Immunologic Research (2020) 68:422–435
https://doi.org/10.1007/s12026-020-09165-1

ORIGINAL ARTICLE

Effects of whole-body cryotherapy on the innate and adaptive immune response in cyclists and runners

Milena Nasi[1] · Elena Bianchini[1] · Domenico Lo Tartaro[2] · Sara De Biasi[3] · Marco Mattioli[3] · Annamaria Paolini[1] · Lara Gibellini[3] · Marcello Pinti[4] · Anna De Gaetano[4] · Roberta D'Alisera[5] · Laura Roli[6] · Johanna Chester[1] · Anna Vittoria Mattioli[1,7] · Tomassina Polverari[8] · Pasqualino Maietta[9] · Ferdinando Tripi[10] · Omar Stefani[10] · Emanuele Guerra[5] · Gustavo Savino[5] · Tommaso Trenti[6] · Andrea Cossarizza[3,7]

Received: 7 September 2020 / Accepted: 30 October 2020 / Published online: 7 November 2020

Abstract

The study aimed to identify the effects of whole-body cryotherapy (WBC) on immunological, hormonal, and metabolic responses of non-professional male athletes. Ten cyclists and ten middle-distance runners received 3 once-a-day sessions of WBC. Before initiating and after the final WBC session, a full set of hematologic parameters, serum chemistry profile, hormones, circulating mitochondrial (mt) DNA levels, cytokines, and chemokines concentration were evaluated. The phenotype of monocyte, T cells, and B cells was analyzed. mRNA expression of 6 genes involved in inflammasome activation (NAIP, AIM2, NLRP3, PYCARD, IL-1β, and IL-18) was quantified. WBC reduced glucose and C and S protein and increased HDL, urea, insulin-like growth factor (IGF)-1, follicle-stimulating hormone, IL-18, IL-1RA, CCL2, and CXCL8. Intermediate and non-classical monocyte percentages decreