without exacerbating GvHD in human leukemia bearing xenogeneic mice. Exercise may serve as an effective and economical adjuvant to increase the GvL effects of allogeneic cell therapies without intensifying GvHD.

KEYWORDS

exercise immunology, NSG mice, single cell transcriptomics, donor lymphocyte infusions, adoptive cell therapy, cancer, immunotherapy, graft-versus-host disease

1 Introduction

Regular physical activity is widely regarded as contributing to lower cancer risk, improving outcomes in cancer survivors, and acting as an adjuvant for several types of cancer therapy (1–3). This assertion has come from a plethora of epidemiological studies linking physical activity to a reduction in cancer occurrence, randomized controlled trials demonstrating beneficial effects of exercise on treatment associated adverse effects, and preclinical studies showing increased tumor suppression in rodents exposed to exercise (4, 5).

Each bout of exercise evokes a catecholamine dependent mobilization and redistribution of effector lymphocytes (e.g. Natural Killer-cells, γδ T-cells, CD8+ T-cells) that has been purported to improve long-term immunosurveillance by increasing recognition and destruction of premalignant cells in the initial stages of tumor development, and contributing to the suppression of tumor growth (6, 7). Indeed, exercise has been shown to increase NK-cell and CD8+ T-cell tumor infiltration and suppress tumor growth in several murine cancer models (8, 9). These tumor infiltrating lymphocytes are similar in phenotype to those mobilized to blood with each exercise bout, indicating that exercise-mobilized lymphocytes could be harnessed for adoptive cell therapies (1,4). Donor lymphocyte infusions (DLI) are commonly delivered after allogeneic stem cell transplantation (allo-HSCT) to prevent and/or treat leukemic relapse. Unfortunately, the success rate of DLI to induce remission remains low (10) and the procedure also increases the risk of graft-versus-host disease (GvHD) – a potentially fatal condition whereby donor T-cells attack healthy tissues in the host causing significant morbidity (11). As such, using exercise mobilized DLI could have therapeutic ramifications if they are found to exert enhanced graft versus leukemia (GvL) effects without increased GvHD.

The characterization of exercise-mobilized lymphocytes has hitherto been limited to just a few surface markers or simple *in vitro* assays to determine function (e.g. cytotoxicity, proliferation), with the latter oftentimes confounded by cell composition shifts that have been evoked by exercise (3). No study, to our knowledge, has transcriptionally profiled exercise-mobilized lymphocytes in humans at single cell resolution or assessed their ability to control human leukemic growth in a whole organism.

The aim of this study, therefore, was to deeply interrogate the transcriptional and phenotypic profiles of exercise mobilized lymphocytes in humans and test their ability to reduce leukemic burden and xenogeneic GvHD in immunodeficient (NSG) mice. We hypothesized that (i) exercise would preferentially mobilize effector lymphocytes to the blood compartment with an enrichment of multiple transcriptional programs associated with anti-tumor activity; and (ii) that exercise mobilized lymphocytes would extend survival and reduce tumor burden without increasing GvHD after adoptive transfer to human leukemia bearing NSG mice. We report for the first time that exercise-mobilized peripheral blood mononuclear cells have differential gene expression (DGEs) and enriched gene sets associated with anti-tumor activity, particularly within effector memory subsets of CD8+ T-cells and NK-cells. Moreover, adoptively transferring these cells into human leukemia-bearing NSG mice prolonged survival by augmenting the GvL effect without exacerbating GvHD, underscoring the potential for exercise-mobilized lymphocytes to enhance the efficacy of allogeneic adoptive cell therapies.

2 Materials and methods

2.1 Participants

Sixteen healthy adults, (6 females, 10 males) participated in this study (Mean ± SD: age: 26.8 ± 5.2 yr; weight: 69.7 ± 13.6 kg; Height: 171.2 ± 15 cm; BMI: 23.2 ± 2 (kg/m^2). Written informed consent and medical history were obtained from each volunteer after proper explanation of the procedures and risks. Only participants classified as 'low risk' in accordance with the risk stratification guidelines published by the American Heart Association and the American College of Sports Medicine (AHA/ACSM criteria) were enrolled (12). The study was approved by the Human Subjects Protection Program at the University of Arizona (#1801161041).

2.2 Exercise trials and blood sampling

All participants were asked to visit the laboratory on two separate occasions with one-two weeks separating each visit. Both laboratory visits occurred between 08:00 and 10:00 local time. Participants were instructed to arrive at the lab following an 8-12h overnight fast (water could be consumed during the fasting period). Additionally, participants were required to refrain from engaging in vigorous physical activity/exercise for 24 hours prior to each visit.

For Visit 1, subjects were asked to complete a maximal exercise protocol on an indoor stationary bicycle (Velotron, Quarq Technology, San Diego, CA). Heart rate, ECG activity and respiratory gas exchange was recoded continuously throughout the test (Cosmed CPET, Rome, Italy). Subjects began cycling at 50 Watts for females and 75 Watts for males, and the power was increased by 15 Watts every minute until volitional exhaustion. Ventilatory threshold, determined as the point corresponding to a rise in the ventilatory equivalent of VO_2 (V_E/VO_2) without a concurrent rise in the ventilatory equivalent of VCO_2 (V_E/VCO_2), and the determined VO_{2max} were used to assign workload for Visit 2.

For Visit 2, an intravenous catheter was inserted and blood was collected before exercise in vacutainer tubes treated with ethylene-diamine-tetra-acetic acid (EDTA) or acid citrate dextrose (ACD) (Becton-Dickinson, USA). Subjects were prepared for continuous heart rate, ECG and respiratory gas exchange collections then asked to cycle at either a power wattage corresponding to 15% above the ventilatory threshold for 30-minutes, or to perform 20-minutes of graded exercise, consisting of four incremental 5-minute stages at power outputs corresponding to 50%, 60%, 70%, and 80% of the individual VO_{2max}. A final venous blood sample was collected during the last 3-5 minutes of the exercise protocol (during-exercise).

2.3 Blood processing and analysis

Complete blood counts were performed on blood collected in EDTA tubes using an automated hematology analyzer (Beckman Coulter, Indianapolis, IN). Whole blood samples from EDTA tubes were labeled with monoclonal antibodies to detect and enumerate leukocyte and progenitor cell subpopulations in blood by flow cytometry (MACSQuant 10, Miltenyi Biotec, San Diego, CA). All immune cell phenotypes were determined within the peripheral blood mononuclear cell (PBMC) gate based on forward and side scatter using FlowLogic (Inivai Technologies, Mentone Victoria, Australia). Single color tubes on whole blood samples were used for electronic color compensation and Fluorescence Minus One (FMOs) were used to set proper gating. A minimum of 50,000 PBMCs were collected for analysis. The flow cytometry antibody panels used are shown in Supplementary Table 1. Peripheral blood mononuclear cells (PBMCs) were isolated from blood collected in ACD tubes and cryopreserved in liquid nitrogen at a concentration of 10x10^6 cells/mL freezing media (90% FBS, 10% DMSO) until further use in animal experiments.

2.4 RNAseq

Isolated PBMCs were resuspended in a PBS/RNAlater solution and delivered to the University of Arizona Genetics Core for single cell RNA sequencing (scRNAseq) analysis using the 10x Genomics platform. 5' RNA whole transcriptome libraries were generated using the "10xGenomics Chromium Next GEM Single Cell 5' reagents kit v2", following recommended guidelines. The gene expression libraries were quantified, normalized, pooled, and sequenced on an Illumina NextSeq500 sequencer. FastQ files were converted into expression matrices using the "cellranger count" function provided by Cell Ranger (10x Genomics Cell Ranger 6.0.1) and unfiltered matrices were imported into R, version 4.1.0. Empty droplets were identified and removed using the emptyDrops function found in the DropletUtils package. Reads with a high percentage of mitochondrial content were identified and removed using the perCellQCMetrics function provided by scuttle. To analyze and visualize gene expression on a per cell basis, principal component analysis (PCA) and uniform manifold approximation and projection (UMAP) clustering was performed using Seurat, version 4.0.5. Differentially expressed genes were then detected using the FindMarkers function, with a log2 fold cutoff of 0, in Seurat. For each differential expression analysis comparison, gene set enrichment analysis (GSEA), with a false discovery rate of (0.25), was performed and annotated to both Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) terms.

2.5 Animal experiments

The experiments detailed here were approved by the University of Arizona IACUC (protocol 17-338). Eight-twelve (8-12) week old NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(IL15)1Sz/SzJ (NSG-Tg(Hu-IL15)) (Jackson Labs, Stock No: 030890) were used for xenotransplantation of K562-luc2 tumor cells (ATCC, Manassas, VA) and human PBMCs. NSG-Tg(Hu-IL15) mice include the human IL-15 transgene allowing endogenous expression of physiological levels of human IL-15 (7.1 ± 0.3 pg/mL). Additional GvHD and human immune cell engraftment experiments were performed using standard NSG mice without

human IL-15 knock-in (Jackson Labs, Stock No: 005557). On Day -2, mice were irradiated with 100cGy (Bio5 Cesium 137 Irradiator, Atomic Energy of Canada Ltd) to improve engraftment of human cells. On Day -1, mice were injected intravenously through the lateral tail vein with $1x10^7$ PBMCs either from resting or during-exercise samples. On day 0, mice were injected in the lateral tail vein with $1x10^6$ K562-luc2 cells. Bioluminescent imaging (BLI) began on day +1 post-tumor injection and repeated every 3-4 days to track tumor progression using the LargoX bioluminescence imager (Spectral Instruments Imaging, Tucson, AZ). Mice not receiving tumor or PBMCs were instead injected with an equal amount of saline. All animals were monitored daily until sacrifice. All PBMC samples were injected in duplicate or triplicate (2-3 mice, per human PBMC sample).

2.5.1 K562-luc2 and PBMC preparation for injection

K562-luc2 cells were thawed from cryopreservation 48h before tumor injections and maintained at 37°C, 5% CO_2 in Iscove's DMEM supplemented with 10% FBS and 8μg/mL blasticidin for 2 days. On the day of tumor injections, K562-luc2 cells were removed from culture, washed three times with sterile PBS, and resuspended at a concentration of $1x10^6$ cells/200μL in sterile saline. Cryopreserved PBMCs were thawed in a 37°C water bath for two minutes or until a small ball of ice remained in solution. PBMCs were resuspended in 5mL RPMI + 10% FBS. A small aliquot (50 μL) was taken, and cells were labeled with monoclonal antibodies (CD8-VioBlue, CD14-VioGreen, CD3-FITC, CD4-PE, CD20-PerCP, CD45-APC, CD56-APC-Vio770 (Miltenyi Biotec) for surface staining prior to injection. The remaining cells were supplemented with IL-15 (0.1mg/mL) and incubated for 1 hour at 37°C, 5% CO_2 to improve activation and recovery of NK cells functions. After incubation, PBMCs were washed three times with PBS to remove all RPMI media and then resuspended at a concentration of $10x10^6$ PBMCs/200μL sterile filtered saline for injections.

2.5.2 PBMC engraftment

Human immune cell engraftment was tracked weekly. A sample of blood (50-100μL) was collected from each mouse using the submental method. 25μL of whole blood samples were stained and analyzed by flow cytometry using the following directly conjugated antibodies: CD8-VioBlue, CD14-VioGreen, CD3-FITC, CD4-PE, CD20-PerCP, CD45mouse-PE-Vio770, CD45human-APC, CD56-APC-Vio770 (Miltenyi Biotec). All antibodies, except CD45mouse, were reactive with human antigens. CD45mouse was used to exclude mouse leukocytes from the analysis.

2.5.3 BLI and morbidity score

All mice were imaged every 3-4 days to monitor tumor progression on the LargoX bioluminescent imager. Briefly, mice were injected intraperitoneally with D-luciferin, potassium salt reconstituted in Dulbecco's phosphate-buffered saline (15mg/ml) (Gold Biotechnologies, St. Louis, MO) at a concentration of 10μL/g body weight (BW). Bioluminescent images were gathered by 5-minute, 1 minute, 10 second, 5 second, or 1 second exposures. Bioluminescent data is expressed as photons/s.

On the same day of imaging, mice were weighed and scored for symptoms of xenogeneic GvHD. Scores were assessed based on the following symptoms of clinical GvHD: Skin Integrity (0-2): 0 = normal, healthy skin; 1 = Scaling of paws/tail; 2 = dehydrated, obvious areas of denuded skin; Fur Integrity (0-2): 0 = normal, fluffy, and elastic fur; 1 = mild to moderate ruffling; 2 = soiled, stiff, and rough fur; Posture (0-2): 0 = normal posture; 1 = hunching only at rest; 2 = severe hunching, sunken or distended abdomen; Activity (0-2): 0 = normal, responsive and vocal; 1 = mild to moderately decreased; 2 = unresponsive, separates from group, circling, head pressing; Weight Loss (0-2): 0 = <10%; 1 = 10% to 20%; 2 = >20%; and Diarrhea (0-1): 0 = no; 1 = yes.

Any multiple combinations of the clinical signs are indicative of a moribund condition and mice were euthanized accordingly. Because actual tumor volume cannot be determined with intravenous administration of K562, euthanasia occurred when any of the following ocurred: GvHD ≥ 8, ≥20% BW loss, hind limb paralysis and/or impaired ambulation.

2.6 Statistical analysis

Paired T-tests were used to detect significant differences in the total numbers of immune cell subtypes measured in blood before and after exercise. Linear mixed models with Bonferroni correction for multiple comparisons were used to detect main effects of time, group and interaction effects (time x group) for human leukocyte engraftment, GvHD, and leukemic burden (BLI). Simple survival analysis (Kaplan-Meier) was used to detect differences in overall survival, tumor-free survival, and GvHD-free survival. The percentage of starting mice that were alive and of low leukemic burden (defined as a BLI score below the 95% confidence interval of the corresponding control mice receiving only K562) were compared weekly between groups by chi-square. Significance was accepted at $p<0.05$. For DGE analysis by scRNAseq, fold changes in individual gene expression within each cluster was considered significant after adjusting for multiple hypothesis testing (Padj<0.05). For GSEA, the false discovery rate (FDR) was used separately for each database (GO and KEGG) to correct for multiple hypothesis testing. Given the exploratory nature of our analysis, we selected an FDR threshold of <0.25, which denotes the confidence of 'possible' or 'hypothesis', while an FDR < 0.05 denotes 'high confidence' or 'statistical significance' (13). We used the less stringent FDR for our GSEA analysis to avoid overlooking potentially meaningful changes in enriched gene sets in response to exercise.

3 Results

3.1 Exercise preferentially mobilizes effector lymphocytes with phenotypic and transcriptomic profiles associated with cytotoxicity, differentiation, migration and effector cytokine signaling

To enumerate circulating immune cells and determine their magnitude of change in response to exercise, we used an extensive

immunophenotyping panel (Table S1) with 8-color flow cytometry on whole blood samples collected before and during exercise. Total cell numbers are shown in Table 1, with fold changes in the absolute cell number from rest to during exercise shown in Figure 1. We observed the archetypal exercise-induced mobilization of lymphocytes with a phenotypic profile consistent with cytotoxicity and differentiation. Consistent with previous observations (6, 14), NK-cells, γδ T-cells and CD45RA+ effector memory (EMRA) CD4+ and CD8+ T-cells were among the most exercise responsive subsets (Figure 1A). In contrast, suppressor cells such as CD4+ regulatory T-cells (Treg) were not significantly mobilized with exercise. A novel observation reported here is that T-cells expressing PD-1, particularly within more differentiated subsets (e.g., EMRAs) were highly responsive to the exercise stimulus. PD-1 plays an important immunoregulatory role and its expression is upregulated in activated T-cells (15), although it is associated with exhaustion and decreased cytotoxicity in CD8+ T-cells (16). Within the NK-cell population, there was a greater mobilization of $CD56^{dim}$ cells, which are more cytotoxic, compared to the $CD56^{bright}$ subset, which has an immunoregulatory role. We also observed a preferential mobilization of NK-cells expressing the activating receptor NKG2D relative to NK-cells expressing the inhibitory receptor NKG2A. NKG2D is a receptor that increases NK-cell expansion, cytotoxic activity, and survival, contributing to a stronger antitumor response (17). NKG2A, on the other hand, inhibits NK cell function and has become a target for cancer immune checkpoint inhibition therapy (18). This indicates that NK-cells mobilized to blood during exercise may have enhanced cytotoxic properties. There was a trend for Vδ1 T-cells to be mobilized with exercise at a greater magnitude compared to the Vδ2 subset. Vδ2 are the most prevalent subset in the circulation and possess well-established anti-tumor effector function (19). Vδ1 are a minor subset of γδ T-cells with evidence of cytotoxic, immunosuppressive and regulatory roles (20).

To investigate the effects of exercise on the transcriptome within PBMCs, we performed single-cell RNA sequencing (scRNAseq) and identified 26 cell clusters in accordance with the Azimuth map for human PBMCs (Figure 1B) following the methods described by Hao and Hao et al. (21). DGE between rest and exercise-mobilized PBMCs were observed in 7 cell types (Figure 1D). A description of each DGE in accordance with GeneCards® is provided in Table S2.

TABLE 1 The total number (cells/μL) of Leukocyte subsets present in peripheral blood at rest and during exercise.

Leukocyte subsets	Rest	Exercise	P Value
Granulocytes	2681.61 ± 1010.11	4264.69 ± 1462.87	4.28E-06
Monocytes	332.37 ± 68.90	623.55 ± 175.88	4.58E-07
$CD14^{bright}CD16^{dim}$	12.56 ± 8.34	18.32 ± 17.08	0.05
$CD14^{bright}$CD16-	256.27 ± 104.02	445.38 ± 210.52	1.90E-05
$CD14^{dim}CD16^{bright}$	222.29 ± 85.85	501.67 ± 358.02	4.03E-03
Lymphocytes	1879.03 ± 282.27	3795.99 ± 1032.27	1.26E-06
B cells	146.24 ± 52.45	253.75 ± 90.62	4.93E-08
NK Cells	383.18 ± 156.75	1276.99 ± 475.70	3.26E-08
$CD56^{dim}$ NK cells	383.83 ± 163.21	1292.98 ± 493.63	7.34E-07
$CD56^{bright}$ NK cells	18.97 ± 10.31	38.55 ± 22.55	8.59E-04
NKG2D+NKG2A-	241.85 ± 124.25	866.22 ± 363.44	1.74E-06
NKG2C+2A-57+	23.92 ± 53.55	73.58 ± 154.10	0.09
NKG2C+2A-	60.07 ± 78.58	181.46 ± 217.21	0.01
NKG2D+2C+2A-	55.20 ± 76.69	165.85 ± 215.04	0.01
NKG2D+NKG2A+	127.46 ± 110.79	379.05 ± 350.37	1.54E-03
NKG2D+2A-57+	54.22 ± 65.95	205.53 ± 221.10	3.83E-03
NKG2D+2C+	68.82 ± 80.44	198.39 ± 224.69	4.35E-03
NKG2D-NKG2A+	6.86 ± 6.45	15.02 ± 12.23	0.01
NKG2D+2C+2A-57+	21.92 ± 52.39	66.14 ± 147.87	0.11
NKG2D-NKG2A-	26.36 ± 17.66	59.11 ± 35.63	1.55E-04
CD3 T Cells	1237.02 ± 247.17	2014.60 ± 712.14	8.93E-06
CD4+ T Cells	872.17 ± 736.74	939.69 ± 1044.46	1.48E-04

(Continued)

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TABLE 1 Continued

Leukocyte subsets	Rest	Exercise	P Value
Naive CD4+	399.45 ± 174.48	539.83 ± 284.52	2.03E-03
CM CD4+	224.28 ± 118.18	305.62 ± 179.20	0.02
EM CD4+	87.49 ± 62.46	123.45 ± 122.32	0.15
EMRA CD4+	33.36 ± 26.80	75.54 ± 89.82	0.06
Naive T CD4+PD1+	304.47 ± 156.05	412.60 ± 255.23	0.01
CM T CD4+PD1+	167.76 ± 66.60	235.79 ± 145.97	0.02
EM T CD4+PD1+	72.65 ± 49.18	109.68 ± 122.17	0.12
EMRA T CD4+PD1+	22.24 ± 16.47	47.36 ± 43.81	0.02
CD8+ T Cells	393.07 ± 88.21	756.73 ± 331.33	2.05E-05
Naive CD8+	82.41 ± 43.04	128.24 ± 86.64	0.02
CM CD8+	47.99 ± 27.01	86.82 ± 96.71	0.06
EM CD8+	137.64 ± 63.12	302.32 ± 246.15	4.96E-03
EMRA CD8+	128.61 ± 91.00	259.46 ± 185.51	4.74E-04
Naive T CD8+PD1+	47.29 ± 49.24	77.30 ± 88.14	0.07
CM T CD8+PD1+	27.82 ± 16.14	47.88 ± 46.93	0.05
EM T CD8+PD1+	75.95 ± 40.51	162.48 ± 124.07	3.35E-03
EMRA T CD8+PD1+	61.93 ± 72.20	123.65 ± 159.67	0.03
CD4-CD8- T Cells	179.86 ± 94.54	440.26 ± 189.65	6.71E-06
Naive CD4-CD8-	11.92 ± 13.19	13.72 ± 18.59	0.70
CM CD4-CD8-	26.82 ± 27.78	55.63 ± 68.68	0.03
EM CD4-CD8-	38.23 ± 42.97	81.39 ± 95.66	0.01
EMRA CD4-CD8-	24.63 ± 32.57	54.57 ± 68.92	0.01
Naive CD4-CD8-PD1+	9.82 ± 13.58	10.97 ± 19.21	0.79
CM CD4-CD8-PD1+	17.48 ± 21.44	34.24 ± 48.21	0.04
EM CD4-CD8-PD1+	15.02 ± 18.96	28.18 ± 36.32	0.02
EMRA CD4-CD8-PD1+	9.52 ± 16.48	21.54 ± 41.90	0.10
Vδ1+ γδ T Cells	20.96 ± 20.38	52.99 ± 55.25	0.01
Vδ2+ γδ T Cells	91.74 ± 75.79	183.59 ± 157.15	1.03E-03
CD45+CD3+iNKT Cells	15.99 ± 14.21	120.99 ± 384.92	0.30
CD4+CD25^{hi}CD127^{dim} Treg	28.10 ± 23.53	33.72 ± 33.98	0.22

P values are displayed in the right column. Significance P ≤ 0.05.

We found a greater number of DGEs in response to exercise within the CD8+ T-cell subset. Genes associated with increased cytotoxicity (GZMB, GAMH, PRF1- (22)), IFN-γ production (ANXA1- (23)), cellular adhesion and migration (ITGB1- (24), ITGB2, CCL5- (25)) and anti-tumor activity (HLA-DPA1, NKG7- (26)) were enriched, while genes encoding proteins that suppress immune function and differentiation (MALAT1- (27), TXNIP - (28), FOXP1- (29)) were downregulated. The majority of these DGEs occurred within the EM subset of CD8+ T-cells. These results re-enforce the concept that exercise preferentially mobilizes effector lymphocytes with phenotypic and transcriptomic profiles associated with cytotoxicity, differentiation, migration, and effector cytokine signaling.

3.2 scRNAseq reveals enrichment of gene sets associated with anti-tumor activity in exercise mobilized lymphocytes

To identify biological processes associated with our DGE data, we performed functional annotation and enrichment analysis using both GO and KEGG terms (Figure 1E). The

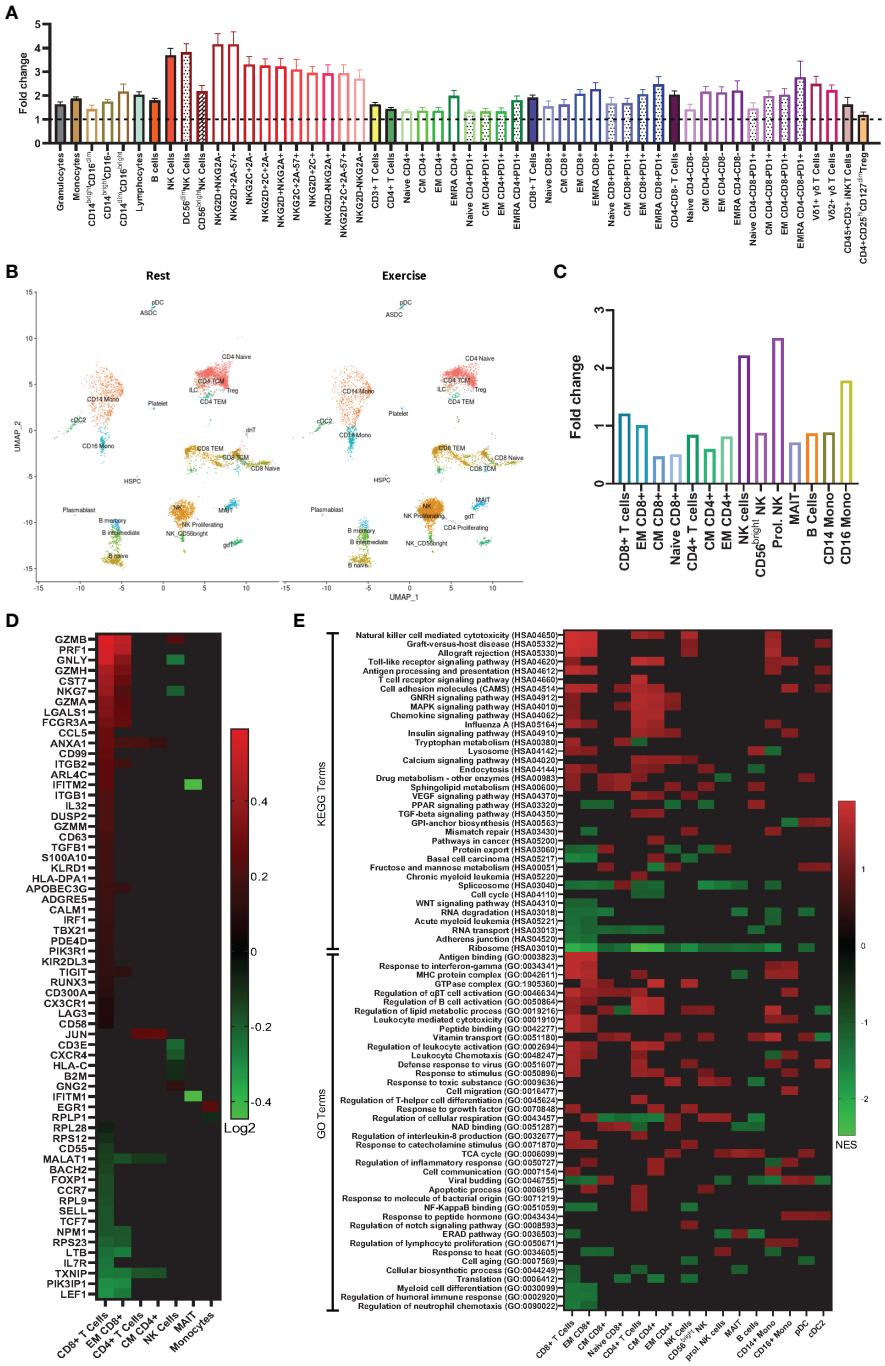

FIGURE 1
Exercise preferentially mobilizes effector lymphocytes with phenotypic and gene expression profiles associated with anti-tumor immunity. **(A)** Fold changes in absolute number of immune cell subsets in human whole blood from rest to during exercise, using the values presented in Table 1 (exercise/rest) (n=16). **(B)** Azimuth map for human PBMCs showing the 26 clusters identified by scRNAseq at rest and during exercise. (n=3) **(C)** Fold changes in proportion of the single cells analyzed from rest to during exercise (exercise/rest)(n=3). **(D)** Heat map showing differentially expressed genes (DEG) from rest to during exercise in 7 immune cell subsets (n=3). **(E)** Gene sets annotated to Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms that were enriched after exercise (n=3). Statistical significance: $p \leq 0.05$ for DEG; and FDR ≤ 0.25 for the gene set enrichment analysis.

greatest number of gene set enrichments were found in CD8+ T-cells, with an upregulation of gene sets associated with cytotoxicity, cellular adhesion and migration, cellular proliferation, and anti-tumor activity. These were mostly driven by the EM population, with a smaller contribution of central memory (CM) and naïve CD8+ T-cells. We observed an upregulation of gene sets involved in GvHD and allograft rejection within CD8+ T-cells, NK-cells, monocytes, and dendritic cells. This was driven mostly by HLA and KIR genes (Figures 2A, B), which are also involved in GvL effects (30). Gene

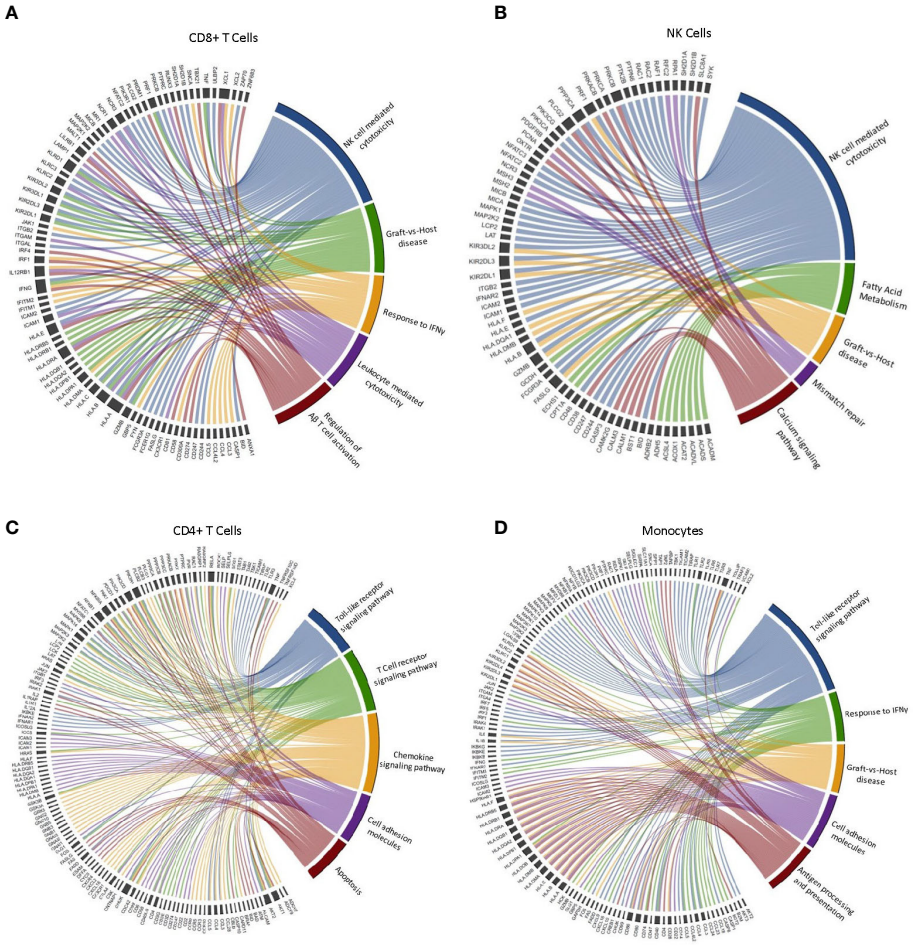

FIGURE 2
Leading-edge genes driving the enrichment of selected GO and KEGG terms in CD4+ T-cells, CD8+ T-cells, NK-cells and monocytes after exercise. Chord diagram displaying the leading-edge genes driving the enrichment of terms associated with cytotoxicity and anti-tumor immune activity in **(A)** CD8+ T cells, **(B)** NK cells, **(C)** CD4+ T cells, and **(D)** Monocytes. (n=3).

sets involved in MAPK signaling were enriched in total and naïve CD8+ T-cells, as well as total, CM, and EM CD4+ T-cells. MAPK pathways play an important role in the regulation of T-cell proliferation and differentiation, although, the signaling cascade might differ between naïve and antigen-experienced T-cells (31). Gene sets involved in calcium and insulin signaling were enriched in CD4+ T-cells and CD16+ monocytes, while the sphingolipid metabolism gene set was enriched in most lymphocytes and downregulated in plasmacytoid dendritic cells (pDCs). Sphingolipids are a major component of cellular membranes, and their metabolism is associated with T-cell viability and function (32). Interestingly, gene sets involved in protein synthesis (e.g., 'ribosome' and 'translation') were downregulated in almost all cell types in response to exercise (Figure 2). Gene sets included in the terms 'defense response to virus' and 'antigen processing and presentation' were enriched in some lymphocytes (CD8+, CD4+, and NK-Cells) and antigen presenting cells (APCs; CD14+ monocytes, and cDC2s). Gene sets associated with cellular respiration were enriched in EM CD4+ and CD8+ T-cells, and NK-cells, but downregulated in naïve and CM T-cells, which might be explained by the higher ATP demand of effector and cytotoxic cells (33). Similarly, Gene sets associated with tricarboxylic acid (TCA) cycle were enriched on CM CD4+ T-cells, proliferating NK-cells, MAIT, B-cells, and pDCs.

We selected five GO/KEGG terms associated with antitumor immune activity to display the leading-edge genes driving the enrichment within each major cell type (Figure 2). Interestingly, we found variability in the leading-edge genes enriching the selected terms across all cell types, with CD8+ T-cells presenting a higher number of the KIR and HLA family genes enriching NK-cell mediated cytotoxicity, when compared to NK-cells. However, there is a high level of overlap in gene sets enriching the selected terms within the same cell type. For example, GvHD and NK-cell mediated cytotoxicity are mostly driven by the same genes. Similarities can also be seen in the leading-edge genes driving enrichment of the 'response to IFN-γ' and 'Toll-like receptor signaling' terms. Overall, our results indicate that PBMCs mobilized to blood during exercise are more alloreactive and cytotoxic and should, therefore, have greater potential to elicit GvL effects if used as a DLI.

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3.3 Exercise-mobilized PBMCs extend survival and reduce leukemic burden in xenogeneic mice

We found that PBMCs mobilized to blood during exercise have surface phenotypes and transcriptomes associated with anti-tumor immunity, including cytotoxicity, migration/chemotaxis, antigen binding, cytokine responsiveness and alloreactivity (e.g. GvHD/GvL). Our next step was to determine if DLI comprised of exercise-mobilized PBMCs would evoke greater GvL effects *in vivo* without exacerbating xenogeneic GvHD. We used the highly immunodeficient NSG mouse strain coupled with a human IL-15 knock-in to support persistence of adoptively transferred NK-cells as well as T-cells. Mice received resting (PBMC-R) or exercise-mobilized (PBMC-E) PBMCs on day -1, while a set of mice were injected with vehicle (saline). Mice were then injected with a luciferase-tagged human chronic myeloid leukemia (CML) cell line (K562-luc2) on day 1. To track GvHD in the absence of tumor, sets of mice were injected with PBMC-R or PBMC-E only (Figure 3A). All mice received an equal number of $1x10^7$ PBMCs, however, as expected, exercise altered the composition of cells in the xenograft. Mice injected with exercise-mobilized PBMCs received

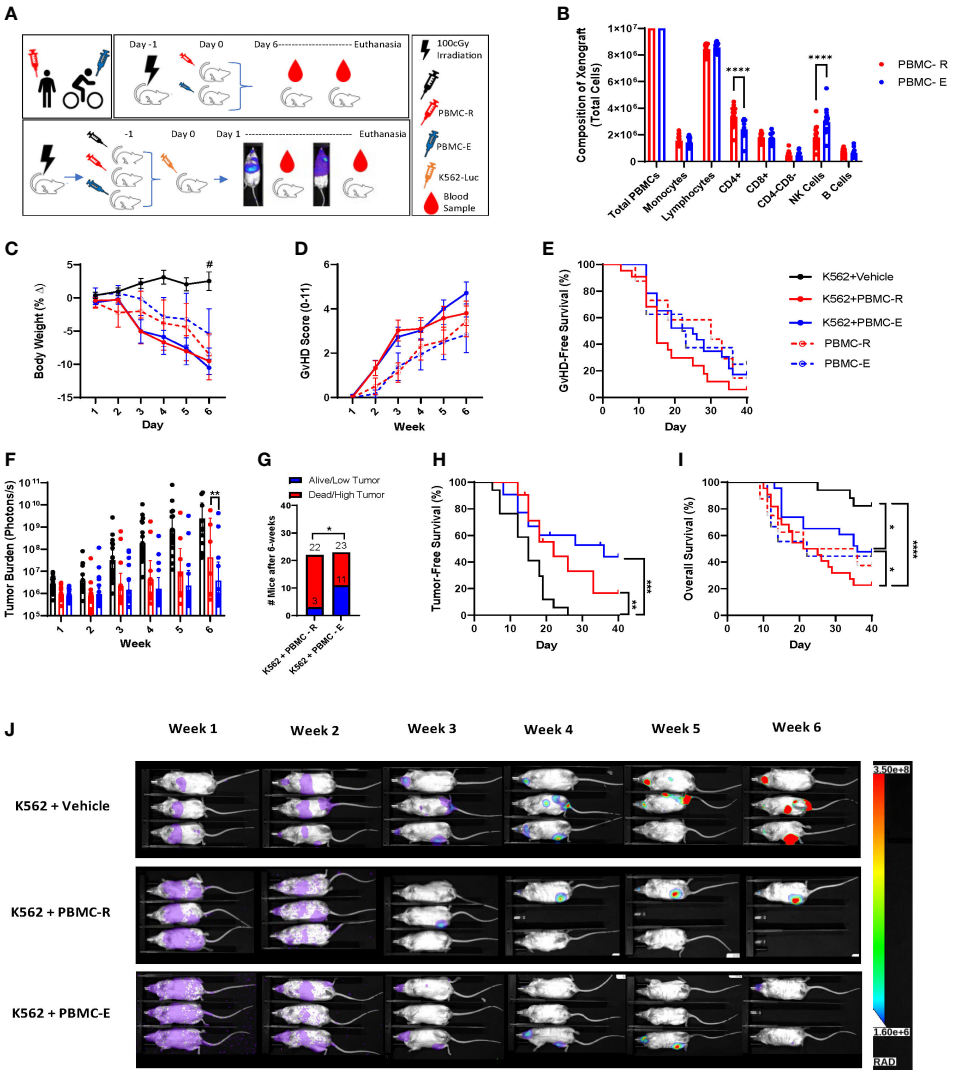

FIGURE 3
Exercise-mobilized PBMCs extend survival and reduce leukemic burden in xenogeneic mice. To determine the GvL effect of the PBMCs mobilized by exercise, NSG-IL15 mice were injected with PBMCs ($10x10^6$) collected either at rest (PBMC-R) or during (PBMC-E) exercise and challenged with $1x10^6$ luciferase tagged human chronic myeloid leukemia cells (K562-luc). **(A)** Illustration of the experiment design. **(B)** composition of the PBMC-R and PBMC-E xenografts. **(C)** body weight and **(D)** GvHD score measured twice weekly. **(E)** GvHD-free survival determined using a composite score of ≥4. **(F)** tumor burden measured twice weekly *via* BLI quantification (photons/s). **(G)** low tumor burden was defined as a BLI score <95% confidence interval of K562+vehicle at the corresponding time point. **(H)** Tumor-free survival. Mice were considered tumor-free until the BLI score surpassed the individual value recorded on day 1. **(I)** Overall survival of leukemia-bearing mice that received vehicle (K562+vehicle, solid black line), resting PBMCs (K562+PBMC-R, solid red line) or exercise PBMCs (K562+PBMC-E, solid blue line); and in non-tumor bearing mice that received resting (PBMC-R, dashed red line) or exercise (PBMC-E, dashed blue line) PBMCs. **(J)** representative bioluminescence image of leukaemia-bearing mice that received vehicle (top row), resting PBMCs (middle row), or exercise PBMC (bottom row), showking BLI intensity on a scale from low (purple) to high (red). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, #Different from K562+Vehicle P ≤ 0.05. (n=20-23 mice/group).

significantly less CD4+ T-cells and more NK-cells (Figure 3B). To track human cell engraftment, murine blood was collected weekly and assessed for the presence of major human lymphocyte populations by flow cytometry (Table S1). No major difference in human immune cell engraftment was found between the groups (Figure 4). Mice achieved ~50% human PBMC engraftment by week 2 with no differences between the groups (Figure 4A). Similarly, engraftment of lymphocyte subtypes did not differ significantly between mice that received resting or exercise-mobilized PBMCs. However, at week 1, NK-cell frequencies trended higher in the K562+PBMC-E group compared to mice that received vehicle+PBMC-E. There was also significantly more CD4+ T-cells at week 1, and CD3+/CD4-/CD8- 'double-negative' (DN) T-cells at week 2 when comparing these groups, indicating a potential interaction between K562 stimulation and the expansion/engraftment of exercise-mobilized PBMCs *in vivo*.

All mice that received PBMCs had a greater reduction in BW compared to mice receiving K562+vehicle due to xenogeneic GvHD (Figure 3C); however, no difference in GvHD score or GvHD-free survival (using a composite score of ≥4) was observed between groups (Figures 3D, E). As expected, mice that did not receive PBMCs and were injected only with K562+vehicle had the highest tumor burden (Figure 3F), demonstrating that the adoptively transferred PBMCs helped limit leukemic progression *in vivo*. By week 3, mice injected with exercise-mobilized PBMCs started to display lower leukemic burden compared to the group injected with resting PBMCs, which reached statistical significance at week 6 (Figures 3F, J). Also, 11 of 23 mice in the K562+PBMC-E group were alive and/or had low tumor (defined as a BLI score <95% confidence interval of K562+vehicle at the corresponding time point) at week 6, compared to only 3 of 22 mice in the K562+PBMC-R group, (Figure 3G). which was reflected by improved, but not significant, tumor-free survival (Figure 3H) and significantly higher overall survival (Figures 3I, J). Finally, we used the standard NSG mouse model, which is known to have slower GvHD manifestation kinetics, to see if there were differences in human cell engraftment and xenogeneic GvHD between resting and exercise-mobilized PBMCs in a less severe model. Similar to the NSG-Tg (Hu-IL15) model, we found no differences in overall survival, GvHD score, GvHD survival, body weight, or engraftment with human immune cells (Supplemental Figure 1). Our results confirmed our hypothesis that DLI collected during exercise would enhance the GvL effect without intensifying GvHD as we observed a slower rate of leukemic progression, improved overall survival, and a trend toward improved GvHD-free survival in the mice that received exercise mobilized PBMCs.

4 Discussion

Every bout of dynamic exercise evokes the mobilization and redistribution of effector lymphocytes, which may play a role in improving cancer immune surveillance in habitual exercisers. This is the first study, to our knowledge, to provide a comprehensive single cell transcriptomic analysis of human mononuclear cells mobilized to blood with exercise, and the first to assess anti-leukemic effects of these mobilized cells in a xenogeneic mouse model of human leukemia. We found that effector lymphocytes mobilized with exercise, particularly effector-memory CD8+ T-cells and NK-cells, have differentially expressed genes and enriched gene sets associated with anti-tumor activity such as cytotoxicity, migration/chemotaxis, antigen binding, cytokine responsiveness and alloreactivity (e.g. graft-versus-host/leukemia). Additionally,

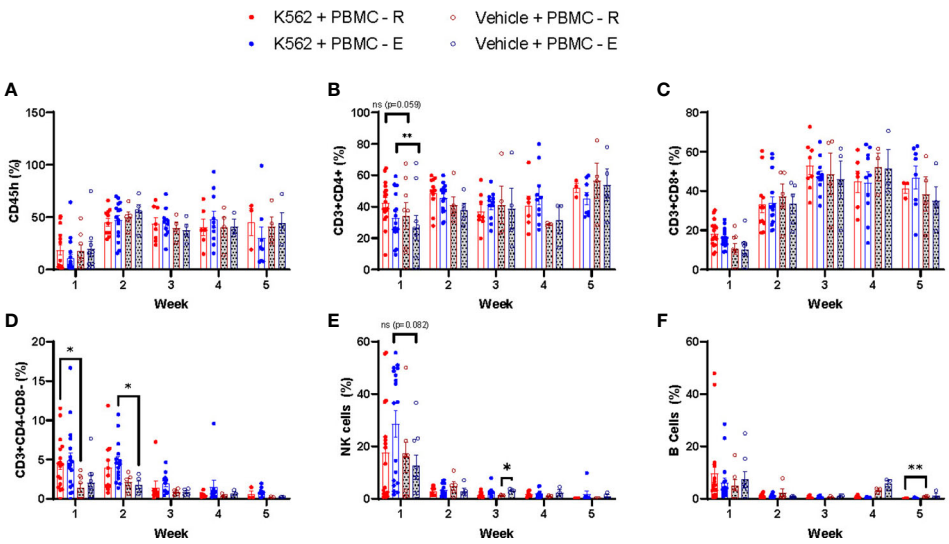

FIGURE 4
Human leukemia expands effector lymphocytes in xenogeneic mice receiving exercise-mobilized but not resting PBMCs. To track the human cells engraftment, mice blood was collected weekly, being the first samples collected 7 days after the PBMC injections, and whole blood was labeled with monoclonal antibodies for the analysis of the following cell types: **(A)** CD45h, **(B)** CD4+ T cells, **(C)** CD8+ T cells, **(D)** DN T cells **(E)** NK cells, and **(F)** B cells in leukemia-bearing mice that received resting PBMCs (K562+PBMC-R, solid red dot) or exercise PBMCs (K562+PBMC-E, solid blue dot); and in non-tumor bearing mice that received resting (PBMC-R, open red dot) or exercise (PBMC-E, open blue dot) PBMCs. *P ≤ 0.05, **P ≤ 0.01. (n=11-23 mice/group).

when used as a donor lymphocyte infusion (DLI), exercise-mobilized cells extended survival and reduced K562 leukemic burden in xenogeneic mice without exacerbating GvHD.

Single exercise bouts are known to preferentially mobilize effector subsets of lymphocytes and monocytes to the blood compartment. NK-cells, γδ T-cells, and CD8+ T-cells are among the most exercise-responsive lymphocytes, while CD16+ 'non-classical' monocytes are mobilized in relatively greater numbers than their 'classical' counterparts (14). We performed extended flow cytometry to enumerate multiple subsets of human lymphocytes and monocytes before and after exercise. Confirming previous findings (1), we saw a step-wise preferential mobilization of CD8 + T-cells in accordance with differentiation status and that EMRA subsets of CD4+ T-cells were preferentially mobilized over their less differentiated CD4+ counterparts. We also noted that subsets of CD8+ T-cells expressing the immune checkpoint and marker of T-cell exhaustion, PD-1 (34), are highly responsive to exercise. This may have important implications for studies attempting to combine exercise with immune checkpoint inhibitors to enhance the therapeutic response (35). Suppressor cells such as CD4+ T-regs are not significantly mobilized with exercise, thus increasing the ratio of effector to suppressor cells among lymphocytes in response to exercise, which is considered beneficial for adoptively transferred cell therapies (3).

We have provided the most comprehensive single cell gene expression profile of exercise-mobilized lymphocytes and monocytes in humans to date. Clustering cells by their transcriptomic signature in accordance with the Azimuth PBMC reference atlas, and using a targeted gene expression panel curated for human immunology, allowed us to identify over 9000 genes and 64 differentially expressed genes in 26 discrete subsets of lymphocytes and monocytes (21). Consistent with the premise that exercise preferentially mobilizes lymphocytes with enhanced cytotoxic potential, we found an upregulation of genes coding for cytolytic granules (e.g. granzyme A, B, H and M; perforin, NKG7) within CD8+ T-cells, an effect that was largely driven by gene expression changes within the CD8+ EM subset. Genes coding for proteins involved in the regulation of T-cell and NK-cell cytotoxicity (e.g. CST7, FCGR3A) (36) were also enriched within CD8+ EM cells, as were genes coding for proteins involved in chemotaxis and migration (e.g. CCL5, CD99) (25), as well as activation and apoptosis (e.g. ANXA1, LGALS1) (23). Interestingly, despite NK-cells being the most 'exercise-responsive' lymphocyte subset, fewer gene expression changes were seen in NK-cells compared to CD8+ T-cells. This could be due to greater heterogeneity among the CD8+ T-cell compartment and the propensity for exercise to mobilize only the most differentiated and cytotoxic CD8+ subsets. We also found that expression levels of certain genes changed divergently with exercise dependent on cell type. For instance, NKG7 gene expression was enriched with exercise in CD8+ T-cells but downregulated in NK-cells. This could have implications for cancer patients on immune checkpoint inhibitors, as low NKG7 expression in CD8+ T-cells has been shown to predict poor responders to anti PD-1 therapy (37). Furthermore, NKG7 expression in CD8+ T-cells regulates the translocation of CD107a to the membrane enhancing their ability to kill malignant cells (26). It is possible that exercise may increase the cytotoxic effects of CD8 + T-cells directly through NKG7 transcription and the concomitant upregulation of cytolytic granules and calcium release, but this remains to be determined. Conversely, genes involved in the development of suppressor functions (e.g. TCF1, LEF1) (38), glucose metabolism, and inhibition and cell quiescence (e.g. TXNIP, FOXP1) (32,43) were downregulated, indicating that exercise-mobilized CD8+ T-cells have increased metabolic activity and are more permissive upon activation.

Functional annotation and enrichment analyses revealed an upregulation of gene sets associated with anti-tumor immunity, especially within subsets of CD8+ T-cells and to a lesser extent CD4 + T-cells. These included GO and KEGG terms related to antigen binding and processing, cytotoxicity, alloreactivity (e.g. GvHD), adhesion/migration, cytokine/chemokine responsiveness and toll-like receptor signaling. Similar terms were enriched among the APCs (e.g. monocyte and dendritic cell subsets). Interestingly, alloreactivity/GvHD was enriched in multiple cell types after exercise including total CD8+ T-cells, EM CD8+ T-cells, NK-cells, classical monocytes and cDC2 cells. While these findings indicate that exercise mobilized lymphocytes and APCs are more alloreactive and could increase risk of GvHD in the allogeneic adoptive cell therapy setting, it is important to note that the GvL effects of these cells is also likely to be greater (39, 40). We also found a large number of gene sets enriched within exercise responsive monocytes. Antigen presentation/processing, cytotoxicity, cytokine responsiveness, fatty acid metabolism, vitamin transport and defense response to virus were enriched in 'classical' monocytes after exercise. The preferentially mobilized 'non-classical' monocytes had an upregulation of gene sets involved in T-cell activation and proliferation, migration/chemotaxis and responsiveness to cytokines, carbohydrates, insulin and hormones. Changes within pDCs were largely related to macronutrient metabolism. Overall, this detailed single cell transcriptomic analysis has revealed exercise mobilized monocytes and lymphocytes (particularly CD8+ T-cells) to have increased gene expression profiles associated with enhanced cytotoxic, migration, activation, cytokine responsiveness, and alloreactive functions. This indicates that exercise-mobilized lymphocytes would be highly effective as an allogeneic cell therapeutic product (e.g. DLI) for the treatment of hematologic malignancies, providing that exacerbation of GvHD is not a limiting factor.

To test whether exercise mobilized lymphocytes have the potential to improve DLI anti-leukemic effects, we engrafted xenogeneic mice with PBMCs collected before and during exercise prior to challenging the mice with K562 cells. By the end of the experiment, more mice that received exercise-mobilized cells (~48%) were alive and had lower tumor burden than mice that received resting cells (~14%) from the same donors. Encouragingly, GvHD effects were not different between NSG-Tg (Hu-IL15) or standard NSG mice receiving resting or exercise-mobilized lymphocytes in the absence of tumor. It is possible that the altered composition of the xenografts evoked by exercise are responsible for the enhanced GvL effects without concomitant increases in GvHD. As expected, exercise lowered the total number of CD4+ T-cells and increased the number of NK-cells

without affecting the total number of other cell types in the graft (1). CD4+ T-cells are known to be highly alloreactive and can cause severe GvHD (41), whereas NK-cells are minimally alloreactive and can exert strong GvL effects (42), particularly against the K562 cell line which is HLA-deficient and a known NK-cell sensitive target (43). Furthermore, increased NK-cell cytotoxicity after exercise is associated with an elevated ratio of activating to inhibitory receptor expression particularly if the target cells express ligands for NK-cell activating receptors (44). The increased GvL effects seen in xenogeneic mice receiving exercise-mobilized DLI is most likely driven by the greater numbers of NK-cells in the graft, as we have shown previously that exercise-mobilized lymphocytes have similar cytotoxic activity against K562 cells *in vitro* after adjusting for NK-cell numbers (44). It is possible, however, that some of our observed transcriptomic changes could contribute to increased cytotoxicity of exercise-mobilized NK-cells *in vivo*, but this requires further investigation.

The human IL-15 knock in strain of the highly immunodeficient NSG mouse was used here to support persistence of the exercise-mobilized NK-cells after adoptive transfer (45). This is particularly important as NK-cell infiltration has been purported to play a mechanistic role in reducing B16 melanoma burden in mice exposed to voluntary wheel running (8), while IL-15 responsive CD8+ T-cells were recently shown to suppress tumor growth in a mouse exercise model of pancreatic ductal adenocarcinoma (46). Our data indicate that NK-cells are also involved in the anti-leukemic effects of exercise, as K562 challenge tended to increase the numbers of human NK-cells and significantly increased 'double negative' (DN) T-cells (which largely consist of γδ T-cells and MAIT cells) in mouse peripheral blood within the first 1-2 weeks, but only if they received exercise mobilized lymphocytes. Although we did not observe differences in IL15R gene expression between resting and exercise mobilized NK-cells, the preferential mobilization of NKG2D+ NK-cells by exercise may have provided a greater number of IL-15 responsive NK-cells in the exercise mobilized grafts, as NK-cells bearing this receptor are more responsive to IL-15 stimulation (47). We have previously shown that exercise mobilized T-cells and γδ T-cells proliferate better *ex vivo* in response to tumor antigen (e.g. WT1, PRAME) peptide loaded dendritic cells and phosphate antigens, respectively (48, 49). Moreover, γδ T-cells expanded after exercise have heightened expression of several activating receptors involved in anti-tumor immunity and are superior killers of multiple hematologic cancer cell lines *in vitro* (49). This indicates that exercise mobilized NK-cells and DN T-cells are proliferating/persisting more readily in response to K562 challenge compared to their counterparts in resting blood, although future studies are required to determine if they play a mechanistic role in reducing leukemic burden after adoptive transfer. It will also be important to determine if exercise-mobilized lymphocytes can curtail growth of pre-established leukemias and to evaluate the response across different hematologic malignancies. Finally, future studies should determine if the gene expression changes and/or graft composition shifts reported herein play a mechanistic role in enhancing the GvL effects of exercise-mobilized lymphocytes.

We conclude that exercise in humans mobilizes effector lymphocytes with an anti-tumor transcriptomic profile and that their use as a DLI extends survival and enhances the GvL effect without exacerbating GvHD in human leukemia bearing xenogeneic mice. These findings provide mechanistic insight into how human lymphocyte and monocyte redistribution with exercise might contribute to the elimination of premalignant and malignant cells in the host, whilst highlighting the potential for exercise-mobilized lymphocytes to be used as an allogeneic cell therapy to effectively and economically increase the GvL effects without exacerbating GvHD.

Data availability statement

The data presented in the study are deposited in the GEO repository, accession number GSE212740. Data can also be found at the link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE212740.

Ethics statement

The studies involving human participants were reviewed and approved by Human Subjects Protection Program at the University of Arizona (#1801161041). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by University of Arizona IACUC (protocol 17-338).

Author contributions

Conception or design of the work – DD, EK, and RS. Data collection – HB, DD, GN, FB, KS, TZ, PM, and MS. Data analysis and interpretation.– HB, DD, FB, BL, EK, and RS. Drafting the article – HB, RS, and EK. Critical revision of the article - HB, DD, GN, FB, KS, TZ, PM, MS, BL, EL, MG, EK, and RS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1067369/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Exercise-mobilized PBMCs have no effect on survival, GvHD or human immune cell engraftment in non-tumor bearing standard NSG mice. To determine the GvHD effect of the PBMCs mobilized by exercise in a less severe model, standard NSG mice were injected with PBMCs ($10x10^6$) collected either at rest (PBMC-R) or during (PBMC-E) exercise. **(A)** Illustration of the experimental design. **(B)** Overall survival. **(C)** GvHD score measured twice weekly. **(D)** GvHD-free survival determined using a composite score of ≥4. **(E)** body weight. To track the human immune cell engraftment, mouse blood was collected weekly starting at Day+7 and whole blood was labeled with monoclonal antibodies to enumerate the following human cell types: **(F)** CD45h, **(G)** CD4+ T cells, **(H)** CD8+ T cells, **(I)** DN T cells **(J)** NK cells, and (K) B cells. There were no significant differences between PBMC-R and PBMC-E ($p>0.05$). (n=11 mice/group).

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Characterization of transitional memory CD4+ and CD8+ T-cell mobilization during and after an acute bout of exercise

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T-cell subsets, including naïve (NA), central memory (CM), transitional memory (TM), effector memory (EM), and RA + effector memory (EMRA), differ in phenotype and function. T-cells are mobilized by exercise, with differences in the magnitude of mobilization between subsets. However, the response of TM T-cells to exercise has not yet been described. Further, T-cells expressing the late differentiation marker CD57 are known to be highly responsive to exercise, but the relative response of CD57 + and CD57- within T-cell subsets is unknown. We therefore aimed to characterize the exercise-induced mobilization of TM T-cells, as well as to compare the exercise response of CD57 + and CD57- cells within T-cell subsets.

Methods: Seventeen participants (7 female; aged 18–40 years) cycled 30 min at 80% of their estimated maximum heart rate. Venous blood obtained pre, post, and 1H post-exercise was analyzed by flow cytometry. CD45RA, CCR7, and CD28 expression within CD4 + and CD8+ T-cells identified NA, CM, TM, EM, and EMRA subsets. CD57 expression within EM, EMRA, and CD28+ T-cells was also quantified. The relative mobilization of each subset was compared by calculating fold change in cell concentration during (ingress, post/pre) and after exercise (egress,1H post/post). Cytomegalovirus (CMV) serostatus was determined by ELISA and was considered in models.

Results: TM CD8+ T-cell concentration was greater post-exercise than pre-exercise (138.59 ± 56.42 cells/μl vs. 98.51 ± 39.68 cells/μl, $p < 0.05$), and the proportion of CD8 + with a TM phenotype was elevated 1H post-exercise (1H: 32.44 ± 10.38% vs. Pre: 30.15 ± 8.77%, $p < 0.05$). The relative mobilization during and after exercise of TM T-cells did not differ from NA and CM but was less than EM and EMRA subsets. Similar results were observed within CD4+ T-cells. CD57 + subsets of CD28+ T-cells and of EM and EMRA CD8+ T-cells exhibited a greater relative mobilization than CD57- subsets (all $p < 0.05$).

Conclusion: These results indicate TM CD4 + and CD8+ T-cells are transiently mobilized into the blood with exercise, but not to as great of an extent as later differentiated EM and EMRA T-cells. Results also indicate CD57 identifies highly exercise responsive cells within CD8+ T-cell subsets.

KEYWORDS

lymphocytes, physical activity, leukocyte differentiation, exercise response, immunity

1. Introduction

T-cell subsets differ in phenotype and function and can be identified by the combination of various proteins found on the cell surface. Mature naïve (NA) T-cells express CD45RA, CCR7, and CD28. Naive cells emerge from the thymus and become effector cells upon cognate antigen exposure; a portion of these cells then become subsets of memory T-cells (1). CD45RA-CCR7 + CD28 + central memory (CM) T-cells are earlier differentiated memory cells with longer telomeres than CD45RA-CCR7-CD28- effector memory (EM) T-cells; EM are later differentiated memory cells and have more immediate cytotoxic capacity (1, 2). Transitional memory (TM) T-cells, defined as CD45RA-CCR7-CD28+, have an intermediate differentiation status between CM and EM, with intermediate telomere length, proliferative potential, and cytotoxic function (3–5). Terminal effector memory cells that re-express the naïve marker (EMRA; defined as CD45RA + CCR7-CD28-) T-cells have the shortest telomere length of these T-cell subsets and exhibit low proliferation and low functional capacity (3, 5, 6). Many EMRA and EM T-cells also express the surface protein CD57, a marker associated with the lowest proliferative ability and increased susceptibility to apoptosis (4, 6). These CD28-CD57 + T-cells are frequently characterized as senescent, a designation suggesting DNA damage and the production of inflammatory proteins (7). Interestingly, CD28+ T-cells may also express CD57; these cells have increased effector functionality and proliferation ability compared to CD28-CD57+ (7).

Acute vigorous endurance exercise preferentially mobilizes later differentiated T-cells into peripheral blood (8–14). For example, using CD45RA in combination with CD27, CD62l, or CCR7, Campbell et al. (10) demonstrated a greater relative increase in EM and EMRA CD8+ T-cells in peripheral blood following 20 min of cycling exercise compared to NA and CM CD8+ T-cells. Similarly, Turner et al. (12) demonstrated a greater increase in late differentiated CD27-CD28- CD8+ T-cells following 60 min of vigorous running exercise than less differentiated cells. CD28-CD57+ T-cells are also significantly mobilized by exercise (13). This selective mobilization of later differentiated T-cells is due to the greater expression of type 2 beta-adrenergic receptors by highly differentiated T-cells, as epinephrine binding to these receptors promotes cell extravasation into the bloodstream (11, 15). After exercise ends, T-cells quickly egress from peripheral blood. Similar to the ingress during exercise, highly differentiated cells typically display a relatively larger egress out of peripheral blood than earlier differentiated cells (9–12, 14). The preferential mobilization of later differentiated cells means that individuals with a greater number of later differentiated cells display a larger cell mobilization response to exercise. This includes people infected with cytomegalovirus (CMV), a β-herpesvirus that leads to the accumulation of later differentiated T-cells (16).

Although the T-cell response to acute dynamic exercise has been fairly well characterized, there are still gaps in our knowledge. While TM T-cells are prevalent in healthy human subjects and are functionally distinct from CM and EM (3), their response to exercise has not been reported. Given the enhanced effector properties of TM relative to CM, the enhanced proliferative capacity of TM relative to EM, and the fact that TM T-cells comprise a substantial portion of the T-cells present in the blood (5), describing the mobilization of these cells by acute exercise is important to fully understanding the changes in T-cells that occur with exercise Further, while CD28-CD57+ T-cells are known to be mobilized by exercise (13), their relative mobilization compared to later differentiated subsets (EM, EMRA) lacking CD57 is not yet known. Finally, to our knowledge, there are no reports of the mobilization of CD28 + CD57+ T-cells with exercise. Given the enhanced functional and proliferative properties of these cells relative to CD28-CD57 + cells, understanding their degree of mobilization with exercise will provide further insight into the subsets of exercise-responsive T-cells. The practical importance of fully characterizing T-cell mobilization with exercise is highlighted by recent interest in the use of exercise as a tool to enhance immunotherapy (17–20).

The aims of our study were two-fold: first, to characterize the mobilization into and out of the peripheral blood of TM CD4 + and CD8+ T-cells relative to NA, CM, EM, and EMRA T-cells with exercise; and second, to compare the exercise response of CD57 + and CD57- cells within the T cell subsets. As latent infection with cytomegalovirus (CMV) has been associated with the heightened mobilization of T-cells into and out of the blood, we also considered the effects of CMV serostatus on the exercise-response of each T cell subset (16). We hypothesized that the relative mobilization of T-cells into the blood with exercise would increase with progressive differentiation; namely, NA < CM < TM < EM < EMRA. We also hypothesized that subsets expressing CD57 would show a greater mobilization compared to subsets lacking CD57, as later differentiated cells are highly mobilized by exercise and commonly express CD57.

2. Materials and methods

2.1. Experimental design and participants

This study was a secondary analysis of a larger investigation which consisted of 20 participants (10 female) between the ages of 18 and 40 years old who performed a cycling exercise in the Laboratory of Integrated Physiology at the University of Houston. All participants were recruited from Houston, TX. From this group, this secondary analysis examined 17 participants (seven female), from whom lymphocyte analyses were available. Participant characteristics are shown in **Table 1**. Participants were screened to ensure they were non-smokers (>10 years), exercised between one and six hours per week on average for the last six months, and met the American College of Sports Medicine criteria for participation in exercise (21). Exercise of any type was accepted for inclusion criteria and it should be noted that not all participants were trained cyclists. Participants were excluded if they reported a history of immune disease, or the regular use of medication known to affect the immune system. All participants gave written informed consent before

TABLE 1 Participant characteristics and exercise performance measures. Values are presented as means ± SD.

Characteristics	All subjects ($n = 17$; 7 Female)
Age (years)	26.3 ± 4.5
Height (cm)	163.2 ± 13.2
Body Mass (kg)	72.7 ± 17.3
BMI (kg/m^2)	24.1 ± 3.0
During Exercise:	
Mean Heart Rate (beats/min)	145 ± 4
Percent Maximum Heart rate[a] (%)	80.6 ± 0.0
RPE	13 ± 2
Power (Watts)	137.6 ± 48.6
Relative Power Output (Watts/kg)	1.7 ± 0.4

[a]Maximum heart rate estimated by (191.5-(0.007x(Age)^2))(24). Mean heart rate (% max) estimated by max heart rate reached during exercise divided by age-predicted maximum heart rate.
[b]Rating of perceived exertion, borg 6–20 scale.

participating in the study. This study was approved by the Institutional Review Board at the University of Houston (STUDY00000990).

For the current study, participants visited the laboratory twice. Participants with symptoms of upper-respiratory illness in the prior two weeks were rescheduled. Participant visits occurred between 6am and 10am. Participants were asked to refrain from strenuous exercise for 48 h, alcohol for 24 h, and food eight hours prior to their visit. During visit one, participants were screened and consented, and completed an incremental submaximal exercise test on a stationary bicycle. The results of this test were used to calculate the resistance corresponding to 80% of estimated maximal heart rate. During visit two, participants performed 30 min of bicycling exercise at this resistance.

2.2. Exercise bouts and blood collection

During visit one, participants completed a modified Åstrand test on a stationary bicycle (Velotron, RacerMate Inc., Seattle, Washington) (22). Briefly, participants cycled at a cadence of 70–90 revolutions per minute (RPM) at an initial resistance of 75–100 watts. Resistance was increased five to 10 watts every three minutes. Heart rate (HR; FT7 Polar, Polar United States) and rating of perceived exertion (RPE; Borg 6–20 scale) were recorded every minute (23). The test was completed when the participants surpassed 80% of their estimated maximum HR (calculated with the equation (191.5-(0.007x(Age)^2)) (24), for a full three-minute interval. During the second visit, participants cycled for 30 min at the resistance found in visit one to correspond to 80% of their estimated maximal heart rate. Heart rate, RPE, and RPM were monitored throughout the trial, and resistance was altered as needed to maintain intensity within +/- 5 bpm of target heart rate. Participants were encouraged to drink water throughout the exercise session. Exercise performance measures are shown in **Table 1**.

Venous blood was collected from a vein in the antecubital space at three time points: pre-exercise, post-exercise, and 1 h (1H) post-exercise. Pre-exercise blood was collected following a 10 min rest prior to any cycling activity. Post-exercise blood was collected within two minutes of exercise cessation. Participants rested quietly until the 1H post-exercise sample was drawn. Blood was collected into 10 ml vacuum-sealed tubes treated with ethylene-diamine-tetra-acetic acid (EDTA; Becton, Dickinson, and Co., Franklin Lakes, NJ). An additional 10 ml blood sample was collected at rest using a serum collection tube (Vacutainer, BD). Blood was processed within three hours of collection.

2.3. Flow cytometry

Blood was treated with red blood cell lysis buffer (eBiosciences Inc., San Diego, CA) and stained with fluorescently tagged monoclonal antibodies against cell-surface antigens. Direct immunofluorescence assays were performed to identify proportions of T-cells using one of two panels. Panel one included Vioblue-anti-CD45RA (IgG2b, T6D11), Viogreen-anti-CD3 (IgG2ak, REA615), FITC- anti-CD4 (IgG2ak, VIT4) PE-anti-CCR7 (recombinant human (REA) IgG1, REA546), PE-Vio615-anti-KLRG1 (REA IgG1, REA261), PE-Vio770-anti-CD62l (IgG1κ, 145/15), APC-conjugated anti-CD28 (IgG1, REA612), and APC Vio770-conjugated anti-CD57 (IgMk, TB03). Panel two was identical to panel one except FITC-anti-CD8 (IgG2ak, BW135/80) was used in place of CD4. All antibodies were purchased from Milteny Biotech Inc. (Bergisch, Gladbach, Germany). Cells were incubated with 2 µl of the antibody in the dark at room temperature for 20 min, then washed and resuspended in 200 µl of phosphate buffered solution (Mediatech Inc., Manassas, VA). Cells were directly analyzed with MACSQuant analyzer flow cytometer (Milteny Biotec Inc., Bergisch, Gladbach, Germany). Compensation beads (Milteny Biotec Inc., Bergisch, Gladbach, Germany) were used to compensate for spectral overlap. Single color tubes were used to identify positive and negative staining by each antibody. Lymphocytes were gated electronically using MACSQuantify™ software. Within the lymphocyte gate, CD3 + CD4 + and CD3 + CD8+ T-cells were identified and further subdivided based on CCR7, CD45RA, CD28 and CD57. **Figure 1** provides an example of the gating strategy. **Table 2** summarizes CD4 and CD8 T cell subset phenotypes.

2.4. Determination of viral serostatus

Serum samples were obtained by centrifugation and stored at −80°C until analysis. Viral serology (IgG) was determined for cytomegalovirus (CMV) in all subjects by ELISA following the manufacturer's instructions (BioCheck Inc., San Francisco, CA, United States). Results were read at 450 nm using a 96-well microplate reader (Molecular Devices, Sunnyvale, CA) and serum samples above the cut-off index were determined to be seropositive resulting in 12 seropositive subjects (6 female).

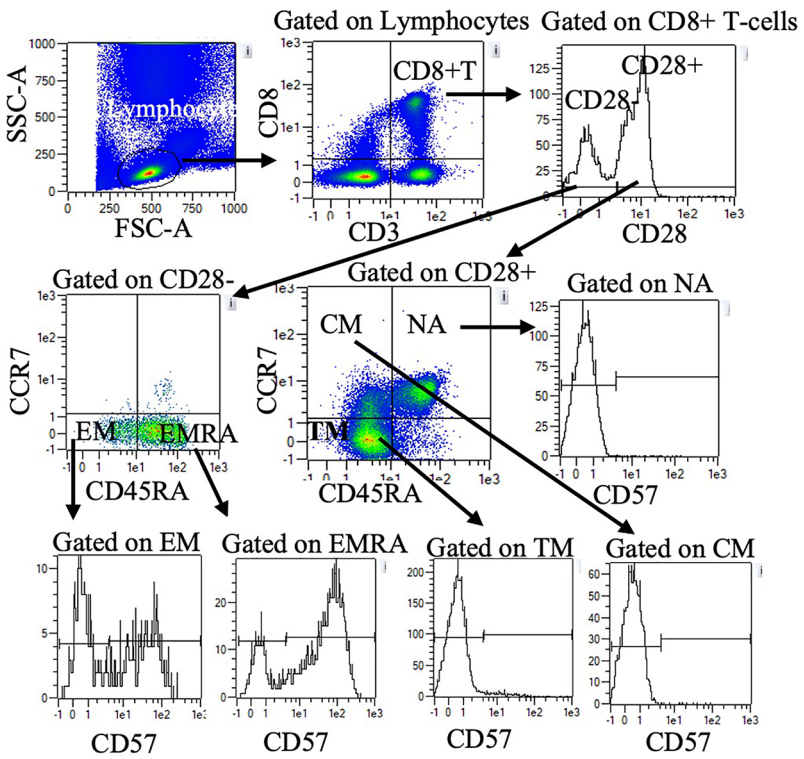

FIGURE 1
Representative flow cytometry data to illustrate gating strategy. Plots demonstrate sequential gating of lymphocytes, CD8+ T-cells, and CD28 + or CD28- CD8+ T-cells. Within CD28 + cells, NA, CM, and TM were identified by CD45RA and CCR7 expression. Within CD28- cells, EM, and EMRA were identified by CD45RA and CCR7 expression. CD57 + and CD57-cells within NA, CM, TM, EM, and EMRA were further quantified. A similar gating strategy was applied for CD4+ T-cells (data not shown). NA: naïve, CM: central memory, TM: transitional memory, EM: effector memory, EMRA: RA + effector memory.

2.5. Statistical analysis

Prior research has indicated a medium to large effect of 30 min of cycling exercise on the concentration of peripheral blood leukocytes (14). Thus, $n = 17$ was expected to provide 80% power to detect differences in peripheral blood leukocytes due to exercise at $p < 0.05$.

Prior to analysis, data were screened for outliers (values >2.5SD from mean) and to assure all assumptions of normality and multicollinearity were met. Skewed data were transformed using log transformations where necessary. The concentrations of T cell subsets present in the blood at each time point were analyzed using random-intercepts maximum likelihood mixed models with a variance components covariance structure. Models included time (three time points) as the independent variable (16). Sex, CMV serostatus, time × sex, and time × CMV serostatus as independent variables were also considered for inclusion in models. Model selection was based on the minimization of Schwarz's Bayesian Criterion. Final models included time, CMV serostatus, and time × CMV serostatus. Significant results were assessed by pairwise comparison of estimated marginal means, adjusted for multiple comparisons by the method of Sidak. Effect sizes are reported as η^2.

The fold change in T-cell subsets (concentration of cells post-exercise/concentration of cells pre-exercise; concentration of cells 1H post-exercise/concentration of cells post-exercise) were also analyzed using random-intercepts maximum likelihood mixed models with a variance components covariance structure. Models included subset (five subsets: NA, CM, TM, EM, EMRA; or two subsets: CD57+, CD57-) as independent variable. Sex, CMV serostatus, subset × sex, and subset × CMV serostatus as independent variables were also considered for inclusion in models; model selection was based on minimization of Schwarz's Bayesian Criterion. Final models included subset, CMV serostatus, and time × CMV serostatus as independent variables. Significant results were assessed by pairwise comparison of estimated marginal means, adjusted for multiple comparisons by the method of Sidak.

All analyses were completed using IBM SPSS Statistics for Windows, Version 28. $p < 0.05$ was accepted as significant.

3. Results

3.1. Exercise mobilizes TM T-cells into peripheral blood, independently of CMV serostatus

Exercise transiently mobilized CD4 + and CD8+ T-cells into peripheral blood (**Table 3**). The concentration of TM CD4 + and CD8+ T-cells in peripheral blood was significantly greater post-exercise than pre-exercise; values returned to baseline by 1H

TABLE 2 Phenotypic identification of T cell subsets and functional properties.

Subset	Identification	Effector Properties	References
Naïve (NA)	CD45RA + CCR7 + CD28+	–	(3,4)
Central Memory (CM)	CD45RA-CCR7 + CD28+	–	(2–5)
Transitional Memory (TM)	CD45RA-CCR7- CD28+	+/-	(3–5)
Effector Memory (EM)	CD45RA-CCR7- CD28-	+	(2–5)
CD45RA + Effector Memory (EMRA)	CD45RA + CCR7- CD28-	+	(3–5)

post-exercise. The concentration of NA, CM, EM, and EMRA CD4 + T-cells and CM, EM, and EMRA CD8+ T-cells was also significantly and transiently elevated with exercise.

CMV serostatus impacted the concentration of CM CD4 + and CD8+ T-cells and EM CD4+ T-cells. CMV seropositive participants had a significantly lower concentration of CM CD4+ T-cells ($F_{(1,16)} = 8.344$, $p = 0.011$, $\eta^2 = 0.343$) and CM CD8+ T-cells ($F_{(1,17)} = 5.812$, $p = 0.028$, $\eta^2 = 0.255$). CMV seropositive participants had a greater concentration of EM CD4+ T-cells ($F_{(1,16)} = 4.561$, $p = 0.049$, $\eta^2 = 0.222$). CMV serostatus did not impact the concentration of TM CD4+ T-cells ($F_{(1,16)} = 0.461$, $p = 0.507$, $\eta^2 = 0.028$) nor TM CD8+ T-cells ($F_{(1,17)} = 0.611$, $p = 0.445$, $\eta^2 = 0.035$).

Exercise also significantly changed the proportions of CD4 + and CD8+ T-cell subsets in peripheral blood (**Table 4**). The proportion of TM CD4 + and CD8+ T-cells did not differ post-exercise relative to pre-exercise, but was significantly greater 1H post-exercise than both pre-exercise and post-exercise. The proportion of EM and EMRA CD4 + and CD8+ T-cells was significantly greater post-exercise and returned to baseline values 1H post-exercise. In contrast, the proportion of NA CD8+ T-cells was significantly decreased post-exercise and returned to baseline values 1H post-exercise.

CMV serostatus also impacted the proportions of CM CD4 + and CD8+ T-cells and EM CD4+ T-cells. CMV seropositive participants had a significantly lower proportion of CM CD4+ T-cells than seronegative participants ($F_{(1,16)} = 5.961$, $p = 0.027$, $\eta^2 = 0.271$), as well as a lower proportion of CM CD8+ T-cells ($F_{(1,17)} = 7.901$, $p = 0.012$, $\eta^2 = 0.317$). CMV serostatus did not impact the proportion of TM T-cells ($F_{(1,16)} = 0.092$, $p = 0.766$, $\eta^2 = 0.006$). Although not significant, there was a trend for a lower proportion of TM CD8+ T-cells amongst CMV seropositive (mean ± standard error: 37.171 ± 3.795% vs. 27.743 ± 2.450%; $F_{(1,17)} = 4.347$, $p = 0.052$, $\eta^2 = 0.204$).

A significant interaction effect between time and CMV serostatus was observed with the proportion of NA CD4 + T-cells. Post-hoc pairwise analyses indicate that among seropositive participants, the proportion of NA cells pre-exercise was significantly greater than post-exercise and 1H post-exercise (mean ± standard error: Pre: 35.7 ± 2.5% vs. Post: 33.5 ± 2.5% and 1H Post: 32.6 ± 2.5%; $p < 0.01$), whereas in CMV seronegative participants the proportion of NA cells pre-exercise was greater than 1H post exercise (Pre: 28.1 ± 4.3% vs. 1H Post: 25.7 ± 4.3%, $p = 0.034$) but not post-exercise (28.7 ± 4.3%, $p = 0.894$).

3.2. TM mobilization is similar to that of NA and CM T-cell subsets

We next asked if the relative mobilization into the blood post-exercise (fold change; ingress) and out of the blood 1H post-exercise (fold change; egress) differed between the CD4 + and CD8+ T cell subsets. Significant effects of subset were observed during ingress (CD4+ T-cells: $F_{(4,64)} = 43.776$, $p < 0.001$, $\eta^2 = 0.732$; CD8+ T-cells: $F_{(4,\ 68)} = 44.505$, $p < 0.001$, $\eta^2 = 0.724$) and egress (CD4+ T-cells: $F_{(4,\ 64)} = 72.119$, $p < 0.001$, $\eta^2 = 0.818$; CD8 + T-cells: $F_{(4,\ 68)} = 50.190$, $p < 0.001$, $\eta^2 = 0.747$). Post-hoc analyses revealed that among both CD4 + and CD8+ T-cells, the ingress and egress of TM CD4 + and CD8+ T-cells did not differ

TABLE 3 The concentration (cells/μl) of CD4 + and CD8+ T cell subsets present in peripheral blood Pre-, post-, and 1H post-exercise. Values are means ± S.D.; Main effects are F-statistic (*p*-value).

Cell subset:	Time			Main effect		Interaction effect
	Pre	Post	1H Post	Time	CMV	Time × CMV
CD4+ T Cells	627 ± 168	770 ± 181*,**	608 ± 120	**10.044 (<.001)**	1.435 (.248)	0.074 (.929)
NA	214 ± 88	253 ± 104*,**	189 ± 65	**8.979 (<.001)**	0.217 (.647)	0.085 (.919)
CM	259 ± 102	305 ± 101*,**	253 ± 76	**11.454 (<.001)**	**8.344 (.011)**	1.234 (.305)
TM	134 ± 44	168 ± 50*	147 ± 56	**5.073 (.012)**	0.461 (.507)	0.203 (.818)
EM	11 ± 11	25 ± 31*,**	10 ± 11	**38.857 (<.001)**	**14.549 (.002)**	0.681 (.513)
EMRA	4 ± 11	13 ± 35*,**	4 ± 11	**38.591 (<.001)**	3.514 (.079)	0.760 (.476)
CD8+ T Cells	339 ± 120	512 ± 214*,**	302 ± 79	**15.438 (<.001)**	1.429 (.248)	0.800 (.458)
NA	106 ± 39	117 ± 41**	94 ± 31	**8.988 (<.001)**	0.173 (.682)	0.775 (.469)
CM	24 ± 12	29 ± 14*,**	23 ± 10	**10.309 (<.001)**	**5.812 (.028)**	2.166 (.130)
TM	99 ± 40	139 ± 56*,**	98 ± 41	**14.755 (<.001)**	0.611 (.445)	0.300 (.743)
EM	45 ± 35	92 ± 80*,**	33 ± 18	**50.788 (<.001)**	3.220 (.091)	0.003 (.997)
EMRA	53 ± 46	118 ± 93*,**	43 ± 40	**56.112 (<.001)**	4.277 (.054)	0.036 (.964)

Significance ($p < .05$) indicated by bold.
*(Differs from Pre).
**(Differs from 1H Post).

TABLE 4 The proportions of CD4 + and CD8+ T-cell subsets in peripheral blood Pre-, post-, and 1H post-exercise. Values are means ± S.D.; Main and interaction effects are F-statistic (*p*-value).

Cell subset:	Time			Main effect		Interaction effect
	Pre	Post	1H Post	Time	CMV	Time × CMV
CD4+ T Cells						
NA	34 ± 9	32 ± 9**	31 ± 10*	**14.717 (<.001)**	1.675 (.214)	**3.583 (.039)**
CM	41 ± 10	40 ± 10	42 ± 10	3.208 (.054)	**5.961 (.027)**	2.449 (.102)
TM	22 ± 7	22 ± 8**	24 ± 7*	**19.813 (<.001)**	0.092 (.766)	2.772 (.078)
EM	2 ± 2	3 ± 4***	2 ± 2	**33.223 (<.001)**	**16.632 (<.001)**	1.124 (.338)
EMRA	1 ± 2	2 ± 4***	1 ± 2	**33.930 (<.001)**	4.135 (.059)	1.112 (.341)
CD8+ T Cells						
NA	32 ± 6	24 ± 7***	31 ± 6	**31.212 (<.001)**	0.738 (.402)	2.565 (.092)
CM	8 ± 4	6 ± 4***	8 ± 4	**26.964 (<.001)**	**7.901 (.012)**	1.462 (.246)
TM	30 ± 9	29 ± 11**	32 ± 10*	**6.831 (.003)**	4.347 (.052)	0.224 (.800)
EM	12 ± 7	16 ± 9***	11 ± 6	**23.156 (<.001)**	3.263 (.089)	0.477 (.625)
EMRA	14 ± 9	21 ± 12***	13 ± 10	**30.585 (<.001)**	4.172 (.057)	0.913 (.411)

Significance ($p < .05$) indicated by bold.
*(Differs from Pre).
**(Differs from 1H Post).

from NA and CM T-cells (Figure 2). Rather, EM and EMRA exhibited a larger fold change increase post-exercise and a larger fold change decrease 1H post-exercise compared to NA, CM, and TM.

No significant effect of CMV serostatus was observed for the ingress and egress of the T cell subsets (data not shown).

3.3. Magnitude of exercise response differs between subsets expressing CD57

The secondary aim of this study was to compare the response to exercise of CD57 + and CD57- cells within T cell subsets. The proportion and concentration of the NA, CM, and TM CD4 + and CD8+ T cell subsets that were CD57 + was very low (Figure 3). Therefore, we summed CD57 + and CD57- cells within the NA, CM, and TM subsets (each of which are CD28+) to analyze the relative mobilization of CD57 + and CD57- subsets of CD28+ T-cells.

Figure 4 displays the ingress and egress of CD4 + and CD8+ T cell subsets by CD57 expression. Among CD28+ CD4 + and CD8+ T-cells, CD57 + cells were mobilized significantly more than CD57- cells during ingress (CD4+ T-cells: $F_{(1,16)} = 8.602$, $p = 0.010$, $\eta^2 = 0.350$; CD8+ T-cells: $F_{(1,17)} = 7.879$, $p = 0.012$, $\eta^2 = 0.317$) and egress (CD4+ T-cells: $F_{(1,16)} = 11.268$, $p = 0.004$, $\eta^2 = 0.413$; CD8 + T-cells: $F_{(1,17)} = 21.027$, $p < 0.001$, $\eta^2 = 0.553$). CD57+ EM CD8 + T-cells were mobilized significantly more than CD57- cells during ingress ($F_{(1,17)} = 14.471$, $p = 0.001$, $\eta^2 = 0.460$) and CD57+ EM CD4 + and CD8+ T-cells displayed a greater egress than CD57- cells (CD4+ T-cells: $F_{(1,16)} = 9.430$, $p = 0.007$, $\eta^2 = 0.371$; CD8+ T-cells: $F_{(1,17)} = 30.521$, $p < 0.001$, $\eta^2 = 0.642$). CD57+ EMRA CD8+ T-cells also displayed a greater mobilization during ingress ($F_{(1,17)} = 16.087$, $p = 0.001$, $\eta^2 = 0.486$) and egress ($F_{(1,17)} = 28.574$, $p < 0.001$, $\eta^2 = 0.627$).

CMV serostatus significantly interacted with CD57 expression in the mobilization of CD28 + CD4+ T-cells (Figure 4). Post-hoc pairwise comparisons revealed that only CMV seropositive participants had a significant difference between CD57 + and CD57- cells in the ingress and egress of CD28+ CD4+ T-cells (interaction ingress: $F_{(1,16)} = 6.609$; $p = 0.021$, $\eta^2 = 0.292$; egress: $F_{(1,16)} = 6.328$; $p = 0.023$, $\eta^2 = 0.283$). A similar interaction effect was found for CD28 + CD8 + T cell egress ($F_{(1,17)} = 5.800$; $p = 0.028$, $\eta^2 = 0.254$).

4. Discussion

The primary aim of this study was to characterize the mobilization of TM CD4 + and CD8+ T-cells by acute exercise. To the best of our knowledge, this subset of T cells, with distinct phenotype and function from CM and EM subsets, has not yet been examined in the context of exercise. We report that the concentration of TM CD4 + and CD8+ T-cells in peripheral blood is significantly increased post-exercise, and that this increase is transient, as TM T-cell concentration 1H post-exercise did not differ from resting values. In contrast, the proportion of CD4 + and CD8+ T-cells with a TM phenotype was significantly elevated 1H post-exercise compared to pre-exercise and post-exercise values. The relative mobilization of TM T-cells did not differ from NA and CM subsets, but was significantly less than EM and EMRA T-cell subsets. This indicates that TM are responsive to exercise, but not to the same extent as later differentiated subsets. The secondary aim of this study was to compare the mobilization of CD57 + and CD57- subsets of memory T-cells with acute exercise. We report that CD57 + subsets of EM and EMRA T-cells are mobilized to a greater extent than CD57- subsets. We further report that the unique subset of CD57 + CD28+ T-cells are mobilized to a greater extent than CD57-CD28 T-cells. Therefore, our results confirm and extend earlier findings that later differentiated T-cells are mobilized to a greater extent than earlier differentiated T-cells by acute exercise.

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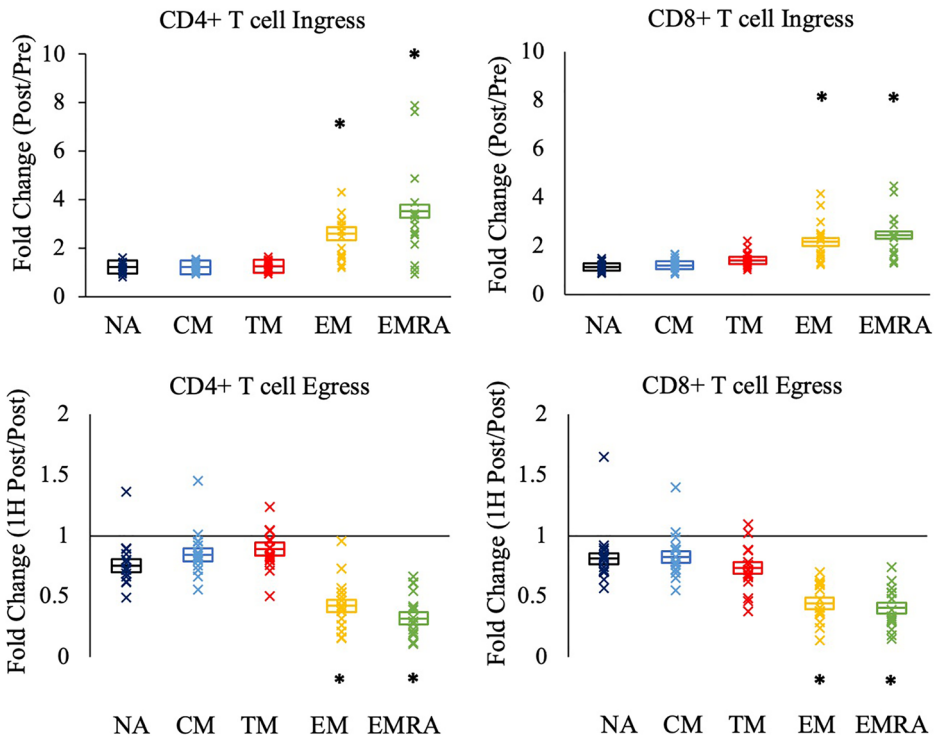

FIGURE 2
The relative mobilization of CD4 + and CD8+ T-cell subsets by an acute bout of exercise. Ingress (upper panels) reflects the fold change in the concentration of cells post-exercise relative to pre-exercise, and egress (lower panels) reflects the fold change 1H post-exercise relative to post-exercise. Data are displayed as estimated marginal means (line) and standard error (box). Individual data points from each participant are also indicated (x). Statistically significant differences ($p < .05$) in fold change from NA, CM, and TM is indicated by *. NA: naïve, CM: central memory, TM: transitional memory, EM: effector memory, EMRA: RA + effector memory.

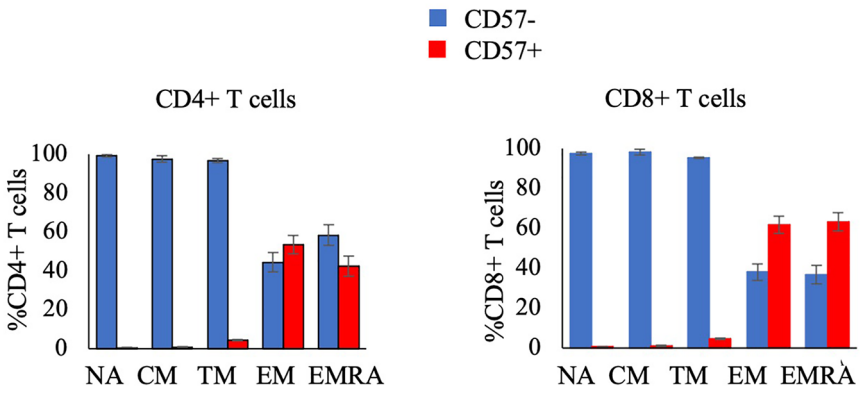

FIGURE 3
The proportion of CD4+ T-cell subsets (left panel) and CD8+ T-cell subsets (right panel) that are CD57- or CD57 + in resting blood. Data are displayed as mean and standard error (error bars).

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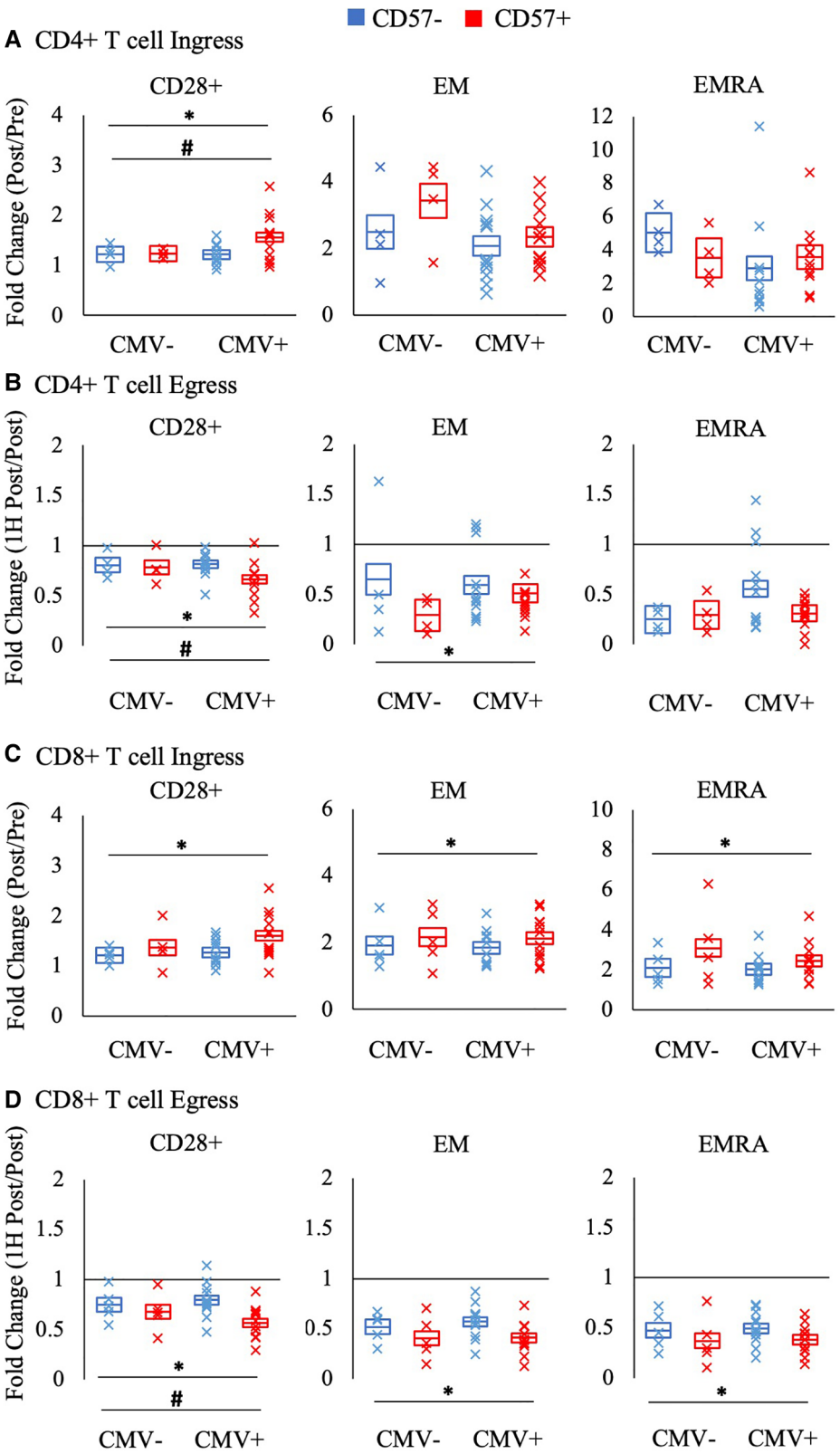

FIGURE 4
The relative mobilization of CD4 + and CD8+ T-cell subsets by CD57 expression. Ingress (panels A and C) reflects the fold change in the concentration of cells post-exercise relative to pre-exercise, and egress (panels B and D) reflects the fold change 1H post-exercise relative to post-exercise. The fold change in CD57 + and CD57- subsets of CD28+, EM, and EMRA CD4 + and CD8+ T-cells amongst CMV seronegative and seropositive participants is shown. Data are displayed as estimated marginal means (line) and standard error (box). Individual data points from each participant are also indicated (x). Significant main effect of CD57 expression indicated by *. Significant interaction effect between CD57 expression and CMV indicated by #. EM: effector memory, EMRA: RA + effector memory, CMV: cytomegalovirus.

Our results are primarily in agreement with previous reports, as we found exercise transiently increases the concentration of T-cells in peripheral blood, particularly later differentiated subsets (25). This study expands upon earlier reports as we for the first time distinguish TM T-cells from EM T-cells. In prior work, the lack of a third surface marker prevented the identification of TM as distinct from EM (10, 12, 14). TM T-cells are an intermediate subset between CM and EM T-cells in terms of their telomere length, proliferation rates, expression of activation and co-stimulation receptors, and cytolytic molecule secretion (3, 4). For example, stimulated TM CD8+ T-cells produce higher levels of IFN-γ and granzyme A and B than CM T-cells, although not as much as EM T-cells. TM T-cells also express intermediate levels of CCR5 and CXCR3, suggesting the capacity to migrate to inflamed tissues (3). Given the intermediate phenotype and function, we also hypothesized that TM T-cells would show an intermediate level of mobilization with exercise, occurring between CM and EM T-cells. In contrast, TM did not differ from NA and CM, but was less than that of EM and EMRA subsets. The preferential mobilization of EM and EMRA has been attributed to the greater concentration of β-adrenergic receptors on the surface of these cells relative to NA and CM T-cells, allowing the enhanced response to exercise-induced increases in epinephrine (15). Although not measured in the current study, these findings suggest that TM T-cells do not express more β-adrenergic receptors than NA and CM T-cells. One-hour post-exercise, the proportion of TM CD4 + and CD8+ T-cells was significantly greater than pre-exercise. This was unique amongst the T-cell phenotypes examined, as NA, CM, EM, and EMRA cells had all returned to resting values or below. Indeed, we found TM T-cells comprised nearly one quarter of CD4 + T-cells and nearly one third of CD8+ T-cells 1H post-exercise. This indicates that the TM subset is an important component of the T-cell pool during the recovery from exercise, and ought to be considered and reported in future research.

This study also expands upon the existing literature characterizing the mobilization of CD57+ T-cells. Earlier work examining the mobilization of CD28-CD57 + or KLRG1 + CD57+ T-cells typically concluded that these cells were increased immediately post-exercise, with their concentration returning to resting values 1–3H post-exercise (25–28). In the current study, we examined CD57 expression within T -cell subsets defined by CD28, CCR7, and CD45RA. Not surprisingly, the expression of CD57 within the CD28 + subsets (NA, CM, and TM) was very low; thus we grouped these subtypes together for analyses (3, 4). Our results indicate that CD57 is a reliable marker of highly exercise-responsive CD8+ T-cells, even in the context of other phenotypic markers. While CD57 identified highly responsive CD28+ CD4+ T-cells, the mobilization of EM and EMRA CD4+ T-cells did not differ by CD57 expression. CD57 has been described as a marker of T-cell senescence, as it strongly associates with shortened telomeres and therefore reduced proliferative potential (4). However, CD57 expression also associates with high effector function, including expression of cytolytic molecules (29). Thus, the mobilization of CD57+ EM and EMRA CD8 + T-cells with exercise may indicate an increase in blood T-cells capable of rapidly responding to threats. Along these lines, post-exercise blood has been shown to contain a greater concentration of functional, virus-specific T-cells (8, 30). Further, while CD57 expression often coincides with the loss of CD28, a unique population of CD28 + CD57+ T-cells has been described (7). These double-positive T-cells appear to be activated, and have greater expression of regulatory molecules including IL-10, PD-1, and CTLA-4 compared to CD28 + CD57- and CD28-CD57+ T-cells. Thus, the increase in these double positive T-cells with exercise observed in the current study may indicate greater regulation of the immune system. However, the proportion of these cells was low in comparison to CD57+ EM and EMRA T-cells.

Due to persistent antigen exposure, latent infection with CMV can drive the accumulation of CD28-CD57+ T-cells (7, 31, 32). Given that these cells are preferentially mobilized with exercise, it follows that CMV seropositive individuals demonstrate a greater mobilization of CD8+ T-cells and T-cell subsets (12, 14, 16, 33, 34). We therefore considered CMV serostatus in the current study; however, although CMV seropositive participants had an elevated concentration and proportion of EM CD4+ T-cells, no significant differences due to CMV serostatus were noted among EM CD8+ T-cells or EMRA T-cells. This lack of a CMV effect on cell concentration likely explains the lack of effect on relative mobilization, as we found no differences between CMV seropositive and seronegative participants in the magnitude of mobilization of the EM and EMRA T-cells. There was a non-significant trend for CMV seropositive participants to have a lower proportion of TM CD8+ T-cells overall relative to CMV seronegative participants. This, alongside the trend for CMV seropositive participants to have a greater proportion of EMRA CD8+ T-cells and slight trend for greater proportion of EM CD8 + T-cells suggests that CMV infection leads to the accumulation of the later differentiated effector memory cells rather than and at the expense of the TM CD8+ T-cell compartment. It is possible that our trending data may have reached the threshold for significance if we had a larger sample size. However, earlier studies reporting an effect of CMV on T-cell mobilization with exercise employed a similar sample size. We speculate that the differences between the current study and earlier work regarding the influence of CMV may be due to our inclusion of both males and females in the current study; a feature that was lacking in the earlier work that recruited only male participants. Sex differences exist in the epinephrine response to aerobic exercise as well as in leukocyte β-adrenergic receptor expression and thus males and females may display differences in the degree of mobilization of T-cell subsets with exercise (35, 36). However, sex was not included as a factor in the current analyses and so this remains speculative. We did consider the addition of sex as an independent variable when building the statistical models, but its addition did not improve model fit and so was not included. We note that this study was not designed to consider sex differences and did not collect data regarding oral contraceptive pill use nor stage of menstrual cycle (35, 36).

In addition to the limitations of not considering oral contraceptive pill use or menstrual cycle phase in female participants, an additional limitation is that we did not measure cell function (i.e., cytokine

expression and cytotoxicity) or telomere length. Thus, we cannot conclude that changes in T-cell subset concentration and/or proportion lead to differences in immune function with exercise. Another limitation is that the number of CMV seropositive and seronegative participants was unequal. This study was a secondary analysis of a separate study that did not include CMV serostatus as a variable of interest, and so we were unable to recruit equal numbers of CMV positive and CMV negative participants.

In conclusion, TM CD4 + and CD8+ T-cells are responsive to acute aerobic exercise. The concentration of TM T-cells is transiently increased immediately post-exercise, whereas the proportion of TM T-cells is elevated 1H post-exercise. TM T-cells display a relative mobilization similar to other CD28 + subsets, NA and CM. Results also indicate CD57 identifies highly exercise responsive cells within CD8+ T-cell subsets, as well as within CD28 + CD4 + T-cells. Collectively, these data expand our understanding of the mobilization of T-cells by exercise.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Committee for the Protection of Human Subjects at the University of Houston. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Experimental design, RH and EL; experimental execution, RH and ME; study supervision, EL, ML, and MM; data analyses, RH and ML; manuscript preparation, RH, ME, MM, ML, and EL. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The effects of exercise training for eight weeks on immune cell characteristics among breast cancer survivors

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Methods: This study examined the effects of exercise training for 8 weeks on blood immune cell characteristics among 20 breast cancer survivors (age 56 ± 6 years, Body Mass Index 25.4 ± 3.0 kg m^2) within two years of treatment. Participants were randomly allocated to a partly-supervised or a remotely-supported exercise group ($n = 10$ each). The partly supervised group undertook 2 supervised (laboratory-based treadmill walking and cycling) and 1 unsupervised session per week (outdoor walking) progressing from 35 to 50 min and 55% to 70% $\dot{V}O_2$max. The remotely-supported group received weekly exercise/outdoor walking targets (progressing from 105 to 150 min per week 55% to 70% $\dot{V}O_2$max) via weekly telephone calls discussing data from a fitness tracker. Immune cell counts were assessed using flow cytometry: CD4+ and CD8+ T cells (Naïve, NA; Central memory, CM; and Effector cells, EM and EMRA; using CD27/CD45RA), Stem cell-like memory T cells (TSCMs; using CD95/CD127), B cells (plasmablasts, memory, immature and naïve cells using CD19/CD27/CD38/CD10) and Natural Killer cells (effector and regulatory cells, using CD56/CD16). T cell function was assessed by unstimulated HLA-DR expression or interferon gamma (IFN-γ) production with Enzyme-linked ImmunoSpot assays following stimulation with virus or tumour-associated antigens.

Results: Total leukocyte counts, lymphocytes, monocytes and neutrophils did not change with training ($p > 0.425$). Most CD4+ and CD8+ T cell subtypes, including TSCMs, and B cell and NK cell subtypes did not change ($p > 0.127$). However, across groups combined, the CD4+ EMRA T cell count was lower after training (cells/μl: 18 ± 33 vs. 12 ± 22, $p = 0.028$) and these cells were less activated on a per cell basis (HLA-DR median fluorescence intensity: 463 ± 138 vs. 420 ± 77, $p = 0.018$). Furthermore, the partly-supervised group showed a significant decrease in the CD4+/CD8+ ratio (3.90 ± 2.98 vs. 2.54 ± 1.29, $p = 0.006$) and a significant increase of regulatory NK cells (cells/μl: 16 ± 8 vs. 21 ± 10, $p = 0.011$). T cell IFN-γ production did not change with exercise training ($p > 0.515$).

Discussion: In summary, most immune cell characteristics are relatively stable with 8 weeks of exercise training among breast cancer survivors. The lower counts and activation of CD4+ EMRA T cells, might reflect an anti-immunosenescence effect of exercise.

KEYWORDS

breast cancer, survivorship, immune profiles, anti-cancer immunity, lifestyle, exercise, body composition

1. Introduction

Breast cancer is the most commonly diagnosed cancer globally (1, 2). Survival over 5-years is close to 90% in most western nations (3–5) due to advances in screening, early detection and treatment (6). As breast cancer is the most prevalent cancer in many countries and the number of people living with and beyond breast cancer is increasing (7), there is a growing need for interventions—such as exercise and physical activity—that reduce the risk of recurrence, mortality and other comorbidities developing. Indeed, this need is recognised globally, as shown by the publication of cancer-specific exercise and physical activity guidelines or position stands by the World Health Organisation (WHO), the American Cancer Society (ACS), the American Society of Clinical Oncology (ASCO), the Clinical Oncology Society of Australia (COSA), the World Cancer Research Fund International (WCRF) and the American College of Sports Medicine (ACSM) (8–13).

There is now good evidence that exercise and physical activity undertaken before or after a cancer diagnosis improves overall health, reduces risk of recurrence, and increases survival (14–18). For example, a study of 959 breast cancer patients showed that 2.5 h or more of brisk walking each week was associated with a 44% lower mortality risk compared with no physical activity (19). Furthermore, a study of 1,340 breast cancer patients showed that those who met physical activity guidelines (150 min of moderate-intensity physical activity each week) both before diagnosis and after treatment had a 55% lower risk of recurrence and a 68% lower risk of all-cause mortality (20). Indeed, the latest WCRF review and meta-analysis as part of the continuous update project, concluded that the most active patients with breast cancer have 42% lower cancer-specific mortality risk than the least active patients (21). However, in most guidelines, the dose of exercise or physical activity, was originally defined in cancer prevention studies, and more research is needed among people living with and beyond cancer (22). In particular, the mechanisms by which exercise or physical activity reduce cancer recurrence and mortality are not understood, although processes that involve changes to inflammation and immunity are possible candidates (18, 23–25).

Chronic inflammation overall and the action of specific inflammatory processes and cytokines can facilitate tumour progression (26). Moreover, inflammation exacerbates ageing, referred to as inflammageing, contributing towards immunosenescence (27). Given that these processes are influenced by exercise and physical activity, countering inflammation and limiting immunosenescence are possible lifestyle-related anti-cancer mechanisms (28–30). In support, regular exercise and physical activity over months and years can modulate immune profiles, for example, by limiting the accumulation of cell populations linked with immunosenescence (31) and reducing inflammation (32). In addition, individual exercise bouts mobilise leukocytes, and sub-populations, enhancing immunosurveillance (33). Many exercise-mobilised cells are differentiated T cells and NK cells that are cytotoxic against tumour cells (34, 35). Given that most breast tumours are immunogenic (36, 37), T cells represent a likely mechanism through which exercise exerts anti-cancer effects (30, 38).

Most studies examining exercise-induced anti-cancer mechanisms have been pre-clinical mouse models. Studies recruiting people living with and beyond cancer that incorporate measurements relevant to cancer immune-surveillance are less common, but some understanding comes from studies among human populations considered to be at high risk of cancer. For example, one study recruited 33 postmenopausal women considered to be at high risk of breast cancer (39). Participants were randomized to either 12 weeks of high intensity interval training (HIIT), moderate-intensity continuous training (MICT) or usual care (UC) and a primary outcome was NK cell cytotoxicity against tumour cell lines. There were no statistically significant differences in NK cell cytotoxicity with exercise training irrespective of group (39). However, across all participants, low baseline cardiorespiratory fitness was negatively associated with exercise-induced increases in NK cell cytotoxicity, implying that less fit individuals might benefit most from exercise (39). In a sub-sample of 24 participants, T cell profiles were also examined (40). It was concluded that exercise alters the frequency of cells associated with immunosenescence, but each exercise mode elicited divergent effects. HIIT was interpreted as invoking pro-immunosenescent effects, by decreasing CD4+ T cells, CD4+ Naïve T cells, CD4+ recent thymic emigrants and the CD4+/CD8+ T cell ratio. However, MICT was interpreted as invoking anti-immunosenescent effects, by increasing total lymphocytes and CD8+ effector memory T cells, and there was a greater positive change in total T cells, CD4+ Naïve T cells, CD4+ central memory T cells, and CD4+ recent thymic emigrants in the MICT group (40).

Further research is needed to understand potential anti-immunosenescence and anti-cancer effects of exercise and physical activity among people living with or beyond cancer, incorporating functional measurements previously shown to correlate with clinical outcomes (41). Therefore, the present study examined the effect of exercise training over eight weeks on the phenotype of circulating T cells, B cells and NK cell populations among breast cancer survivors within 2 years of treatment. Importantly, this study also made functional measurements, including basal unstimulated T cell activation, and T cell recognition of virus and tumour-associated antigens.

2. Methods

2.1. Participants

This study comprised 20 participants. Inclusion criteria were; women aged between 35 and 69 years, body mass index 20–35 kg m^2, post-menopausal (or who had not had a menstrual period for at least 1 year), and a past diagnosis of non-metastatic non-bilateral stage I–III invasive breast cancer. Participants had received their last treatment at least 2 months before enrolment, but no longer than 5 years prior, however women on long-term endocrine therapy were eligible. Participants were free from

TABLE 1 Diagnostic and treatment information.

Clinical summary	% of participants (number or sub-type) or duration
Diagnosis	
Ductal carcinoma *in situ* (DCIS)	30% (6/20)
Multifocal carcinoma	10% (2/20)
Hormone expression (ER+/PR+)	65% (13/20)
HER2 expression (HER2+)	10% (2/20)
Tumour Grade (G)	0% (G1), 45% (G2), 25% (G3)
TNM scoring	
Tumour (T)	35% (T1), 30% (T2), 5% (T3)
Nodes (N)	75% (N0), 15% (N1), 5% (N2), 5% (N3)
Metastasis (M)	100% (M0)
Average time since diagnosis	14 ± 7 months
Treatment	
Surgery	100% (20/20)
Chemotherapy	25% (5/20)
Radiotherapy	75% (15/20)
Hormone therapy	60% (12/20)
Anti- HER2+ therapy	10% (2/20)
Average time since surgery	12 ± 6 months

Data is expressed as percentage of participants or as mean ± SD. Grade refers to the histologic grade (G1, G2, G3, etc). TNM staging defines the characteristics of the tumour 0 to 4 (T) and 0 to 3 (N). ER, Estrogen receptor; HER2, Human Epidermal growth factor Receptor 2, PR, progesterone receptor.

cancer, significant cardiac comorbidity, severe hypertension (>200/120 mmHg) or cardiovascular disease and did not have an active infection at the time of enrolment. Participants self-reported to not undertake regular structured physical activity for more than 30 min on two or more occasions per week. Participants were recruited via an NHS hospital in the UK and provided full and informed consent. The study was approved by an NHS research ethics committee (18/WA/0314).

Participants were 56 ± 6 years of age, with body mass 67 ± 8 kg, Body Mass Index 25.4 ± 3.0 kg m^2 and $\dot{V}O_2$max 29.07 ± 5.55 ml kg^{-1} min^{-1}. Table 1 and Supplementary Table S1 show the diagnostic and treatment history of participants. Upon recruitment, time since diagnosis was 14 ± 7 months and time since surgery was 12 ± 6 months. All participants had received a diagnosis of primary invasive breast cancer, with 30% of participants diagnosed with ductal carcinoma *in situ* and 10% of participants with multifocal carcinoma. 65% of participants had tumours which were positive for Estrogen/Progesterone Receptors (ER+/PR+) and 10% were positive for Human Epidermal Growth Factor Receptor 2 (HER2). None of the patients had advanced metastatic disease. All participants had surgery, and some underwent radiotherapy (75%), hormone therapy (60%), chemotherapy (25%) or immunotherapy (10%).

2.2. Study design

Participants were randomised to one of two intervention groups (allocation ratio 1:1, block size 6) by an independent researcher using a computer-generated randomisation list. Randomisation was stratified for previous chemotherapy treatment (yes/no) and BMI (<25 kg m^2 or >25 kg m^2). The two intervention groups were either an eight-week partly-supervised exercise group, or an eight-week remotely-supported exercise group (see Section 2.5).

2.3. Participant characterisation

Measurements were made before the intervention (within 7 days) and at least 24 h after the last exercise bout (within 7 days). Participants visited a research laboratory in the morning (between 07:00 and 11:00) after fasting overnight (since 22:00) and having refrained from alcohol, caffeine or exercise in the previous 24 h. After a 10 min seated rest, blood pressure was measured with an automated sphygmomanometer (Diagnostec EW3106, Panasonic, Japan) in the contralateral arm to the affected breast. Height was assessed with a wall mounted stadiometer (Holtain Ltd, UK), body mass was assessed using electronic scales (BC-543 Monitor, Tanita, Tokyo, Japan) and waist and hip circumference was measured using a tape (SECA 201, Hamburg, Germany). Body composition was assessed using a dual energy x-ray absorptiometry (DEXA) whole body scan (QDR, Discovery W, Hologic, Bedford, UK).

Cardiorespiratory fitness was assessed using a treadmill-based maximal walking test to exhaustion (HP Cosmos Saturn, Nußdorf, Germany) comprising 3 min stages, beginning at 2.7 kph with a 1% gradient, and increasing by 1.3 kph until 6.6 kph, with further intensity increments via increasing gradient by 2%. During the final minute of each stage, heart rate was measured using telemetry (Polar heart rate monitor RS400, Kempele, Finland), rating of perceived exertion (RPE) was recorded using the Borg scale, and an expired air sample was collected using Douglas bags. Oxygen and Carbon dioxide concentrations were assessed with a Servomex 5200 Multi-Purpose HF gas analyser (Servomex; Sussex, UK). The volume of air was measured with a dry gas meter (Harvard Apparatus, Cambridge, UK).

Physical function was assessed using three tests. First, for the 6 min walk test, participants walked as far as possible in 6 min between two cones placed 7 meters apart (42). Second, for the sit-to-stand test, participants performed as many sit-to-stands in 30 s (seated on a standard chair, rising to reach full knee extension, and return to seated, with arms folded across the chest) (43, 44). Third, for the 8-foot get-up-and-go test, participants rose from a seated position on a standard chair, walked 8-feet, returning to a seated position as quickly as possible (45, 46).

2.4. Blood sample collection, processing and storage

After a 10 min seated rest, approximately 30 ml of blood was collected into a syringe containing sodium heparin (4 IU/ml) for isolation of peripheral blood mononuclear cells (PBMCs). A further 5 ml of blood was collected into an anticoagulant-free serum separation tube and left to clot at room temperature for 30 min, and 5 ml of blood was collected into a potassium–ethylenediaminetetraacetic acid (K_3–EDTA) tube (BD Vacutainer, BD Biosciences, Oxford, UK) for preparation of serum and

plasma respectively. Tubes for serum and plasma were centrifuged for 10 min at 2,000 × *g* and 4°C, and the supernatant was collected and stored at −80°C until analysis. PBMCs were isolated using density gradient centrifugation. Blood treated with sodium heparin was diluted 1:1 with sterile RPMI (Sigma-Aldrich, Gillingham, UK), layered onto Ficoll-Paque^{TM} plus (GE Healthcare, Buckinghamshire, UK) and centrifuged for 25 min at 500 × *g* and 21°C. PBMCs were washed in RPMI, by centrifuging for 10 min at 400 × *g* and 21°C and 7 min at 300 × *g* and 21°C. Platelets were removed by centrifuging for 7 min at 200 × *g* and 21°C. PBMCs were counted in Trypan blue (1.5% acetic acid) with a haemocytometer and a light microscope. PBMCs were resuspended in freezing mix (70% RPMI, 20% FCS and 10% DMSO), and frozen at −1°C/min in a freezing container (Mr Frosty, Nalgene) in a −80°C freezer.

2.5. Exercise interventions

2.5.1. Partly-supervised exercise group

The partly supervised group undertook 2 supervised (laboratory-based treadmill walking and cycling) and 1 unsupervised session per week (e.g., outdoor walking) progressing from 35 to 50 min and 55% to 70% $\dot{V}O_2$max (Table 2). By week 7, the exercise prescription aligned with common physical activity recommendations (i.e., 150 min per week of moderate-vigorous intensity activity). During supervised laboratory sessions, intensity was confirmed and adjusted using indirect calorimetry. The intensity of unsupervised sessions was recorded using a chest-worn heart rate monitor (Wahoo Fitness, Atlanta, Georgia, USA).

2.5.2. Remotely-supported exercise group

The remotely-supported group received a target for a total duration of exercise each week (e.g., outdoor walking) progressing in duration from 105 to 150 min and progressing in intensity from 55% to 70% $\dot{V}O_2$max (Table 2). By week 7, the exercise prescription aligned with common physical activity recommendations (i.e., 150 min per week of moderate-vigorous intensity activity). Participants were advised how they could breakdown their target into manageable bouts (e.g., 3 × 35 min walks = 105 min in week 1) and were instructed to accumulate their exercise with a minimum bout-length of 10 min. Intensity was checked by participants using heart-rate thresholds that corresponded to their cardiorespiratory fitness. Participants took part in a weekly 30 min telephone call to discuss the exercise they completed, as documented by a web-based data visualisation platform with data input from a wrist worn fitness tracker (Polar A370, Polar Electro, Kempele, Finland) that recorded accelerometery data and heart rate via photoplethysmography.

2.5.3. Adherence

In the partly-supervised group, adherence was assessed by attendance and completion of each supervised session and participant verbal confirmation of completing the unsupervised session, verified via data interpretation of chest-worn heart-rate recording (Wahoo Fitness, Atlanta, Georgia, USA). In the remotely-supported group, adherence was assessed by participant verbal confirmation of reaching the weekly exercise target (duration and intensity) verified via data interpretation from wrist-worn heart rate-recording and accelerometery (Polar A370 fitness tracker (Polar Electro, Kempele, Finland).

2.6. Sample preparation prior to assays

PBMCs were cryopreserved for 6 ± 4 months and thawed rapidly in a 37°C water bath. PBMCs were washed in media (RPMI, 10% Foetal Calf Serum, and 1% penicillin-streptomycin)

TABLE 2 Exercise prescription.

	Week 1 & 2		Week 3 & 4		Week 5 & 6		Week 7 & 8	
	Duration (min)	Intensity (%$\dot{V}O_2$max)	Duration (min)	Intensity (%$\dot{V}O_2$max)	Duration (min)	Intensity (%$\dot{V}O_2$max)	Duration (min)	Intensity (%$\dot{V}O_2$max)
Partly-supervised group	105	55	120	60	135	65	150	70
Remotely-supported group	105	55	120	60	135	65	150	70
Breakdown								
Partly-supervised group	min		min		min		min	
Treadmill	20		25		30		35	
Bike	15		15		15		15	
Total after 1 session each week	35		40		45		50	
Total after 2 sessions each week	70		80		90		100	
Unsupervised walking	35		40		45		50	
Remotely-supported group	Bouts × min		Bouts × min		Bouts × min		Bouts × min	
Advice for achieving duration	3 × 35		3 × 40		3 × 45		3 × 50	
Minimum bout-length	≈11 × 10		12 × 10		≈14 × 10		15 × 10	

For the partly-supervised group, the exercise prescription was divided into 2 supervised and 1 unsupervised sessions per week. The supervised session took place in a laboratory and was further divided into treadmill walking and stationary cycle ergometer exercise. During supervised sessions, intensity was confirmed and adjusted using indirect calorimetry. Unsupervised sessions generally consisted of outdoor walking and intensity was prescribed using target heart rate thresholds corresponding to %$\dot{V}O_2$max.The remotely-supported group were asked to achieve the target duration and intensity (prescribed using target heart rate thresholds corresponding to %$\dot{V}O_2$max) across a given week in exercise bouts no shorter than 10 min bouts. The remotely-supported group were advised how they could breakdown their target into manageable bouts (e.g., 3 × 35 min walks = 105 min in week 1) and exercise generally consisted of outdoor walking.

by centrifuging for 7 min at 300 × *g* and 21°C. If clumping was seen, PBMCs were treated with 0.3 µl of benzonase nuclease (250 U/µl, HC, Novogen) for 5 min, washed with media and centrifuged for 7 min at 300 × *g* and 21°C. PBMCs were counted, resuspended in media at a concentration of 2 million cells per ml and rested overnight for approximately 16 h in tubes that enabled gas exchange, at 37°C and 5% CO_2. After resting overnight, PBMCs were counted and used for flow cytometry (see Section 2.7) and ELISpot assays (see Section 2.8).

2.7. Antibody panels, flow cytometry and data analysis

Two antibody panels were prepared; with 400,000 PBMCs added to a T cell panel and a B cell and Natural Killer (NK) cells panel. An additional isotype control panel was prepared to inform gating of T cell HLA-DR expression. PBMCs were stained with a Fixable Viability Stain (FVS) conjugated to v450 (Beckton Dickinson; Oxford, UK). The T cell panel included anti-CD3—PERCP clone SK7, anti-CD4—PE-Cy7 clone L200, anti-CD8—APC clone SK1, anti-CD45RA—FITC clone HI100, anti-CD127—APC-Cy7 clone A019D5, anti-HLA-DR—V500 clone G46-6, anti-CD27—PE clone M-T271 and anti-CD95—BV605 clone DX2. The B cell and NK cell panel included anti-CD3—PE-Cy7 clone SK7, anti-CD19—PERCP clone 4G7, anti-CD10—APC-Cy7 clone HI10a, anti-CD27—APC clone L128, anti-CD38—FITC clone HB7, anti-CD16—V500 clone 3G8 and anti-CD56—PE clone B159. The isotype control panel included HLA-DR—V500 isotype control (IgG2a, κ, clone G155-178) and anti-CD3—PERCP clone SK7. Antibodies were purchased from BD Biosciences (Beckton Dickinson; Oxford, UK) with the exception of anti-CD127—APC-Cy7, anti-CD95—BV605 and anti-CD10—APC-Cy7 (Biolegend, California, US).

Samples were analysed using a FACSAria III flow cytometer (Beckton Dickinson; Oxford, UK), within two hours of preparation. Voltages were optimised and maintained for all participants and all samples and acquisition flow rate was also maintained. Single-stained tubes containing positive and negative compensation beads (Beckton Dickinson; Oxford, UK) were used to perform compensation each day and calculated automatically (BD FACS DIVA™, Beckton Dickinson; Oxford, UK). Approximately 70,000–100,000 events (T cell panel), 50,000–75,000 events (B cell panel) and 15,000 events (isotype control tube) were recorded from the lymphocyte gate. Data were analysed using Flowjo v10.7.1 (FlowJo. LLC, BD Biosiences, Beckton Dickinson; Oxford, UK).

After excluding doublets via forward-scatter (height) vs. forward-scatter (area) lymphocytes were gated via side-scatter vs. forward-scatter, and viability was assessed (typically >90%). T cells (CD3+) were divided into CD4+ and CD8+ and further defined as Naïve (NA: CD27+CD45RA+), Central Memory (CM: CD27+CD45RA−), Effector Memory (EM: CD27−CD45RA−) and Effector Memory expressing CD45RA (EMRA: CD27−CD45RA+). Using the isotype control, activated T cells were defined as HLA-DR+, and Median Fluorescence Intensity (MFI) was used to examine HLA-DR expression density. Stem cell like memory T cells (TSCMs) were identified as CD95+CD127+ from the CD4+ and CD8+ Naïve T cell pools (48–50). B cells (CD3−CD19+) were divided into Plasmablasts/Plasma cells (CD3−CD19+CD27+CD38+), Memory B cells (CD3−CD19+CD27+CD38−), Naïve B cells (CD3−CD19+CD27−CD10−) and Immature B cells (CD3−CD19+CD27−CD10+). Natural Killer cells (CD3−CD19−CD56+) were further defined into Effector (CD3−CD19−CD56+CD16+) and Regulatory cells (CD3−CD19−CD56+CD16−). Absolute cell counts were computed using the leukocyte differential determined in fresh whole K_3–EDTA blood on the day of sampling (Sysmex Cell Counter Kx 21; Sysmex Europe, Germany) and the proportions of cells computed by the FlowJo software. The CD4+/CD8+ ratio was calculated to examine inverted or high ratios (<1 or >2.5), which have been linked to immunosenescence and chronic inflammation (51–53), and normal range was assumed between ≥1 and ≤2.5 (54).

2.8. ELISpot assays and data analysis

Enzyme-Linked ImmunoSpot (ELISPot) interferon-gamma (IFN-γ) assays were undertaken in a subset of participants with available samples (partly-supervised; $n = 2$; remotely-supported $n = 8$). Under sterile conditions, samples from both the pre- and post-intervention time points from the same participant were assayed on a single 96-well PVDF membrane plate, which had been activated for 30 s with 70% ethanol, washed three times with PBS, and incubated with an anti-IFN-γ antibody (7.5 µg/ml; clone 1D1K, Mabtech, Stockholm, Sweeden) for approximately 16 h at 4°C. After incubation, wells were washed three times with PBS, blocked with 100 µl of media for 1 h, and PBMCs added in 100 µl of media. Wells challenged with tumour-associated antigens contained 500,000 PBMCs, and wells challenged with virus antigens, or the positive control (anti-CD3 OKT3) or the negative control (DMSO) contained 300,000 PBMCs. Occasionally, for participants with low PBMC yields ($n = 3$) a lower number of PBMCs were added to wells challenged with tumour-associated antigens (minimum 300,000) and results adjusted accordingly.

The following antigens, depending on cell yields for each participant, were added to wells at 1 µg/ml (JPT peptide PepMixes; Berlin, Germany). Virus antigens; Cytomegalovirus (CMV) pp65, Varicella Zoster Virus (VZV) IE63 ($n = 8$ participants). Tumour-associated antigens: Mammaglobin, Survivin, Mucin-1 ($n = 10$ participants). Plates were incubated for approximately 16 h at 37°C and 5% CO_2. After incubation, plates were washed eight times with PBS 0.05% TWEEN 20 (200 µl per well) and incubated for three hours with an anti-IFN-γ antibody (1 µg/ml; clone 7-B6-1, Mabtech, Stockholm, Sweeden). Plates were washed eight times with PBS 0.05% TWEEN 20 (200 µl per well) and wells incubated with Streptavidin-Alkaline Phosphatase (diluted 1:1,000; Mabtech, Stockholm, Sweeden) for 1.5 h. Plates were washed eight times with PBS 0.05% TWEEN 20 (200 µl per well) and a further three times with PBS only, before a chromogen substrate (Alkaline phosphatase conjugate substrate kit, Bio-Rad Laboratories Inc.; Watford, UK) was added following manufacturer's instructions. The reaction was stopped after 45–60 min by washing the plate with tap water. The plate was left to dry for at least 24 h before counting spots on an AID classic ELISpot reader [AID software,

Autoimmun Diagnostika GmbH (AID), Strassberg, Germany]. Camera and counting settings were optimized and maintained for all samples and all participants. Data were expressed as spots per million PBMCs.

2.9. Assessment of cytomegalovirus (CMV) serostatus

CMV-specific IgG antibodies were measured in serum using enzyme-linked immunosorbent assays (ELISAs) according to manufacturer instructions (ENZY-WELL, Diesse Diagnostica, Italy). A SPECTROstar Nano plate reader (BMG Labtech Ltd., UK) was used and absorbance was determined at 450 nm. Values were blank corrected, and a 4-parameter curve was used to calculate concentrations. CMV+ was considered to be >1.2 IU/ml, and CMV− was <0.8 IU/ml. Inter and intra assay variation was 1.74% and <5.2% respectively.

2.10. Statistical analysis

Data were examined for normal distribution using descriptive statistics, Shapiro Wilks and Kolmogorov-Smirnov tests. Non-normally distributed data were log10 transformed. Pre- and post-intervention data were examined using repeated measures analyses of variance (ANOVAs) controlling for baseline values and analysing the effect of group. Subsequent post-hoc univariate ANOVAs were conducted for each group separately when appropriate. Effect sizes were reported as eta squared (η^2), where, $\eta^2 = 0.14$ is considered a large effect, $\eta^2 = 0.06$ a medium effect and $\eta^2 = 0.01$ a small effect (55). Statistical significance was considered at $p < 0.05$. Statistical analyses were performed with SPSS v27.0.1.0 (IBM Corp.; New York, US) and figures were created with GraphPad Prism v9.0.0 for Windows (GraphPad Software; California, US).

3. Results

3.1. Body composition and cardiorespiratory fitness did not change with 8 weeks of exercise training, but some measures of functional fitness were improved

Intervention adherence was generally higher in the partly-supervised group compared to the remotely-supported group (88 ± 8% vs. 70 ± 25% of sessions completed) but this difference was not statistically significant ($F_{(1,19)} = 4.150$, $p = 0.056$, $\eta^2 = 0.187$). All sessions were attempted by all participants (i.e., no sessions were missed), therefore the lack of adherence reflects failure to achieve the prescribed duration and intensity of sessions. Body composition and cardiorespiratory fitness did not change with eight weeks of exercise training (main effect: $F_{(1,17)} < 1.144$, $p > 0.300$, $\eta^2 < 0.063$) (**Tables 3**, **4**). However, distance walked in the six minute walk test increased (main effect: $F_{(1,17)} = 12.970$, $p = 0.002$, $\eta^2 = 0.433$) driven by a statistically significant increase in the partly-supervised group, which was 5% greater compared to the remotely-supported group (one-way ANOVA for the partly-supervised group: $F_{(1,8)} = 14.020$, $p = 0.006$, $\eta^2 = 0.637$) (**Table 4**). In addition, the sit to stand score improved (main effect: $F_{(1,17)} = 11.368$, $p = 0.004$, $\eta^2 = 0.401$), with improvements in both groups (partly-supervised; $F_{(1,8)} = 4.301$, $p = 0.072$, $\eta^2 = 0.350$; remotely-supported; $F_{(1,8)} = 6.830$, $p = 0.031$, $\eta^2 = 0.461$). There was also a trend for an improvement for the get up and go test (main effect: $F_{(1,17)} = 3.417$, $p = 0.082$, $\eta^2 = 0.167$). There were no statistically significant time × group interaction effects.

3.2. Total leukocyte counts did not change with 8 weeks of exercise training and CMV-specific IgG declined

There were no significant differences in total leukocytes, lymphocytes, monocytes and neutrophils with eight weeks of exercise training (main effect: $F_{(1,17)} < 0.925$, $p > 0.350$, $\eta^2 > 0.052$), and each group responded similarly (interaction effect: $F_{(1,17)} < 2.629$, $p > 0.123$, $\eta^2 < 0.134$) (**Table 5**). Among CMV + participants, CMV-specific IgG significantly decreased ($F_{(1,6)} = 6.602$, $p = 0.042$, $\eta^2 = 0.524$) but each group responded similarly (interaction effect: $F_{(1,6)} = 1.262$, $p = 0.304$, $\eta^2 = 0.174$). However, the magnitude of the decrease in the partly-supervised group was 7% greater compared to the remotely-supported group and was closer to statistical significance (one-way ANOVA for the partly-supervised group: $F_{(1,3)} = 9.206$, $p = 0.056$, $\eta^2 = 0.754$).

3.3. T cell subset counts were largely unchanged with 8 weeks of exercise training, but CD4+ EMRA T cells declined

CD4+ and CD8+ T cells and most of their sub-types did not change with eight weeks of exercise training (main effect: $F_{(1,17)} < 2.871$, $p > 0.108$, $\eta^2 < 0.144$) (see **Figure 1**, panels A–J). However, there were significantly fewer CD4+ EMRA T cells after training across all participants (main effect: $F_{(1,17)} = 5.985$, $p = 0.026$, $\eta^2 = 0.260$). Both groups responded similarly (interaction effect: $F_{(1,17)} = 2.076$, $p = 0.168$, $\eta^2 = 0.109$), although the partly-supervised group decreased 30% more compared to the remotely-supported group (one-way ANOVA for the partly-supervised group: $F_{(1,8)} = 4.849$, $p = 0.059$, $\eta^2 = 0.377$). There were also fewer CD4+ EM cells after training across all participants but this did not reach statistical significance (main effect: $F_{(1,17)} = 4.399$, $p = 0.051$, $\eta^2 = 0.206$). Although there was no main effect for CD8+ EM, there was a statistically significant group × time interaction effect (interaction effect: $F_{(1,17)} = 7.258$, $p = 0.015$, $\eta^2 = 0.299$). When these analyses were repeated in both study groups separately, the partly-supervised group showed an increase in the CD8+ EM counts following training that was close to being statistically significant (Pre-intervention: 22 ± 22

TABLE 3 Body composition.

	Pre-intervention	Post-intervention	Main effect of time	Time × group interaction effect
Body mass (kg)				
Whole group	66.87 ± 7.90	67.21 ± 8.39	$F_{(1,17)} = 0.820$, $p = 0.378$, $\eta^2 = 0.046$	$F_{(1,17)} = 0.002$, $p = 0.962$, $\eta^2 = 0.000$
Partly-supervised	67.05 ± 8.25	67.38 ± 8.50	$F_{(1,8)} = 0.427$, $p = 0.532$, $\eta^2 = 0.051$	
Remotely-supported	66.68 ± 7.97	67.03 ± 8.73	$F_{(1,8)} = 0.639$, $p = 0.560$, $\eta^2 = 0.044$	
BMI (kg/m^2)				
Whole group	25.38 ± 2.98	25.53 ± 3.13	$F_{(1,17)} = 0.676$, $p = 0.422$, $\eta^2 = 0.038$	$F_{(1,17)} = 0.002$, $p = 0.966$, $\eta^2 = 0.000$
Partly-supervised	25.84 ± 3.31	26.01 ± 3.45	$F_{(1,8)} = 0.285$, $p = 0.608$, $\eta^2 = 0.034$	
Remotely-supported	24.91 ± 2.70	25.05 ± 2.87	$F_{(1,8)} = 0.399$, $p = 0.545$, $\eta^2 = 0.047$	
Body fat (%)				
Whole group	36.18 ± 5.88	36.82 ± 5.49	$F_{(1,17)} = 0.458$, $p = 0.507$, $\eta^2 = 0.026$	$F_{(1,17)} = 0.176$, $p = 0.680$, $\eta^2 = 0.010$
Partly-supervised	37.32 ± 4.50	37.16 ± 4.04	$F_{(1,8)} = 0.140$, $p = 0.718$, $\eta^2 = 0.017$	
Remotely-supported	35.03 ± 7.06	36.48 ± 6.86	$F_{(1,8)} = 0.529$, $p = 0.488$, $\eta^2 = 0.062$	
Lean mass (kg)				
Whole group	41.27 ± 4.01	41.52 ± 4.12	$F_{(1,17)} = 1.144$, $p = 0.300$, $\eta^2 = 0.063$	$F_{(1,17)} = 0.004$, $p = 0.952$, $\eta^2 = 0.000$
Partly-supervised	41.31 ± 4.22	41.55 ± 4.34	$F_{(1,8)} = 0.354$, $p = 0.568$, $\eta^2 = 0.042$	
Remotely-supported	41.22 ± 4.03	41.49 ± 4.12	$F_{(1,8)} = 0.928$, $p = 0.364$, $\eta^2 = 0.104$	
Fat mass (kg)				
Whole group	24.69 ± 5.61	24.80 ± 5.90	$F_{(1,17)} = 0.138$, $p = 0.714$, $\eta^2 = 0.008$	$F_{(1,17)} = 0.271$, $p = 0.609$, $\eta^2 = 0.016$
Partly-supervised	24.92 ± 5.34	25.18 ± 5.37	$F_{(1,8)} = 0.387$, $p = 0.551$, $\eta^2 = 0.046$	
Remotely-supported	24.46 ± 6.15	24.41 ± 6.66	$F_{(1,8)} = 0.016$, $p = 0.904$, $\eta^2 = 0.002$	
Fat mass index (kg/m^2)				
Whole group	9.39 ± 2.22	9.44 ± 2.30	$F_{(1,17)} = 0.128$, $p = 0.725$, $\eta^2 = 0.007$	$F_{(1,17)} = 0.291$, $p = 0.597$, $\eta^2 = 0.017$
Partly-supervised	9.63 ± 2.21	9.74 ± 2.21	$F_{(1,8)} = 0.330$, $p = 0.581$, $\eta^2 = 0.040$	
Remotely-supported	9.16 ± 2.33	9.14 ± 2.47	$F_{(1,8)} = 0.025$, $p = 0.879$, $\eta^2 = 0.003$	

Data are mean ± standard deviation (SD) for breast cancer survivors, as a whole group (n = 20), and also as part of the two different study groups: partly-supervised group (n = 10) and remotely-supported group (n = 10). Repeated measures ANOVA were performed in raw data for variables that were normally distributed (body mass, BMI, lean mass, fat mass, fat mass index), and in log10 transformed data for variables that deviated significantly from the normal distribution (body fat percentage). Statistical significance was considered as $p < 0.05$. Main effect of time indicated as $^*p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$, time × group effect indicated as $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ or $^{\dagger\dagger\dagger}p < 0.001$. BMI, body mass index

TABLE 4 Cardiorespiratory fitness and functional measurements.

	Pre-intervention	Post-intervention	Main effect of time	Time × group interaction effect
$\dot{V}O_2$max (ml kg^{-1} min^{-1})				
Whole group	29.07 ± 5.55	28.25 ± 5.28	$F_{(1,17)} = 0.680$, $p = 0.421$, $\eta^2 = 0.038$	$F_{(1,17)} = 1.029$, $p = 0.325$, $\eta^2 = 0.057$
Partly-supervised	28.11 ± 4.83	28.63 ± 5.49	$F_{(1,8)} = 0.193$, $p = 0.672$, $\eta^2 = 0.024$	
Remotely-supported	30.03 ± 6.30	27.87 ± 5.34	$F_{(1,8)} = 2.467$, $p = 0.155$, $\eta^2 = 0.236$	
Six minute walk (m)				
Whole group	478.0 ± 42.4	508.9 ± 51.3**	$F_{(1,17)} = 12.970$, $p = 0.002$, $\eta^2 = 0.433$	$F_{(1,17)} = 1.783$, $p = 0.199$, $\eta^2 = 0.095$
Partly-supervised	473.6 ± 36.6	516.6 ± 38.9**	$F_{(1,8)} = 14.020$, $p = 0.006$, $\eta^2 = 0.637$	
Remotely-supported	482.5 ± 49.2	501.1 ± 62.6	$F_{(1,8)} = 2.282$, $p = 0.169$, $\eta^2 = 0.222$	
Sit to stand (reps)				
Whole group	16 ± 4	19 ± 5**	$F_{(1,17)} = 11.368$, $p = 0.004$, $\eta^2 = 0.401$	$F_{(1,17)} = 0.000$, $p = 0.998$, $\eta^2 = 0.000$
Partly-supervised	17 ± 4	19 ± 6	$F_{(1,8)} = 4.301$, $p = 0.072$, $\eta^2 = 0.350$	
Remotely-supported	16 ± 3	18 ± 5*	$F_{(1,8)} = 6.830$, $p = 0.031$, $\eta^2 = 0.461$	
Get up and go (s)				
Whole group	5.12 ± 0.67	4.84 ± 0.66	$F_{(1,17)} = 3.417$, $p = 0.082$, $\eta^2 = 0.167$	$F_{(1,17)} = 0.044$, $p = 0.836$, $\eta^2 = 0.003$
Partly-supervised	4.99 ± 0.84	4.85 ± 0.67	$F_{(1,8)} = 0.402$, $p = 0.544$, $\eta^2 = 0.048$	
Remotely-supported	5.26 ± 0.47	4.83 ± 0.67	$F_{(1,8)} = 3.916$, $p = 0.083$, $\eta^2 = 0.329$	

Data are mean ± standard deviation (SD) for breast cancer survivors, as a whole group (n = 20), and also as part of the two different study groups: partly-supervised group (n = 10) and remotely-supported group (n = 10). Repeated measures ANOVA were performed in raw data for variables that were normally distributed (six-minutes' walk and get up and go), and in log10 transformed data for variables that deviated significantly from the normal distribution ($\dot{V}O_2$max and sit to stand). Statistical significance was considered as $p < 0.05$. Main effect of time indicated as $^*p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$, time × group effect indicated as $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ or $^{\dagger\dagger\dagger}p < 0.001$. reps = repetitions, $\dot{V}O_2$max = cardiorespiratory fitness.

TABLE 5 Leukocyte differential and CMV serostatus.

Cell count ($\times 10^9$/L)	Pre-intervention	Post-intervention	Main effect of time controlled for baseline	Time × group interaction effect
Lymphocytes				
Whole group	1.46 ± 0.40	1.41 ± 0.34	$F_{(1,17)} = 0.925$, $p = 0.350$, $\eta^2 = 0.052$	$F_{(1,17)} = 0.220$, $p = 0.645$, $\eta^2 = 0.013$
Partly-supervised	1.52 ± 0.36	1.47 ± 0.32	$F_{(1,8)} = 0.387$, $p = 0.551$, $\eta^2 = 0.046$	
Remotely-supported	1.39 ± 0.45	1.34 ± 0.36	$F_{(1,8)} = 0.503$, $p = 0.498$, $\eta^2 = 0.059$	
Monocytes				
Whole group	0.44 ± 0.19	0.45 ± 0.19	$F_{(1,17)} = 0.087$, $p = 0.772$, $\eta^2 = 0.005$	$F_{(1,17)} = 2.629$, $p = 0.123$, $\eta^2 = 0.134$
Partly-supervised	0.51 ± 0.20	0.54 ± 0.14	$F_{(1,8)} = 0.659$, $p = 0.441$, $\eta^2 = 0.076$	
Remotely-supported	0.36 ± 0.14	0.35 ± 0.19	$F_{(1,8)} = 0.028$, $p = 0.870$, $\eta^2 = 0.004$	
Neutrophils				
Whole group	3.15 ± 1.15	3.10 ± 1.08	$F_{(1,17)} = 0.084$, $p = 0.776$, $\eta^2 = 0.005$	$F_{(1,17)} = 1.466$, $p = 0.242$, $\eta^2 = 0.079$
Partly-supervised	3.61 ± 1.36	3.16 ± 1.08	$F_{(1,8)} = 3.214$, $p = 0.111$, $\eta^2 = 0.287$	
Remotely-supported	2.69 ± 0.68	3.03 ± 1.14	$F_{(1,8)} = 1.523$, $p = 0.252$, $\eta^2 = 0.160$	
CMV IgG (IU/ml)[2]				
Whole group ($n = 9$)	18.11 ± 4.37	16.59 ± 4.47*	$F_{(1,6)} = 6.602$, $p = 0.042$, $\eta^2 = 0.524$	$F_{(1,6)} = 1.262$, $p = 0.304$, $\eta^2 = 0.174$
Partly-supervised ($n = 5$)	16.15 ± 4.89	14.23 ± 3.92	$F_{(1,3)} = 9.206$, $p = 0.056$, $\eta^2 = 0.754$	
Remotely-supported ($n = 4$)	20.57 ± 2.17	19.53 ± 3.47	$F_{(1,2)} = 2.236$, $p = 0.273$, $\eta^2 = 0.528$	

Data are mean standard ± deviation (SD) for breast cancer survivors, as a whole group ($n = 20$), and also as part of the two different study groups: partly-supervised group ($n = 10$) and remotely-supported ($n = 10$). Repeated measures ANOVAs were performed in raw data, as this was normally distributed. Monocytes refer to the "Mixed cells' fraction from an automated haematology analyser: <10% correspond to basophils and eosinophils. [2]CMV IgG concentration for CMV seropositive individuals (partly-supervised group: $n = 5$; remotely-supported group: $n = 4$). Statistical significance was considered as $p < 0.05$. Main effect of time indicated as *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$, time × group effect indicated as $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ or $^{\dagger\dagger\dagger}p < 0.001$. CMV, Cytomegalovirus, IgG, Immunoglobulin G.

cells/μl vs. Post-intervention: 32 ± 29 cells/μl; one-way ANOVA: $F_{(1,8)} = 4.682$, $p = 0.062$, $\eta^2 = 0.369$).

3.4. T cell subset activation was largely unchanged with 8 weeks of exercise training, but CD4+ EMRA T cell activation declined

Overall, after eight weeks of exercise training across groups, there were no changes to basal unstimulated T cell activation, assessed by HLA-DR expression density ($F_{(1,15)} < 1.496$, $p > 0.240$, $\eta^2 < 0.091$). However, for CD4+ EMRA T cells, activation significantly declined following training across all participants (main effect: $F_{(1,15)} = 7.003$, $p = 0.018$, $\eta^2 = 0.318$). Both groups responded similarly (interaction effect: $F_{(1,15)} = 1.413$, $p = 0.253$, $\eta^2 = 0.086$) (see **Figure 1**, panels K–T). The proportion of T cells and their subtypes expressing the activation marker HLA-DR was also assessed as an alternative measure of activation, but this did not change following training ($F_{(1,15)} < 0.841$, $p > 0.374$, $\eta^2 < 0.053$, **data not shown**).

3.5. Partly-supervised exercise significantly reduced the CD4+/CD8+ ratio towards more normal values

Overall across groups, there was no change to the CD4+/CD8+ ratio following eight weeks of exercise training (main effect: $F_{(1,17)} = 2.885$, $p = 0.108$, $\eta^2 = 0.145$) (see **Table 6**), but there was a statistically significant group × time interaction effect (interaction effect: $F_{(1,17)} = 6.525$, $p = 0.021$, $\eta^2 = 0.277$). When these analyses were repeated for both study groups separately, the partly-supervised group showed a decrease in the CD4+/CD8+ ratio following training that was statistically significant (Pre-intervention: 3.90 ± 2.98 vs. Post-intervention: 2.54 ± 1.29; one-way ANOVA: $F_{(1,8)} = 13.795$, $p = 0.006$, $\eta^2 = 0.633$).

Pre-intervention, across all participants, 35% had a normal CD4+/CD8+ ratio (≥1 or ≤2.5) and 65% had a high ratio (>2.5). Post-intervention, the percentage of participants with a normal ratio increased to 45% and the percentage of participants with a high ratio decreased to 55% (Pearson Chi square: 0.417 $p_{2-sided} = 0.748$, **data not shown**). When groups were examined separately, each group exhibited divergent responses. In the remotely-supported group, 30% of participants had a normal ratio and 70% had a high ratio pre-intervention and this distribution was unchanged post-intervention (Pearson Chi square: 0.000 $p_{2-sided} = 1.000$). However, in the partly-supervised group, 40% of the participants had a normal ratio and 60% had a high ratio pre-intervention, but this distribution inverted post-intervention: 60% of partly-supervised participants had a normal ratio and 40% had a high ratio (Pearson Chi square: 0.800 $p_{2-sided} = 0.656$).

3.6. TSCM, B cell and NK cell counts were largely unchanged by 8 weeks of exercise training

The counts of CD4+ and CD8+ TSCMs did not change following eight weeks of exercise training (main effects: $F_{(1,17)} < 1.017$, $p > 0.327$, $\eta^2 < 0.056$) (see **Table 6**) and there were no

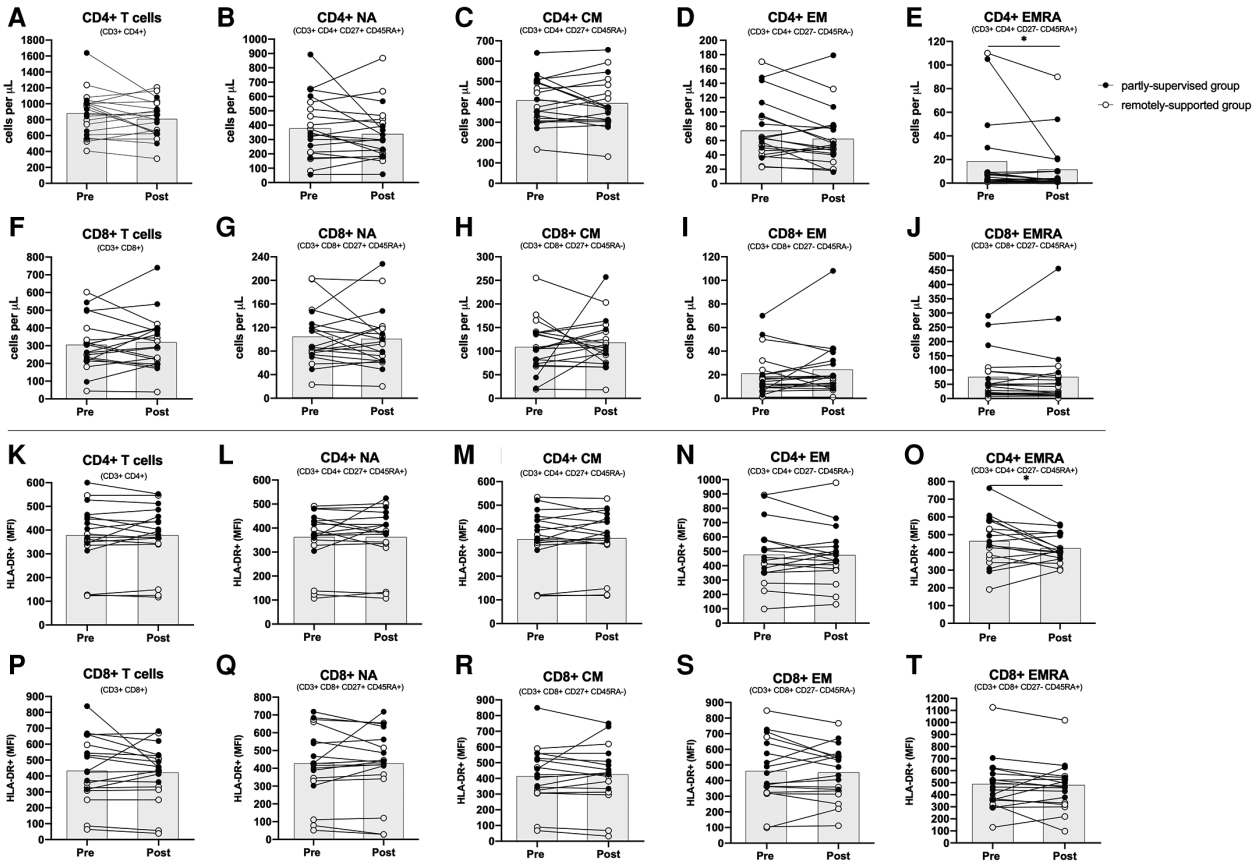

FIGURE 1
Absolute counts and HLA-DR expression density among CD4+ and CD8+ T cell subsets. Absolute counts (**panels A–J**) and HLA-DR expression density expressed as median fluorescence intensity (MFI) (**panels K–T**) for CD4+ and CD8+ T cell subsets: Naïve (NA), Central Memory (CM), Effector Memory (EM) and Effector Memory expressing CD45RA (EMRA). ($n = 20$ for absolute counts; $n = 18$ for activation). Data are displayed for each participant and from both study groups: partly-supervised group (black data points, $n = 10$ for absolute counts and activation) and remotely-supported group (white data points, $n = 10$ for absolute counts; $n = 8$ for activation). Datapoints from the same participant have been joined with lines and the group mean is displayed with the grey bars. Statistical significance from repeated measures ANOVAs using log10 transformed data in the whole group is shown. Statistical significance was considered as $p < 0.05$ (*$p < 0.05$).

statistically significant group × time interaction effects ($F_{(1,17)} < 0.226$, $p > 0.641$, $\eta^2 < 0.013$). Furthermore, the counts of B cells and their subtypes (Plasmablasts/Plasma cells, Memory B cells, Immature B cells and Naïve B cells) did not change with training (main effects: $F_{(1,17)} < 0.865$, $p > 0.365$, $\eta^2 < 0.048$) and there were no statistically significant group × time interaction effects ($F_{(1,17)} < 3.743$, $p > 0.070$, $\eta^2 < 0.180$). In addition, the counts of NK cells and NK cell subtypes (CD16+ effector and CD16− regulatory cells) did not significantly change following training (main effects: $F_{(1,17)} < 1.566$, $p > 0.228$, $\eta^2 < 0.084$). However, there was a statistically significant group × time interaction effect for total NK cells ($F_{(1,17)} = 4.473$, $p = 0.049$, $\eta^2 = 0.208$) and CD16− Regulatory cells ($F_{(1,17)} = 7.755$, $p = 0.013$, $\eta^2 = 0.313$) and the group × time interaction effect for CD16+ effector cells was close to significance ($F_{(1,17)} = 3.647$, $p = 0.073$, $\eta^2 = 0.177$). When these analyses were repeated for both study groups separately, there was a statistically significant increase in CD16− Regulatory cells in the partly-supervised group with training (Pre-intervention: 16 ± 8 cells/µl vs. Post-intervention: 21 ± 10 cells/µl; one-way ANOVA: $F_{(1,8)} = 10.667$, $p = 0.011$, $\eta^2 = 0.571$).

3.7. T cell IFN-γ production in response to stimulation with virus and tumour associated antigens

T cell IFN-γ production in response to stimulation with VZV IE63 did not significantly change with eight weeks of exercise training ($n = 8$, main effect: $F_{(1,5)} = 0.228$, $p = 0.653$, $\eta^2 = 0.044$) and there was no statistically significant group × time interaction effect (interaction effect: $F_{(1,5)} = 5.018$, $p = 0.075$, $\eta^2 = 0.501$). However, this VZV-specific response increased among the partly-supervised group (Pre-intervention; 438 ± 158 spots vs. post-intervention; 811 ± 78 spots) and decreased among the remotely-supported group with training (Pre-intervention; 235 ± 463 spots vs. post-intervention; 129 ± 274 spots) (see **Figure 2**).

T cell IFN-γ production in response to stimulation with CMV pp65 did not significantly change with eight weeks of exercise training ($n = 8$, main effect: $F_{(1,5)} = 0.288$, $p = 0.615$, $\eta^2 = 0.054$), and there was no significant group × time interaction effect ($F_{(1,5)} = 0.001$, $p = 0.982$, $\eta^2 = 0.000$). However, this response decreased among the partly-supervised group (Pre-intervention; 1,364 ± 662 spots vs. post-intervention; 1,063 ± 108 spots) and

TABLE 6 CD4+/CD8+ ratio and absolute counts for TSCMs, B cell and NK cell subsets.

Cells per µl	Pre-intervention	Post-intervention	Main effect of time controlled for baseline	Time × group interaction effect
CD4+/CD8+ ratio				
Whole group	3.85 ± 3.03	3.40 ± 2.89†	$F_{(1,17)} = 2.885$, $p = 0.108$, $\eta^2 = 0.145$	$F_{(1,17)} = 6.525$, $p = 0.021$, $\eta^2 = 0.277$
Partly-supervised	3.90 ± 2.98	2.54 ± 1.29**	$F_{(1,8)} = 13.795$, $p = 0.006$, $\eta^2 = 0.633$	
Remotely-monitored	3.80 ± 3.24	4.26 ± 3.79	$F_{(1,8)} = 0.780$, $p = 0.403$, $\eta^2 = 0.089$	
CD4+ TSCMs				
Whole group	2.35 ± 1.04	2.60 ± 1.60	$F_{(1,17)} = 1.017$, $p = 0.327$, $\eta^2 = 0.056$	$F_{(1,17)} = 0.226$, $p = 0.641$, $\eta^2 = 0.013$
Partly-supervised	2.30 ± 1.16	2.40 ± 1.27	$F_{(1,8)} = 0.191$, $p = 0.674$, $\eta^2 = 0.023$	
Remotely-supported	2.40 ± 0.97	2.80 ± 1.93	$F_{(1,8)} = 0.832$, $p = 0.388$, $\eta^2 = 0.094$	
CD8+ TSCMs				
Whole group	0.30 ± 0.47	0.35 ± 0.49	$F_{(1,17)} = 0.230$, $p = 0.637$, $\eta^2 = 0.013$	$F_{(1,17)} = 0.002$, $p = 0.963$, $\eta^2 = 0.000$
Partly-supervised	0.40 ± 0.52	0.40 ± 0.52	$F_{(1,8)} = 0.000$, $p = 1.000$, $\eta^2 = 0.000$	
Remotely-supported	0.20 ± 0.42	0.30 ± 0.48	$F_{(1,8)} = 0.400$, $p = 0.545$, $\eta^2 = 0.048$	
CD19+ total (B cells)				
Whole group	28.30 ± 28.51	31.20 ± 28.68	$F_{(1,17)} = 0.528$, $p = 0.477$, $\eta^2 = 0.030$	$F_{(1,17)} = 0.012$, $p = 0.914$, $\eta^2 = 0.001$
Partly-supervised	21.70 ± 11.20	27.90 ± 27.27	$F_{(1,8)} = 0.334$, $p = 0.579$, $\eta^2 = 0.040$	
Remotely-supported	34.90 ± 38.66	34.50 ± 31.12	$F_{(1,8)} = 0.163$, $p = 0.697$, $\eta^2 = 0.020$	
Plasmablasts				
Whole group	1.20 ± 1.24	1.10 ± 1.12	$F_{(1,17)} = 0.865$, $p = 0.365$, $\eta^2 = 0.048$	$F_{(1,17)} = 3.743$, $p = 0.070$, $\eta^2 = 0.180$
Partly-supervised	1.10 ± 1.52	0.80 ± 1.32	$F_{(1,8)} = 2.688$, $p = 0.140$, $\eta^2 = 0.251$	
Remotely-supported	1.30 ± 0.95	1.40 ± 0.84	$F_{(1,8)} = 0.689$, $p = 0.431$, $\eta^2 = 0.079$	
Memory B cells				
Whole group	6.00 ± 9.67	5.95 ± 6.52	$F_{(1,17)} = 0.273$, $p = 0.608$, $\eta^2 = 0.016$	$F_{(1,17)} = 0.122$, $p = 0.731$, $\eta^2 = 0.007$
Partly-supervised	2.20 ± 2.82	3.70 ± 6.34	$F_{(1,8)} = 0.366$, $p = 0.562$, $\eta^2 = 0.044$	
Remotely-supported	9.80 ± 12.54	8.20 ± 6.18	$F_{(1,8)} = 0.007$, $p = 0.937$, $\eta^2 = 0.001$	
Immature B cells				
Whole group	1.25 ± 1.59	1.35 ± 1.81	$F_{(1,17)} = 0.000$, $p = 1.000$, $\eta^2 = 0.000$	$F_{(1,17)} = 0.002$, $p = 0.963$, $\eta^2 = 0.001$
Partly-supervised	0.90 ± 0.74	1.30 ± 2.11	$F_{(1,8)} = 0.021$, $p = 0.889$, $\eta^2 = 0.003$	
Remotely-supported	1.60 ± 2.12	1.40 ± 1.58	$F_{(1,8)} = 0.073$, $p = 0.794$, $\eta^2 = 0.009$	
Naive B cells				
Whole group	19.65 ± 17.54	22.55 ± 23.75	$F_{(1,17)} = 0.075$, $p = 0.788$, $\eta^2 = 0.004$	$F_{(1,17)} = 0.744$, $p = 0.400$, $\eta^2 = 0.042$
Partly-supervised	17.00 ± 8.33	21.60 ± 22.19	$F_{(1,8)} = 0.504$, $p = 0.498$, $\eta^2 = 0.059$	
Remotely-supported	22.30 ± 23.76	23.50 ± 26.40	$F_{(1,8)} = 0.112$, $p = 0.746$, $\eta^2 = 0.014$	
CD56+ total (NK cells)				
Whole group	117.65 ± 78.47	120.00 ± 70.26†	$F_{(1,17)} = 1.278$, $p = 0.274$, $\eta^2 = 0.070$	$F_{(1,17)} = 4.473$, $p = 0.049$, $\eta^2 = 0.208$
Partly-supervised	121.00 ± 102.81	136.70 ± 89.65	$F_{(1,8)} = 2.685$, $p = 0.140$, $\eta^2 = 0.251$	
Remotely-supported	114.30 ± 49.01	103.30 ± 42.02	$F_{(1,8)} = 1.359$, $p = 0.277$, $\eta^2 = 0.145$	
CD16+ Effector cells				
Whole group	98.25 ± 75.71	100.35 ± 67.80	$F_{(1,17)} = 1.357$, $p = 0.260$, $\eta^2 = 0.074$	$F_{(1,17)} = 3.647$, $p = 0.073$, $\eta^2 = 0.177$
Partly-supervised	105.10 ± 99.44	116.30 ± 86.79	$F_{(1,8)} = 2.234$, $p = 0.173$, $\eta^2 = 0.218$	
Remotely-supported	91.40 ± 45.91	84.40 ± 40.09	$F_{(1,8)} = 0.555$, $p = 0.478$, $\eta^2 = 0.065$	
CD16– Regulatory cells				
Whole group	19.30 ± 10.37	19.60 ± 8.99†	$F_{(1,17)} = 1.566$, $p = 0.228$, $\eta^2 = 0.084$	$F_{(1,17)} = 7.755$, $p = 0.013$, $\eta^2 = 0.313$
Partly-supervised	16.00 ± 7.83	20.50 ± 10.28**	$F_{(1,8)} = 10.667$, $p = 0.011$, $\eta^2 = 0.571$	
Remotely-monitored	22.60 ± 11.89	18.70 ± 7.95	$F_{(1,8)} = 3.742$, $p = 0.089$, $\eta^2 = 0.319$	

Data are mean standard ± deviation (SD) for breast cancer survivors, as a whole group ($n = 20$), and also as part of the two different study groups: partly-supervised group ($n = 10$) and remotely-supported group ($n = 10$). Repeated measures ANOVAs were performed in log10 transformed data (or log10 + 1 in the case of CD4+ and CD8+ TSCMs, Plasmablasts, Memory B cells and Immature B cells) as the majority of variables were not normally distributed. Statistical significance was considered as $p < 0.05$. Main effect of time indicated as *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$, time x group effect indicated as $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ or $^{\dagger\dagger\dagger}p < 0.001$.

increased among the remotely-supported group with training (Pre-intervention; 547 ± 728 spots vs. post-intervention; 581 ± 706 spots).

T cell IFN-γ production in response to stimulation with Mammaglobin did not significantly change with eight weeks of exercise training ($n = 10$, main effect: $F_{(1,7)} = 0.469$, $p = 0.515$, $\eta^2 = 0.063$) and there was no significant group × time interaction effect ($F_{(1,7)} = 0.259$, $p = 0.626$, $\eta^2 = 0.036$). However, this response increased among the partly-supervised group (Pre-intervention; 34 ± 6 spots vs. post-intervention; 56 ± 28 spots) and also the remotely-supported group with training (Pre-intervention; 46 ± 46 spots vs. post intervention; 50 ± 38 spots).

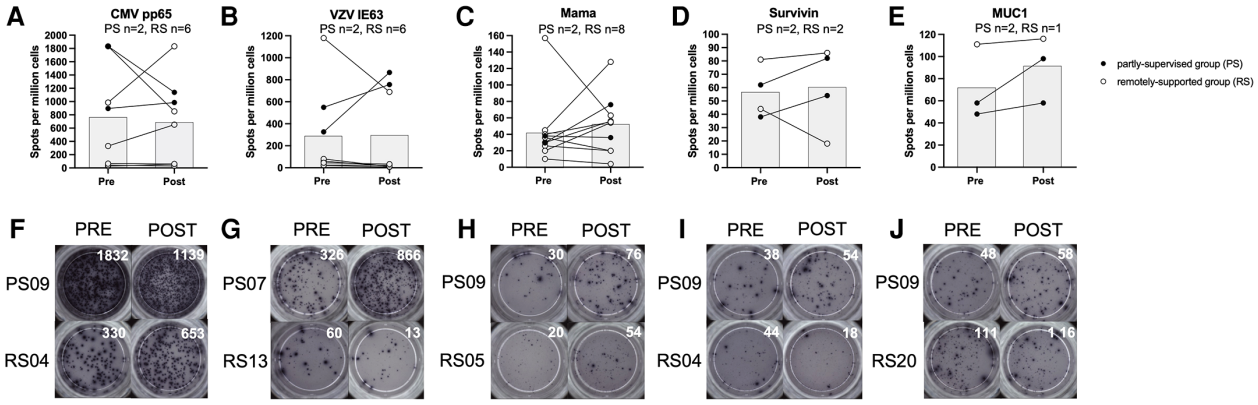

FIGURE 2
T cell interferon-gamma production in response to stimulation with virus and tumour-associated antigens. Data is expressed as spots per million cells and reflects IFN-γ production from T cells as a result of antigen recognition (panels A-E). Individual timepoints for the same participant have been joined with lines and the group mean is displayed with the grey bars. Repeated measures ANOVAs using log10 transformed data were performed for CMV pp65, VZV IE63 and Mama (Mammaglobin). Data for Survivin and MUC1 (Mucin-1) is presented for qualitative interpretation, as statistical comparisons were not appropriate due to the small sample size. Statistical significance was considered as $p < 0.05$. Representative examples of ELISPot wells from individual participants from each study group have been shown for each antigen, pre vs. post intervention (panels F-J).

T cell IFN-γ production in response to stimulation with Survivin and Mucin-1 were not examined statistically due to a small sample size, but data are shown for qualitative comparison and interpretation. However, there was a similar pattern of response as shown for Mammaglobin. For Survivin, the response increased with eight weeks of exercise training in the partly-supervised group (Pre-intervention; 50 ± 17 spots vs. post-intervention; 68 ± 20 spots), and it decreased in the remotely-supported group (Pre-intervention; 63 ± 26 spots vs. post-intervention; 52 ± 48 spots). For Mucin-1, the response increased in the partly-supervised group (Pre-intervention; 53 ± 7 spots vs. post-intervention 78 ± 28 spots), and was largely unchanged in the remotely-supported group ($n = 1$, Pre-intervention; 111 spots vs. post-intervention; 116 spots).

4. Discussion

This study examined immune cell characteristics before and after an eight-week exercise intervention among breast cancer survivors, randomly allocated to a partly-supervised exercise group or a remotely-supported exercise group. Using multi-parameter flow cytometry, we assessed absolute counts of lymphocyte subtypes and basal unstimulated activation levels of T cells in peripheral blood. In addition, using Enzyme-Linked ImmunoSpot (ELISpot) assays, we examined T cell IFN-γ production in response to stimulation with virus and tumour-associated antigens. In summary, we showed that, although total leukocyte counts did not change with eight-weeks of exercise training, changes were evident among some leukocyte subtypes. CD4+ EMRA T cells significantly decreased in number and their activation levels declined across all participants, irrespective of intervention group. In addition, the partly-supervised group exhibited a return of the CD4+/CD8+ ratio towards more normal values and a significant increase in the numbers of CD16– Regulatory NK cells. Eight-weeks of exercise training had no effect on T cell IFN-γ production in response to stimulation with virus antigens from CMV and VZV or tumour-associated antigens including Mammaglobin, Survivin and Mucin-1.

The significant decrease of CD4+ EMRA T cells in blood following exercise training could be interpreted as a sustained redistribution of cells to tissues, as has been demonstrated previously (56, 57). Indeed, it has been hypothesized that repeated bouts of exercise could increase the numbers of effector T cells in tissues, which might facilitate cancer immunosurveillance, even in the absence of a clinically detectable tumour (30). In support, studies across a range of pre-clinical tumour models in mice have shown that compared to non-exercising mice, exercise bouts following tumour challenge lead to a greater tumour infiltration of several immune cell subtypes —including T cells—and smaller/slower tumour growth (58). Mechanistically, this finding could be explained by acute bouts of exercise mobilising tissue-homing cells into blood, which subsequently migrate to tissues in the hours after exercise, most likely being attracted to sites of inflammation, such as tumours. However, a finding which is harder to explain, is that in the same study (58), mice undertaking exercise bouts for 4 weeks prior to tumour challenge (but not after) exhibited a greater tumour-infiltration of immune cells and smaller/slower tumour growth, than non-exercising mice. A possible explanation is a gradual exercise-induced accumulation of effector cells in tissues, so that these cells are ready to respond to malignant transformation if encountered. Indeed, the delivery and accumulation of these immune cells within tissues could be influenced by an exercise-induced increase in pan-tissue vascularization (59, 60). Thus, in the present study, the significant decrease of CD4+ EMRA T cells in blood—which exhibit strong tissue-homing potential—could indicate, in humans, that regular exercise increases the trafficking of immune cells from blood to tissues, as part of immune surveillance (61–64). If proven directly, then this could be one mechanism for how regular exercise reduces the risk of cancer overall, and

prevents cancer recurrence, by facilitating the detection and elimination of pre-malignant or malignant cells in tissues (30).

An alternative interpretation for the fall in CD4+ EMRA T cells in blood after eight weeks of exercise training is an exercise-induced anti-immunosenescence effect, which is thought to be driven by three processes. First, cells of a late-stage differentiated phenotype are mobilised into blood during exercise bouts. Second, these cells extravasate from blood, homing to tissues, where they are exposed to pro-apoptotic stimuli. Third, the naïve T cell pool expands, due to exercise-induced thymopoiesis and/or extrathymic T cell development in tissues such as the liver (65–67). Several studies have provided evidence in support of this hypothesis. For example, a study in 32 healthy young women aged 18–29 years examined exercise-induced changes in T, B and NK cell subtypes in blood (68). Participants undertook either a weekly 90 min session of aerobic-type total-body-shaping workouts (TBSW) or Pilates workouts (PW) for 14 weeks. The TBSW group showed increases in the percentage of naïve B cells and CD8+ NA T cells ($p < 0.0428$), while CD4+ CM T cells and CD8+ EMRA T cells decreased ($p < 0.0363$) (68). Evidence for an anti-immunosenescence effect of exercise training has also been shown in older populations. For example, 100 healthy older (>65 years) women were randomly assigned to strength endurance training (SET, 40% one-repetition maximum), intensive strength training (IST, 80% one-repetition maximum) or a control group (CON, stretching exercises) (69). The SET group exhibited a significant decline in the numbers and proportions of so-called "senescent prone" CD8+ T cells, defined using CD57 and CD28 ($p < 0.05$) (69). In addition, 29 older (>65 years) sedentary women who took part in a 6 week-long functional conditioning gymnastic exercise program exhibited a significant increase in the proportions of CD8+ NA and CM cells ($p < 0.0408$) and a significant decrease of CD8+ EMRA T cells ($p = 0.0238$) (70). Further support is shown by a study that randomized 40 inactive people aged 60–75 years into either a 6-week low-dose combined resistance and endurance training exercise group or a control group (71). Exercise training returned the CD4+/CD8+ ratio to normal values of between 1.5 and 2.5 ($p = 0.043$), whereas the control group exhibited increases in "senescent-prone" CD8+ T cells defined using CD57 and CD28 ($p < 0.006$) (71). Evidence in clinical populations is lacking, other than a small study of 16 men diagnosed as having pre-diabetes (72). In this study, men were randomised to a 3-week concentric exercise (CE) or eccentric exercise (EE) resistance training group. It was shown that both groups exhibited a significant increase in the proportions of CD8+ NA, CD4+ CM and CD8+ CM ($p < 0.047$) and a significant fall in the proportions of CD4+ and CD8+ EMRA T cells ($p < 0.018$) (72). Thus, the present study is novel, by providing evidence of a possible anti-immunosenescence effect of exercise among cancer survivors.

In addition to being a hallmark of an ageing immune system, high numbers of late differentiated T cells have been associated with poor clinical outcomes in cancer settings. For example, in a study recruiting 89 women with metastatic breast cancer, it was shown that higher frequencies of CD8+ CD28− cells was negatively correlated with progression free survival (73). Patients with ≥24.0% of these cells within the CD8+ T cell pool exhibited median survival of 2 months less compared to patients with a lower frequency (<24.0%) ($p < 0.001$) (73). Thus, whether the fall in CD4+ EMRA T cells in the present study is interpreted as either cell trafficking and accumulation in tissues, perhaps facilitating cancer immune-surveillance, *or* whether this finding is interpreted as an anti-immunosenescence effect of exercise, *both* interpretations could have clinical relevance. In other words, if exercise decreases the counts of late-stage differentiated T cells—which could be defined using many overlapping strategies (74, 75)—then this effect could lead to improvements in recurrence and survival. Although the concept of exercise-induced apoptosis of late-stage differentiated T cells in tissues might at first seem to be in stark contrast to an accumulation of tissue-resident effector cells searching for pre-malignant cells, there is an important nuance. It has been proposed that the late-stage differentiated effector cells selectively removed by exercise are not needed. For example, at least some (e.g., up to 10%) of the T cells with a CD4+ EMRA phenotype in the present study will have been specific for the latent herpes virus *Cytomegalovirus* (CMV) (76). If regular exercise helps to limit viral reactivation by promoting redox balance and an anti-inflammatory environment (77), then it could be speculated that fewer CMV-specific T cells are needed. In support, we show a statistically significant fall in CMV-specific IgG after exercise training, which is considered to be a proxy marker of viral reactivation (78, 79). Similarly and in support, a 6-weeks endurance exercise training study in older (>65 years) women found a significant decrease in the number of "senescence-prone" T cells, but also showed a significant positive correlation between the number and the proportion of these cells and the concentration of CMV IgG (80).

Further evidence from this study supports an anti-immunosenescence effect of exercise. We show a statistically significant group × time interaction effect for a change in the CD4+/CD8+ ratio ($p = 0.021$). This effect was driven by a statistically significant fall in the CD4+/CD8+ ratio among members of the partly-supervised exercise group ($p = 0.006$). Indeed, from this group, a third of breast cancer survivors with a high ratio (>2.5) exhibited a return to a normal ratio, of between 1 and 2.5. This is a promising finding, given that an inverted (<1) or high (>2.5) CD4+/CD8+ ratio has been associated with immunosenescence, the immune risk profile (20) and chronic inflammation (51–53). Further, a very high CD4+/CD8+ ratio has been linked with frailty and poor survival (81). Similar findings have been shown in non-cancer settings. For example, a cross sectional study among adults who were 60–90 years of age, classified participants as being physically active or physically inactive using a combination of directly measured cardiorespiratory fitness and lifestyle questionnaires (53). It was shown that there was a lower percentage of people defined as being physically active with inverted (<1) or increased (>2.5) CD4+/CD8+ ratios compared to people being defined as physically inactive group (active: 44% vs. inactive: 55%).

A strength of this investigation is that we examined a range of lymphocyte populations in blood that have received interest in ageing, cancer and exercise settings. For example, stem cell like

memory T cells (TSCMs)—which are thought to have anti-tumour potential (82)—were examined, but showed no differences with eight weeks of exercise training. In addition, total B cells, memory B cells, immature B cells and naïve B cells and plasmablasts were also examined, and exhibited small non-statistically significant fluctuations in number following training, with divergent trends between groups (i.e., the partly-supervised group exhibited increases, whereas the remotely-supported group exhibited decreases). B cells have been shown to have roles in anti-tumour immunity, by facilitating the optimal stimulation and clonal expansion of T cells (83, 84). In addition, low B cell numbers are part of the immune risk profile (20) and B cell number and function generally decline with immunosenescence (85). Finally, we also showed that the numbers of NK cells and their subtypes did not substantially change with eight weeks of exercise training, although there was a trend for these cells to increase at a whole group level and in the partly-supervised group. Furthermore, CD16− Regulatory NK cells were significantly increased in the partly-supervised group following training. Previous studies have shown that NK cell numbers and cytotoxicity are increased after moderate-intensity continuous training and high-intensity interval training among untrained healthy people (86). Indeed, in the context of the present study, higher NK cell counts have been associated with good prognosis in several cancers (87–89). However, a recent systematic review of studies examining the effects of exercise training on the numbers and functions of NK cells among breast cancer survivors could not find conclusive evidence of a consistent and beneficial effect (90).

T cell activation is essential for a targeted immune response (91) and is positively associated with survival in cancer settings (92, 93) and a good response to treatment (94). Our assessments of basal non-stimulated T cell activation using HLA-DR expression generally showed no change with eight weeks of exercise training. However, for CD4+ EMRA T cells, there was a statistically significant decrease in HLA-DR expression density with training, which could be interpreted as an anti-inflammatory response to exercise. Indeed, chronic "non-specific" T cell activation has been linked to inflammatory processes that can contribute to the development of malignancy and cancer recurrence (95). An alternative explanation for the lower HLA-DR expression on CD4+ EMRA T cells in blood following eight weeks of exercise training is the gradual accumulation of cells in tissues, so that the most activated cells are no longer present in blood (61–64).

For the first time, the present study examined whether T cell IFN-γ production in response to stimulation with virus and tumour-associated antigens changed with eight weeks of exercise training among breast cancer survivors. Although there were no statistically significant changes in antigen-specific T cell responses with training, there was an overall trend for an increase in the response to VZV, Mammaglobin, Mucin-1 and Survivin, especially in the partly-supervised exercise group. T cell IFN-γ production in response to stimulation with CMV pp65 was lower following training, aligning with the idea of better anti-viral control with regular exercise, and supporting our finding of lower CMV-specific IgG following training (77, 80). Strong T cell responses to tumour associated antigens measured in blood is positively associated with good clinical outcomes. For example, in a study of 40 patients with breast cancer, those who had HER2-reactive T cells and a lower proportion of Myeloid derived suppressor cells (MDSCs) before treatment exhibited a 100% rate of survival after 5 years, compared to 38% survival among patients without HER2-reactive T cells and higher frequencies of MDSCs ($p = 0.03$). Similarly, 100% survival over 5 years was exhibited by patients with HER2-reactive T cells and lower frequencies of regulatory T cells compared to 50% survival among patients without HER2-reactive T cells and with higher levels of regulatory T cells ($p = 0.03$) (41). Thus, if regular exercise training can increase the numbers of tumour-reactive T cells in blood, or their magnitude of response, this could be one mechanism by which exercise reduces cancer recurrence and mortality.

The present study did not feature a non-exercise control group. Randomised and controlled trials comparing an exercise group to a non-exercise control group have shown that similar immunological measurements to those examined in the present study, are susceptible to exercise-induced change (e.g. 69, 71). Other studies have shown that exercise training among breast cancer survivors, compared to non-exercise controls, can positively modulate other aspects of immunity, including neutrophil function (e.g. 96). The present study examined whether the magnitude of exercise-induced immunological change (compared to pre-intervention values) was greater with a rigorously controlled intervention (i.e., the partly-supervised group) compared to an intervention that would be more feasible for implementation in clinical settings (i.e., the remotely-supported group). Inclusion of a non-exercise control group would have enabled analyses to isolate the effects of exercise *per se* and would eliminate possible confounding by time (e.g., week-by-week variation). However, changes in immune cell characteristics over eight weeks in the absence of a change in lifestyle or disease are likely to be very small in magnitude (97). It should also be considered that cryopreserved cells were examined in the present study, which may have impacted the phenotypic or functional characteristics of cells present in samples. Although cell viability was >90% and results from positive and negative controls were interpreted carefully, the magnitude of IFN-γ production in functional assays is likely to have been smaller than with fresh samples, however it is unlikely that the pattern of change and overall study results would have been affected. Finally, given the small sample size, the results of the present study provide proof-of-principle evidence that immune cell characteristics and function are susceptible to exercise-induced change among breast cancer survivors.

In summary, we showed that eight-weeks of exercise training decreased the counts and activation levels of CD4+ EMRA T cells among breast cancer survivors. In addition, the partly-supervised exercise group exhibited a return of the CD4+/CD8+ ratio towards more normal values and an increase in the numbers of CD16− Regulatory NK cells. Eight-weeks of exercise training had no effect on T cell IFN-γ production in response to stimulation with virus antigens from CMV and VZV or tumour-

associated antigens including Mammaglobin, Survivin and Mucin-1. However, the response to tumour-associated antigens was maintained or at least slightly larger in magnitude following exercise training. Our results add to the large body of literature, suggesting that exercise is beneficial for breast cancer survivors. Our data could be interpreted as providing evidence of an anti-immunosenescence effect of exercise. Indeed, these changes might elicit changes in the immunological landscape that could have positive implications for anti-cancer immunity, giving further impetus for examining whether these exercise-induced changes can be linked to clinical outcomes in cancer settings.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by NHS research ethics committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JET, DT, LS and MB conceived the overall study design. MB and LS recruited the participants and LS contributed to data collection. AAE contributed to study design and data collection. JET and AAE drafted and revised the manuscript. MB, JPC, DT and LS undertook a critical review of the manuscript, edited and provided intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fspor.2023.1163182/full#supplementary-material.

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Acute exercise induces distinct quantitative and phenotypical T cell profiles in men with prostate cancer

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Background: Reduced testosterone levels can influence immune system function, particularly T cells. Exercise during cancer reduces treatment-related side effects and provide a stimulus to mobilize and redistribute immune cells. However, it is unclear how conventional and unconventional T cells (UTC) respond to acute exercise in prostate cancer survivors compared to healthy controls.

Methods: Age-matched prostate cancer survivors on androgen deprivation therapy (ADT) and those without ADT (PCa) along with non-cancer controls (CON) completed ~45 min of intermittent cycling with 3 min at 60% of peak power interspersed by 1.5 min of rest. Fresh, unstimulated immune cell populations and intracellular perforin were assessed before (baseline), immediately following (0 h), 2 h, and 24 h post-exercise.

Results: At 0 h, conventional T cell counts increased by 45%–64% with no differences between groups. T cell frequency decreased by –3.5% for $CD3^+$ and –4.5% for $CD4^+$ cells relative to base at 0 h with $CD8^+$ cells experiencing a delayed decrease of –4.5% at 2 h with no group differences. Compared to CON, the frequency of $CD8^+CD57^+$ cells was –18.1% lower in ADT. Despite a potential decrease in maturity, ADT increased $CD8^+$perforin$^+$ GMFI. $CD3^+V\alpha7.2^+CD161^+$ counts, but not frequencies, increased by 69% post-exercise while $CD3^+CD56^+$ cell counts increased by 127% and were preferentially mobilized (+1.7%) immediately following the acute cycling bout. There were no UTC group differences. Cell counts and frequencies returned to baseline by 24 h.

Conclusion: Following acute exercise, prostate cancer survivors demonstrate normal T cell and UTC responses that were comparable to CON. Independent of exercise, ADT is associated with lower $CD8^+$ cell maturity (CD57) and perforin frequency that suggests a less mature phenotype. However, higher perforin GMFI may attenuate these changes, with the functional implications of this yet to be determined.

KEYWORDS

androgen deprivation therapy (ADT), exercise oncology, exercise immunology, conventional t cells (Tconv), unconventional t cells, exercise induced immunosuppression

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1. Introduction

Prostate cancer is one of the most common malignancies in U.S. men (1). With 5-year survival rates of ~95% in localized disease (1), treatment for prostate cancer is highly effective but is accompanied by many side effects, including alterations in immune cell function (2–9). A robust immune system is critical for immunosurveillance, with regular exercise being one approach to boost immunity (10, 11). Current exercise oncology guidelines exist (12, 13) but do not include recommendations for immune function. This gap is likely the result of a limited number of high-quality studies in these areas.

Prostate cancer treatments for localized disease include prostatectomy, radiation, and hormone therapy. While effective in reducing tumor growth, these treatments include numerous adverse effects. Specifically, androgen deprivation therapy (ADT) has negative impacts on multiple physiological systems (14), with a direct influence on immune function, although the response is complex and paradoxical. Potential benefits of ADT on circulating immune cell function include increased $CD3^+$, $CD4^+$ and $CD8^+$ cell counts (8), greater T cell proliferation (2), and decreased $CD4^+CD25^+$ (Treg) cells (7). Moreover, ADT increases $CD3^+$ and $CD8^+$ cell infiltration within the prostate (3, 6), although comparable increases in $CD4^+CD25^+$ have also been observed (6). In contrast, others report decreased mitogenic response to stimulation in $CD8^+$ cells (5, 7) or only minimal changes in $CD3^+$, $CD4^+$ and $CD8^+$ populations (4, 5), although naïve $CD4^+$ T cell frequency was consistently elevated (5, 9). Additionally, increases in pro-inflammatory cytokines have been linked to ADT (15, 16). While greater numbers, proliferation, and infiltration by T cells may improve management of the tumor burden, these benefits may be offset by elevations in chronic inflammation (17) that reduces cellular immunity and leads to a pro-tumor environment (18).

Exercise is a safe, effective, and non-pharmacological means of stimulating the immune system (19–22). Current exercise oncology guidelines recommend bouts of both aerobic and resistance exercise performed at moderate to vigorous intensities for >150 min per week (12, 13). The immune system of healthy individuals is highly responsive to acute exercise via increases in cell number, cytotoxic function, and changes in circulating frequency that reflect redistribution of cells into the tissues (10, 23). However, in cancer survivors, this response has been far less studied (24, 25). In prostate cancer specifically, there are very few acute exercise and immune studies. Initially, modest increases in complete blood counts are reported following resistance exercise (20). Our group observed that one bout of aerobic exercise increased $CD3^-CD56^+$ natural killer (NK) cell frequency in men with PCa (21), although ADT tended to attenuate exercise-induced mobilization compared to non-cancer controls. Additionally, NK cell IFNγ expression was higher with ADT which suggests a more immature phenotype, despite no differences in maturity ($CD57^+$) or function (perforin) markers. Recently, high intensity interval exercise increased $CD3^-CD16^+CD56^+$ NK cells, along with $CD3^+CD8^+$ T cells and $CD3^+CD56^+$ natural killer-like T (NKT-like) cell counts (22). NK and T cell $CD57^+$ counts and cytotoxic activity also increased, which appears as a more mature phenotype with higher functional capacity. However, a single bout of acute exercise was insufficient to induce NK cell infiltration within the prostate tissue (19). Collectively, these studies provide an overview of the potential benefits (e.g., increased cell numbers, greater cytotoxicity) of acute exercise during prostate cancer, although NK cells appear overrepresented in oncology populations (24, 26). Consequently, the limited examination of other cell types constitutes a gap in the literature with, to our knowledge, only one study that examines non-NK cell populations in men with prostate cancer (22).

To address this gap, we sought to examine the response of T cell populations to acute exercise in prostate cancer survivors. As part of the adaptive immune system, conventional T cells express a diverse repertoire of α and β chains within the T cell receptor (TCR), along with the co-receptors CD4 and CD8 (27). These cells recognize cancer antigens via the major-histocompatibility complex (MHC)−1 (28) and produce cytotoxic proteins (e.g., perforin, granzyme B) and cytokines to enhance the response of other cells. In contrast, unconventional T cells that includes mucosal associated invariant T (MAIT) cells (29) NKT cells (30) that have properties of both the innate and adaptive immune systems (31). MAIT cells are characterized by an invariant Vα7.2 chain within the TCR and are MR-1 restricted (29) while NKT express NK1.1 (CD161) and are restricted via the non-classical MHC-1 protein CD1d (32). MAIT and NKT cells also produce key cytokines and cytotoxic proteins that activate other immune cells and kill tumor cells, respectively (33). While conventional and unconventional T cells are responsive to acute exercise and share functional properties that play critical roles in tumor management (33), these data arise from healthy populations primarily and the response in men with prostate cancer is unknown. Additionally, previous investigations in prostate cancer often lack comparison groups to contextualize the response. We have previously observed impaired stress hormone release (34) and T cell mobilization (35) following acute exercise in breast and prostate cancer survivors, respectively. As such, including non-cancer controls who represent the normal response and separating PCa survivors based on androgen status may help isolate the effects of testosterone suppression on immune function.

Therefore, the purpose of this study was to determine the response of conventional ($CD3^+$, $CD4^+$ and $CD8^+$) and unconventional T cells (MAIT, NKT-like) to acute, moderate intensity aerobic exercise in prostate cancer survivors with and without ADT relative to age-matched controls without cancer. We hypothesized that both conventional and unconventional T cell populations would be mobilized immediately post exercise before returning to resting levels within 24 h of recovery. With the potential of thymic regeneration following prolonged ADT (8), we further hypothesized that men lacking testosterone would exhibit smaller T cell responses to acute exercise and that $CD8^+$ T cell and NKT-like T cells would have reduced CD57 and perforin levels.

2. Materials and methods

2.1. Design

This study was a pre-planned secondary analysis, with the methodology for this study previously published for the primary outcomes (21, 34). In brief, this 4-visit study was completed across 1–2 weeks. Following an initial familiarization session (visit 1), cardiopulmonary exercise testing (CPET, visit 2) was performed. The main testing sessions (visit 3 and 4) were held on subsequent days ∼1 week after visit 2 in the early morning and at the same time of day to minimize the influence of diurnal variations on hormonal concentrations and immune cell circulation in the periphery.

2.2. Participants

Men diagnosed with prostate cancer on ADT [ADT; $n = 11$, 67 (2 years)] and not on ADT [PCa; $n = 14$, 67 (2 years)] were recruited from physician collaborators and support groups in Melbourne, Australia along with non-cancer controls [CON; $n = 8$, 64 (3 years)]. ADT and PCa were pathologically diagnosed with prostate cancer via biopsy, were inactive (no regular exercise except for walking for previous 6 months) and were screened for acute or chronic conditions that would contraindicate participation in aerobic exercise. Men on ADT were treated with luteinizing releasing hormone agonists (91%) and anti-androgen receptor (9%) medications for 3+ months prior to enrolling in the study. CON reported no previous history of cancer and met all inclusion and exclusion criteria.

Exclusion criteria included uncontrolled prostate cancer, symptomatic cardiovascular disease, any conditions that caused severe pain with exertion, Type 1 diabetes, uncontrolled Type 2 diabetes, history of bone fractures, inability to engage safely in moderate exercise, or lack of medical clearance from their oncologist, urologist, general practitioner or specialist physician. All participants provided written informed consent. Local ethics committees at Peter MacCallum Cancer Centre, Victoria University, and Western Health approved this project. All procedures were conducted in accordance with principles set out in the Declaration of Helsinki.

2.3. Visit 1

To familiarize participants with CPET procedures, participants were fitted with a mask to collect expired gases and to the electronically braked cycle ergometer (Lode, Gronigen, Netherlands). Participants completed 3–4 submaximal stages (0 watts up to 60 or 80 watts). Preassessment guidelines for the main testing session were discussed and included: 2+ hours fasted, no exercise in previous 24 h, and no caffeine or alcohol for previous 12 and 48 h, respectively.

2.4. Visit 2

Body composition was determined using dual-energy x-ray absorptiometry (Hologic, Waltham, MA, USA). Quality control checks were completed daily, and all scans were performed and analyzed by the same certified densitometry technician (EH). A CPET was used to determine peak oxygen consumption (VO_2peak) and exercise trial workloads. Participants completed 1-minute stages with 20 watt increases until volitional exhaustion. Expired gases were sampled every 15 s using automated gas analyzers (Moxus Modular VO_2 System, AEI Technologies, Pittsburgh, PA, USA) calibrated prior to each test. VO_2peak was determined as the average oxygen consumption across the last minute of the CPET. Heart rate was assessed continuously via 12 lead electrocardiogram (GE Case Cardiosoft v6.6 ECG Diagnostic Systems, Palatine, IL, USA) and rate of perceived (RPE) exertion using the original Borg scale at the end of each stage.

2.5. Visits 3 and 4

Approximately 1 week later, participants arrived in the laboratory between 0600 and 0900 and preassessment guidelines were confirmed verbally. A venous catheter was inserted, and an initial resting blood sample was obtained. Participants then completed an acute, intermittent exercise bout consisting of 10 intervals of 3 min of cycling at 60% of peak wattage from the CPET followed by 1.5 min of passive recovery (45 min total time), as adapted from studies in breast cancer survivors (36). Respiratory gases were sampled throughout the trial and the last minute of each exercise stage was used to determine oxygen consumption, respiratory exchange ratio, and the percentage of exercise relative to VO_2peak. Heart rate and RPE were obtained in the last 30 s of all stages. Blood samples were also obtained immediately (0 h) and 2 h after exercise. During the recovery period, participants remained seated and consumed water *ad libitum*. Participants went home and returned to the laboratory 24 h after exercise for the last blood sample. They were asked to consume an identical meal prior to visits 3 and 4.

2.6. Hematology analysis

Complete blood counts from each time point were determined in duplicate and averaged (Sysmex KX-21N, Kobe, Japan), with a maximal white blood cell difference of 0.1 cells/μl between replicates. Hemoglobin and hematocrit were used to estimate plasma volume shifts following exercise (37).

2.7. Peripheral blood mononuclear cells isolation and immunofluorescence

Freshly isolated peripheral blood mononuclear cells were labelled as previously described (38, 39). Briefly, whole blood was

diluted in PBS and isolated using SepMate-50 (Stemcell, Vancouver, BC Canada) following manufacturer instructions. Peripheral blood mononuclear cells were washed, counted via hemocytometer and 2×10^6 cells were aliquoted for immunolabelling.

T cell phenotyping was performed via direct immunofluorescence labelling of cells by identifying surface markers with mouse anti-human monoclonal antibodies (Biolegend, San Diego, CA) for 15 min at 4°C in the dark. Lymphocytes were gated using forward and side scatter parameters, with T cells identified as $CD3^+$ (APC-Cy7), along with $CD4^+$ (V500) and $CD8^+$ (PE Cy7). MAIT cells were identified within total T cells using Vα7.2 (PE) and CD161 bright (BV421), along with the $CD8^+$ subpopulation, while NKT-like cells were $CD3^+$and $CD56^+$ (AF647). CD57 (Pacific Blue) was used as a marker of maturity and differentiation status. In addition, intracellular perforin (PE) was assessed as an indicator of function for $CD8^+$ and CD3+CD56+ NKT-like cells, as performed previously (21). Briefly, cells were washed twice in PBS before fixation and permeabilization following manufacturer instructions (Cytofix/Cytoperm kit, BD Biosciences, San Jose, CA). Intracellular perforin (PE, Biolegend, San Diego, CA) was stained at 4°C in the dark for 30 min. Cells were then washed and resuspended prior to flow cytometry analysis.

2.8. Flow cytometry

500,000 events for each sample were acquired and analyzed via flow cytometry on a BD Canto II running FACSDIVA v6.1 (BD Biosciences) software. Flow cytometry analyses were done in duplicate by blinded investigators (EC, GS) using FCS express v7.0 (Pasadena, CA, USA), with the gating strategy shown in **Figure 1**. Total T cell counts (cells/μl) were determined by multiplying the $CD3^+$ cell frequency with the hematology lymphocyte count (38, 39). T cell subpopulation counts were determined by multiplying the T cell count by the frequency of the respective sub-populations. Intracellular perforin expression was quantified as the frequency of cells staining positive along with using the geometric mean fluorescent intensity (GMFI). Fluorescence minus one (FMO) and single color compensation tubes were used with every experiment.

2.9. Hormone analysis

Prostate specific antigen (R & D Systems, Minneapolis, MN, USA) and total testosterone levels (Abnova, Taipei City, Taiwan) were determined in duplicate using ELISA, as described previously (34).

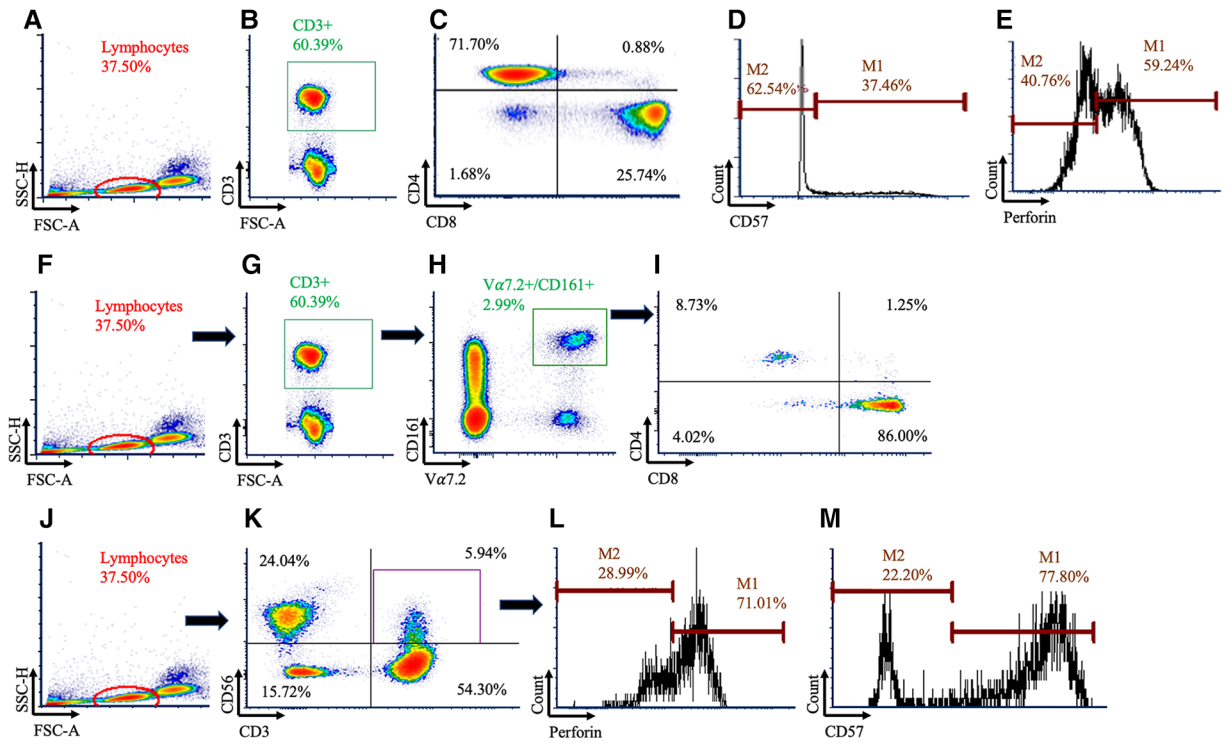

FIGURE 1
Gating strategy used to identify conventional and unconventional T cells. Conventional T cells were identified using (A) lymphocytes, (B) followed by $CD3^+$ cells (C) before being subdivided using $CD4^+$ and $CD8^+$. Within the $CD8^+$ population, histograms were created for (D) CD57 and (E) perforin. Mucosal Associated Invariant T (MAIT) cells were identified using (F) lymphocytes, (G) $CD3^+$, and (H) $V\alpha7.2^+$ and $CD161^+$ cells. (I) MAIT cells were then subdivided using CD4 and CD8. Natural killer (NK)-like T cells were identified using (J) lymphocytes, (K) then $CD3^+CD56^+$ cells, and histograms were created for (L) perforin and (M) CD57.

TABLE 1 Baseline participant characteristics.

	ADT ($n = 11$)	PCa ($n = 14$)	CON ($n = 8$)
Age (y)	67 ± 7	67 ± 7	64 ± 8
Mass (kg)	91.9 ± 19.8[#]	81.0 ± 8.1	75.1 ± 6.4
Height (cm)	173.1 ± 8.1	174.9 ± 6.0	173.1 ± 3.9
% Fat	29.9 ± 6.7[#]	25.3 ± 3.9	21.0 ± 3.5
Comorbidities[a]	1.5 ± 1.1	1.4 ± 1.1	0.6 ± 1.1
PSA (ng/ml)	9.3 ± 26.8	1.6 ± 2.5	1.9 ± 1.2
Total T (ng/dl)	46.3 ± 23.5[†,#]	640.7 ± 354.2	669.2 ± 231.1
Gleason Score	8 ± 1[†]	7 ± 1	–
Diagnosis Days	1,241 ± 1,365	1,307 ± 1,099	–
Prostatectomy (%)	36	43	–
Radiation (%)	55	50	–
Length of ADT (d)	596 ± 383	–	–

Mean ± SD.
[a]Comorbidities is the sum of the following conditions being present: hypertension, hypercholesterolemia, Type II diabetes, smoker, former smoker, and regular alcohol consumption.
[#]$P < 0.05$ vs. CON.
[†]$P < 0.05$ vs. PCa.

2.10. Statistical analysis

Group differences for participant characteristics were assessed using one-way ANOVA with Tukey post-hoc analysis. Immune outcomes were analyzed using a linear mixed model, with group and time as fixed factors and subjects as a random effect (21, 33). Group x time interactions were resolved with simple effects examining the group response at each time point. Non-significant interactions were removed from the model. Raw data are presented as mean ± SD, modelled data are presented as mean ± SE with percent changes and model estimates being expressed relative to baseline or the CON group and include 95% confidence intervals. All data were analyzed using Jamovi v2.25 and figures were created in GraphPad Prism version 9 (La Jolla, CA, USA). Statistical significance was set at $P < 0.05$ for main effects and $P < 0.1$ for group x time interactions, given the exploratory nature of this analysis.

3. Results

3.1. Participants

Men in this study were inactive but relatively healthy otherwise. Men on ADT had higher body mass ($P = 0.019$) and % fat ($P < 0.001$) compared to CON. Apart from total testosterone ($P < 0.001$) and Gleason scores ($P = 0.007$), ADT and PCa were otherwise similar. These data have been published previously (21, 34) and are summarized in **Table 1**.

3.2. Physiological response to acute exercise

Absolute VO_2peak (L/min), maximal HR, and peak power outputs from the CPET were similar between groups with relative VO_2peak (ml/kg/min) being lower for ADT ($P = 0.040$, **Supplementary Table S1**) compared to CON. During the intermittent exercise protocol performed at a standardized load of 60% of peak power output, average heart rate and oxygen consumption during exercise were 83.9 ± 10.2% and 80.7 ± 7.8% of maximum with RPE values that were between 12 and 13 and were similar across groups, as reported previously (34).

3.3. Complete blood count changes

A group x time interaction was present for leukocyte counts ($P = 0.016$, **Supplementary Table S2**). There was an initial increase at 0 h, CON increased by 46%, ADT by 57%, and PCa by 39%, with no group difference. At 2 h, leukocytes decreased slightly from 0 h but remained elevated relative to baseline for CON (33%) and ADT (33%) while PCa increased to 46%, which led to a difference in the change from 0 h to 2 h between ADT and PCa ($+1.7 \times 10^3$ cells/μl; $P = 0.007$). Independent of group, lymphocytes increased by 58% at 0 h before returning to baseline by 2 h and 24 h with similar patterns observed for mixed cells. Neutrophils increased by 46% and 64% at 0 h and 2 h, respectively, but returned to baseline by 24 h. Compared with baseline, plasma volume decreased by −13.4 ± 0.9 ($P < 0.001$) at 0 h, −4.9 ± 1.0 ($P < 0.001$) at 2 h and −2.3 ± 1.0 ($P = 0.039$) at 24 h and were similar across groups.

3.4. Conventional T cell counts and frequencies

There were no group differences for conventional T cell counts or frequencies. At 0 h, $CD3^+$ counts increased by 51% (+457 cells/μl, 95% CI 334, 580; $P < 0.001$, **Figure 2A**), $CD3^+CD4^+$ counts increased by 45% (+273 cells/μl, 95% CI 183, 364; $P < 0.001$, **Figure 2B**), and $CD3^+CD8^+$ counts increased by 64% (+167 cells/μl, 95% CI 113, 221; $P < 0.001$, **Figure 2C**). At 2 h, there was a trend for $CD8^+$ counts to decrease below baseline by 19% (−50 cells/μl, 95% CI −105, 4; $P = 0.074$).

At 0 h, CD3 + cell frequency decreased (−3.5%, 95% CI −6.4, −0.7; $P = 0.018$, **Figure 2D**), while $CD3^+CD4^+$ (−4.5%, 95% CI −7.9, −1.3; $P = 0.008$, **Figure 2E**) decreased initially but then increased at 2 h (+5.6%, 95% CI 2.3, 8.9; $P = 0.001$). $CD3^+CD8^+$ did not increase at 0 h, but decreased below baseline at 2 h (−4.5%, 95% CI −7.6, −1.5, $P = 0.005$, **Figure 2F**).

3.5. Cytotoxic T cell CD57 and perforin counts, frequencies, and expression levels

CD57 was used as a marker of maturity within $CD3^+CD8^+$ cells. At 0 h, $CD3^+CD8^+CD57^+$ counts increased by 75% (+79 cells/μl, 95% CI 31, 127; $P = 0.002$, **Table 2**) with no change in frequency over time. ADT had a lower frequency of $CD3^+CD8^+CD57^+$ cells compared to both PCa (−15.1%, 95% CI −22.5, −7.8; $P < 0.001$) and CON (−18.1%, 95% CI −26.3, −9.9; $P < 0.001$).

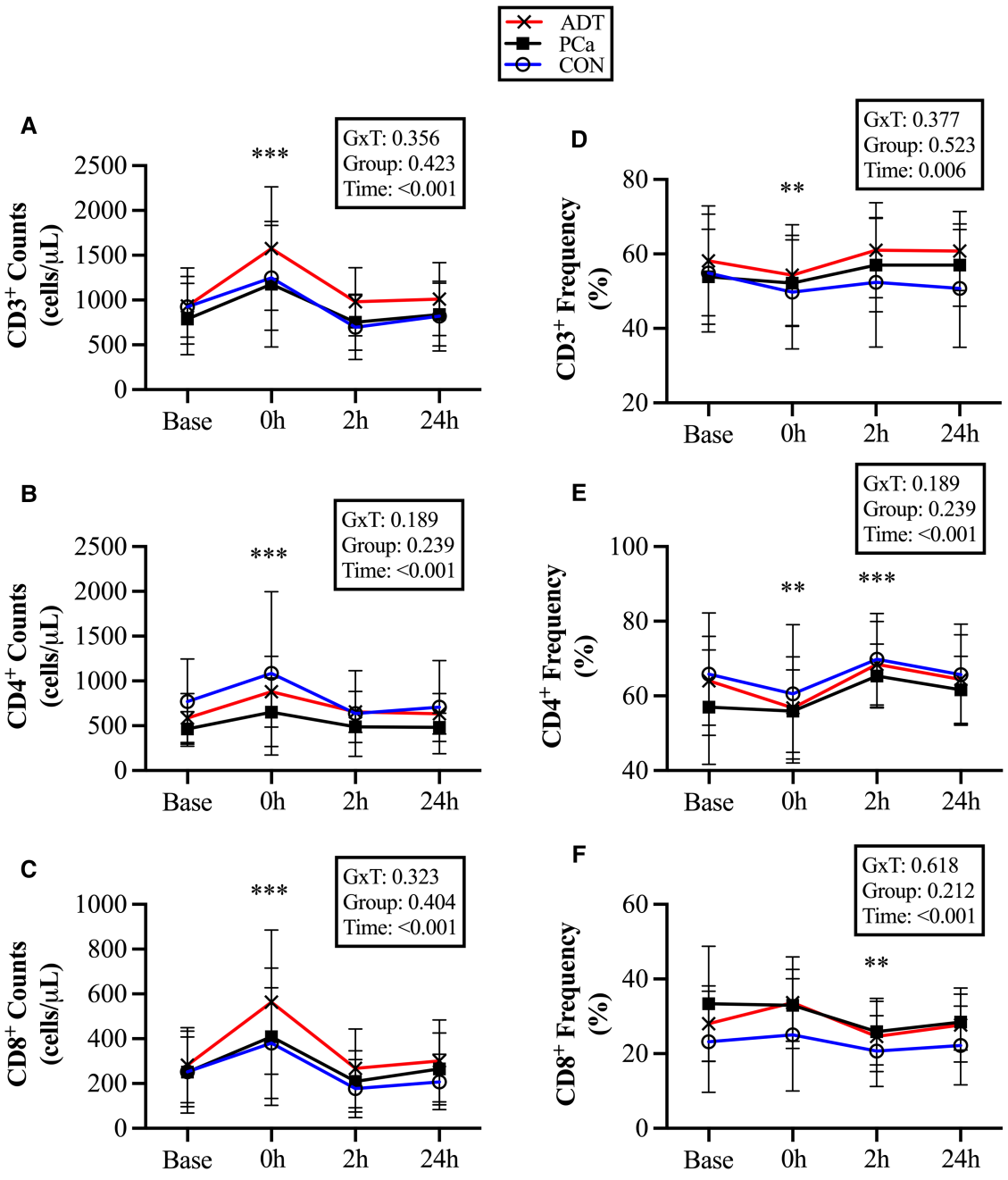

FIGURE 2
Changes in conventional T cell populations at baseline (base) and during recovery from acute aerobic exercise. Cell counts are reported for (A) $CD3^+$, (B) $CD3^+CD4^+$, and (C) $CD3^+CD8^+$ T cells. Cell frequencies reported for (D) $CD3^+$, (E) $CD3^+CD4^+$, and (F) $CD3^+CD8^+$ T cells Data are reported as mean and standard deviation. *, **, *** $P < 0.05$, $P < 0.01$, $P < 0.001$ vs. Baseline.

At 0 h, $CD3^+CD8^+Perforin^+$ counts increased by 68% (+120 cells/µl, 95% CI 60, 181; $P < 0.001$, Table 2) before decreasing below baseline at 2 h (−68 cells/µl, 95% CI −127, −10; $P = 0.026$). For $CD3^+CD8^+Perforin^+$ frequency, there was a group x time interaction ($P = 0.031$). There were no baseline differences. At 0 h, both CON (−26.3%, 95% CI −29.0, −23.6; $P = 0.018$) and PCa decreased (−16.2%, 95% CI −17.0, −15.4; $p = 0.074$), although the latter demonstrated only a trend. ADT was unchanged initially but then decreased below baseline at 2 h (−23.6%, 95% CI 20.9, −26.3, −20.9; $P = 0.008$). For GMFI, ADT $CD3^+CD8^+Perforin^+$ expression was higher than both PCa (+1,851, 95% CI 614, 3,088; $P = 0.007$) and Con (+2,826, 95% CI 1,435, 4,218; $P < 0.001$).

3.6. Unconventional T cell counts and frequencies

At 0 h, $CD3^+V\alpha7.2^+CD161^+$ counts increased by 69% (+17 cells/µl, 95% CI 9, 24; $P < 0.001$, Figure 3A), $CD3^+V\alpha7.2^+CD161^+CD8^+$ counts increased by 69% (+12 cells/µl, 95% CI 7, 17; $P < 0.001$, Figure 3B),

TABLE 2 CD57 and perforin expression in $CD3^+CD8^+$ T cells before and after acute exercise.

		Base	0 h	2 h	24 h
$CD8^+CD57^+$ ($\times10^3$ cells/μl)	Total	105 ± 23	184 ± 24**	83 ± 24	111 ± 23
	ADT	92 ± 36	204 ± 43	90 ± 41	56 ± 38
	PCa	110 ± 38	150 ± 38	68 ± 38	111 ± 38
	CON	113 ± 46	199 ± 46	93 ± 46	166 ± 46
$CD8^+CD57^+$ (%)	Total	42.2 ± 3.2	35.6 ± 3.4	46.3 ± 3.1	42.0 ± 3.3
	ADT	34.3 ± 5.1$^{\#,\dagger}$	26.6 ± 6.3$^{\#,\dagger}$	33.8 ± 5.3 $^{\#,\dagger}$	27.0 ± 5.1 $^{\#,\dagger}$
	PCa	47.6 ± 5.3	44.1 ± 5.1	42.4 ± 5.1	48.2 ± 5.1
	CON	44.7 ± 6.3	36.1 ± 6.3	62.8 ± 5.9	50.7 ± 6.9
$CD8^+Perf^+$ ($\times10^3$ cells/μl)	Total	105 ± 23	184 ± 24**	83 ± 24*	111 ± 23
	ADT	92 ± 36	204 ± 43	90 ± 41	56 ± 38
	PCa	110 ± 38	150 ± 38	68 ± 38	111 ± 38
	CON	113 ± 46	199 ± 46	93 ± 46	166 ± 46
$CD8^+Perf^+$ (%)	Total	71.2 ± 4.0	56.7 ± 4.3	58.7 ± 3.9	52.9 ± 3.9
	ADT	62.5 ± 6.3	61.6 ± 7.9	39.0 ± 6.1*	49.4 ± 6.1
	PCa	76.3 ± 6.3	60.1 ± 6.3	59.5 ± 6.1	60.6 ± 6.1
	CON	74.7 ± 7.9	48.4 ± 7.9*	77.7 ± 7.4	48.8 ± 7.7*
$CD8^+Perf^+$ (GMFI)	Total	2,398 ± 439	3,167 ± 464	3,259 ± 433	3,094 ± 433
	ADT	3,918 ± 688 $^{\#,\dagger}$	4,868 ± 848 $^{\#,\dagger}$	3,735 ± 688 $^{\#,\dagger}$	5,633 ± 688 $^{\#,\dagger}$
	PCa	1,871 ± 718	2,348 ± 688	3,955 ± 688	2,578 ± 688
	CON	1,404 ± 862	2,286 ± 862	2,086 ± 862	1,073 ± 862

Mean ± SE from the estimated marginal mean. When statistical significance is indicated on the Total, this represents a main effect. GMFI, geometric mean fluorescent intensity.

*$P < 0.05$

**$P < 0.01$ vs. baseline.

$^{\#}P < 0.05$ vs. CON.

$^{\dagger}P < 0.05$ vs. PCa.

and $CD3^+CD56^+$counts increased by 127% (+99 cells/μl, 95% CI 68, 131; $P < 0.001$, Figure 3C).

There were no changes in $CD3^+V\alpha7.2^+CD161^+$ or $CD3^+V\alpha7.2^+CD161^+CD8^+$ cell frequency (Figures 3D & 3E), while $CD3^+CD56^+$cells were increased at 0 h (+1.7%, 95% CI 0.8, 2.6; $P < 0.001$, Figure 3F) and 24 h (+1.1%, 95% CI 0.2, 2.1; $P = 0.017$).

3.7. NKT-like cell CD57 and perforin counts, frequencies, and expression levels

At 0 h, $CD3^+CD56^+CD57^+$ counts increased by 138% (+65 cells/μl, 95% CI 43, 88; $P < 0.001$, Table 3) with a non-significant increase in frequency at 0 h followed by a decrease at 2 h (−3.6% 95% CI −9.3, −1.7; $P = 0.006$) compared to baseline.

There was a group x time interaction for $CD3^+CD56^+Perforin^+$ counts, with ADT increasing by 172% at 0 h (+101 cell/uL, 95% CI 60, 142, $P < 0.001$, Table 3) while PCa (+82%, $P = 0.115$) and CON (+92%, $P = 0.295$) experienced smaller, non-significant rises. $CD3^+CD56^+Perforin^+$ frequency revealed an overall effect of time ($P = 0.025$). However, *post hoc* analysis revealed that none of the time points were different from baseline. Perforin GMFI was unchanged across group or time.

4. Discussion

ADT slows tumor progression and increases survival but has many potential side effects, including alterations in the immune system. Regular exercise targets many adverse outcomes, but specifics regarding best practice on enhancing immune function in cancer survivors remains unclear. In the present study, the effects of acute exercise on T cell counts, frequency, and functional markers in men with prostate cancer were examined. We found that (i) the conventional T cell response in prostate cancer survivors is similar to non-cancer controls; (ii) overall CD57 frequency in $CD8^+$ T cells is lower with ADT, suggesting reduced maturation in the absence of testosterone, yet higher perforin expression levels were observed that may represent a compensatory response; and (iii) UTCs demonstrate increased counts with acute exercise in all groups, with NKT-like cells showing preferential mobilization whereas MAIT cells did not. Collectively, these data provide evidence that moderate-vigorous bouts of acute exercise effectively mobilize T cells during PCa, with apparent recovery within 24 h without reduced immune cell counts, frequency, or potential function.

To provide context, the limitations and strengths of the study are presented initially. Limitations include modest sample sizes per group and variable time since diagnosis. Both may have impacted the ability to detect group x time interactions, which is why a value of $P < 0.1$ was used. No direct measurements of cytotoxic function were assessed. Instead, intracellular perforin levels were used as a surrogate measure of cellular function. Finally, NKT-like cells (vs. bona fide NKT cell) were examined, as access to the Cd1D tetramer was not readily available at study inception. The use of NKT-like cells is a common limitation in exercise immunology (33), and likely contributes to the higher-than-expected frequencies observed compared to actual NKT cells (31). Strengths of the study include reporting of all immune outcomes for pooling of data in future analyses (24), examining

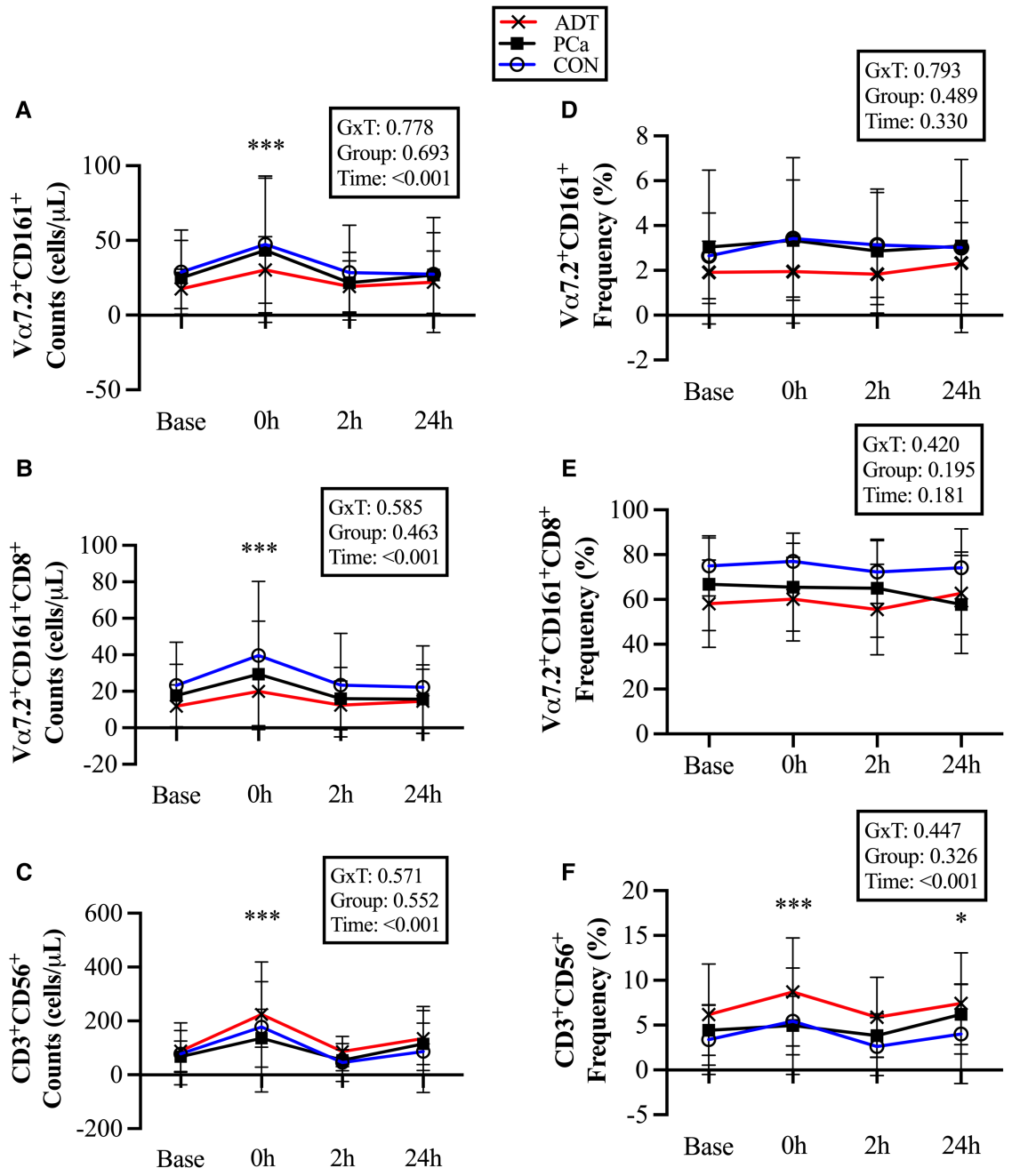

FIGURE 3
Changes in unconventional T cell populations at baseline (base) and during recovery from acute aerobic exercise. Cell counts are reported for (A) $CD3^+V\alpha7.2^+CD161^+$ Mucosal Associated Invariant T (MAIT) cells, (B) $CD3^+V\alpha7.2^+CD161^+CD8^+$ MAIT cells, and (C) $CD3^+CD56^+$ Natural killer (NK)-like T cells. Cell frequencies are reported for (D) $CD3^+V\alpha7.2^+CD161^+$ MAIT cells, (E) $CD3^+V\alpha7.2^+CD161^+CD8^+$ MAIT cells, and (F) $CD3^+CD56^+$ NKT-like cells. Data are reported as mean and standard deviation. *, **, *** $P < 0.05$, $P < 0.01$, $P < 0.001$ vs. Baseline.

PCa and ADT separately to better isolate exercise effects, the use of multiple recovery time points, and the inclusion of more complex phenotyping and investigating UTCs.

In men with prostate cancer, acute exercise produces a typical mobilization of conventional T cells that mirrors that of non-cancer controls. All T cell counts increased at 0 h, with greater relative increases in the $CD8^+$ populations. While $CD3^+$ cell counts increased, the frequency decreased overall as concomitant increases in NK cells occurred at greater rates in these men (21). Additionally, $CD8^+$ T cell frequency increased while $CD4^+$ frequency decreased, with the relative changes of these sub-populations mirroring each other here and elsewhere (35, 39). Overall, these T cell count and frequency changes are consistent with previous work in breast cancer survivors (35) while supporting and extending recent work in prostate cancer survivors (22). A shift towards cells with greater cytotoxicity following adrenergic receptor stimulation is well established, as NK and CD8+ cells demonstrate greater mobilization following

TABLE 3 CD57 and perforin expression in $CD3^+CD56^+$ T cells before and after acute.

		Base	0 h	2 h	24 h
$CD3^+CD56^+CD57^+$ ($\times10^3$ cells/μl)	Total	47 ± 15	112 ± 15***	32 ± 15	62 ± 16
	ADT	48 ± 24	126 ± 24	39 ± 24	64 ± 24
	PCa	38 ± 25	80 ± 25	27 ± 25	54 ± 27
	CON	56 ± 30	131 ± 30	30 ± 30	68 ± 31
$CD3^+CD56^+CD57^+$ (%)	Total	56.6 ± 4.4	59.5 ± 4.4	51.1 ± 4.5**	52.9 ± 4.5
	ADT	52.0 ± 6.8	58.6 ± 6.8	49.3 ± 6.9	46.8 ± 6.8
	PCa	52.5 ± 7.6	55.1 ± 7.6	47.5 ± 7.6	52.8 ± 7.7
	CON	65.3 ± 8.6	64.9 ± 8.6	56.4 ± 8.6	59.2 ± 8.7
$CD3^+CD56^+Perf^+$ ($\times10^3$ cells/μl)	Total	41 ± 14	93 ± 14	29 ± 14	55 ± 14
	ADT	59 ± 21	161 ± 21***	55 ± 22	80 ± 22
	PCa	38 ± 23	69 ± 23	21 ± 23	52 ± 25
	CON	26 ± 27	50 ± 27	11 ± 27	32 ± 28
$CD3^+CD56^+Perf^+$ (%)	Total	51.3 ± 5.7	57.2 ± 5.7	46.7 ± 5.7	47.6 ± 5.8
	ADT	50.8 ± 8.8	62.2 ± 8.8	51.5 ± 8.9	49.5 ± 8.8
	PCa	52.3 ± 9.7	54.3 ± 9.7	42.2 ± 9.7	48.9 ± 10.0
	CON	50.7 ± 11.0	55.0 ± 11.0	46.3 ± 11.0	44.5 ± 11.2
$CD3^+CD56^+Perf^+$ (GMFI)	Total	4,587 ± 517	4,915 ± 512	4,643 ± 520	4,908 ± 525
	ADT	5,012 ± 786	5,281 ± 786	5,268 ± 803	5,664 ± 786
	PCa	4,246 ± 850	4,573 ± 824	4,202 ± 850	4,152 ± 906
	CON	4,504 ± 1,032	4,892 ± 1,032	4,460 ± 1,032	4,908 ± 1,022

Mean ± SE from the estimated marginal mean. When statistical significance is indicated on the Total, this represents a main effect.
**$P < 0.01$
***$P < 0.001$ vs. baseline.

acute exercise, psychological stress, and β-agonist infusion (40). As we previously reported that men with prostate cancer have attenuated epinephrine responses to acute exercise (34), it interesting that neither T cells (current study) or NK cell mobilization (21) were adversely impacted. This suggests that other factors may compensate for the reduced epinephrine response, such as changes in shear stress or myokine release (41).

While conventional T cell mobilization appears to be unimpaired after acute exercise, alterations in CD57 and perforin may have functional implications. We report an increase in $CD8^+CD57^+$ counts post-exercise, which is consistent with previous work in healthy men (42) as acute exercise generally increases mobilization of senescent ($CD28^-CD57^+$) T cells (43). In contrast, the frequency of $CD8^+CD57^+$ cells was unchanged, with a non-significant 6.6% decrease at 0H. The lack of change is similar to our work in NK cells (21), but contrasts recent work using HIIT which found increased CD57 frequency after a graded exercise test (22). Differences in exercise intensity (60% vs. 100% of peak power output in the HIIT study) may provide an explanation. Perhaps more interesting is that ADT decreased the frequency of $CD8^+CD57^+$ cells compared to both PCa and CON, an effect that was independent of exercise, although the total counts were not impacted that suggests no lack of availability of these cells. One interpretation is that castrate levels of testosterone lead to less mature $CD8^+$ T cells, which aligns with our hypothesis. Reports of higher rates of T cell proliferation (2), decreased response to simulation (7) and increases in naïve CD8+ T cells (8) indirectly support this finding. Changes in thymus mass and output may be a potential mechanism explaining the reductions in mature T cells with ADT. Thymic involution occurs with age (44) and increased testosterone levels (45), but can be reversed following castration (8). ADT increased naïve $CD4^+$ T (but not $CD8^+$) cells (5), that supports increased thymic output of less mature cells. However, CD57 was not included in the analysis and the present study did not include $CD4^+CD57^+$ cells, so direct comparisons are limited.

In support of ADT leading to less mature $CD8^+$ cells, perforin frequency was ∼12%–14% lower at baseline. However, the group main effect revealing potential differences between ADT and the other groups was not examined because of the significant group x time interaction. Instead, ADT appeared to delay the decrease in perforin frequency until 2H, whereas CON and PCa both were reduced at 0H, with the reasons behind this currently being unclear. Conversely, perforin GMFI during ADT was increased within the $CD8^+$ cells. A decrease in maturity status (as determined by CD57%) was not anticipated to augment cytotoxic protein expression, as men with prostate cancer report $CD56^{bright}$ NK cells have substantially lower perforin MFI and CD57 expression compared to $CD56^{dim}$ (21). Alternatively, higher perforin expression may be a compensatory shift within these cells. As cytotoxic or stimulation assays were not performed, the functional implications of this remain unknown.

To our knowledge, this is the first instance where the MAIT cell response to acute exercise has been quantified in prostate cancer survivors. There was a 69% increase in counts at 0 h, which falls between the 46% increase reported in breast cancer survivors but less than the 137% of non-cancer controls performing an identical bout of acute exercise (35). Somewhat surprisingly, acute exercise did not preferentially mobilize MAIT cells within the T cell pool. This is contrary to our prior work in healthy and breast cancer populations (35, 38, 39), which report consistent increases of 0.7%–1.7% that occur in an exercise intensity-dependent manner. As the intensity of our intermittent exercise protocol aligns well with other submaximal bouts (35, 38), this

explanation seems unlikely. Alternatively, the proportions of $CD8^+$ MAIT cells was lower (10%–15%) compared to younger men (38, 39). However, this MAIT cell sub-population is less responsive to acute exercise, thereby a lower frequency of these cells argues for (not against) preferential mobilization. Prostate cancer treatment does not appear to alter MAIT cell numbers but decreases proliferation and immune cell IFNγ production (4). However, it is not apparent if this is the direct result of testosterone suppression, as only 67% of patients were on ADT. While not assessed in the current study, acute and chronic exercise have potential to offset ADT-related impairments in MAIT cell cytokine production. For example, TNFα production was greater (with a trend towards increased IFNγ) in healthy young men and older women immediately following a single exercise bout (35, 38). Women with breast cancer improved stimulated TNFα and IFNγ following a 16 week exercise intervention (35), which suggests that training status influences this response.

In NKT-like ($CD3^+CD56^+$) cells, cell counts and frequencies both increased post-exercise. Cell counts for NKT-like cells expressing CD57 and perforin both increased at 0H with only minor changes in frequency. The increased counts were similar to studies in healthy individuals (46) but reduced compared to maximal exercise (22). Together, this indicates there may be an intensity threshold that leads to increased CD57 and Granzyme B frequency (22) that were not observed in our study. With limited work to date, exercise intensity differences seem plausible but need to be investigated within the same study. The mobilization of more mature NKT-like cells with greater cytotoxic protein frequency seems promising, particularly as NKT cells are a target for immunotherapy (47). However, the lack of functional assays using these cells from cancer survivors and the use of CD1d to distinguish classical NKT cells (vs. NKT-like cells) during acute and chronic exercise currently limits our knowledge in this area.

From the findings that the acute exercise response in men with prostate cancer is normal, three implications for consideration regarding this type of physical activity are presented. (i) While a single exercise bout was insufficient (19, 48), multiple sessions enhanced immune cell infiltration of prostate tumors (49) that aligns with data from pre-clinical models (50, 51). (ii) Breast cancer cell culture experiments reveal that acute but not chronic exercise reduce cell viability (52), leading to the hypothesis that "every exercise bout matters" (53). Together, these may be potential mechanisms by which increasing amounts of physical activity reduce prostate cancer specific mortality (54). (iii) Finally, the exercise immune landscape is becoming increasing complex, as more cells are included with greater phenotypic complexity. This reinforces the need to have standardized techniques and outcome reporting (24) such that additional meta-analyses can be performed to better isolate the effects of exercise mode or specific cell types on immune function in cancer survivors (26).

In summary, conventional and unconventional T cell numbers and frequencies in prostate cancer survivors demonstrate a normal response to moderate-vigorous acute exercise, which is consistent with our prior NK cell work. MAIT cells do not undergo preferential mobilization post exercise, with this response in older men conflicting with previous findings. Independent of exercise, ADT had lower $CD8^+$ cell maturity (CD57) and perforin frequency that suggests a less mature phenotype than CON that appears to be influenced by testosterone levels. However, higher perforin MFI may attenuate these changes, although the functional implications of this is yet to be determined. Finally, UTC/T cell responses had normalized by 24H. This implies that consecutive training sessions can be performed with minimal concerns of adverse immune system effects during prostate cancer. However, the response following multiple consecutive bouts of acute exercise needs to be determined to test this hypothesis.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Peter MacCallum Cancer Centre, Victoria University, and Western Health ethics committees.. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Each author contributed to the manuscript in at least two ways. Conception: EH, SS, JV, CB, GK, AH. Data collection: EH, SS, SQ, EK, AH. Data analysis: EH, SS, EC, GS, DB, LS. Drafted manuscript: EH, LBF. Revised and approved the final manuscript: SS, SQ, EC, GS, EK, CB, LS, DB, GK. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fspor.2023.1173377/full#supplementary-material

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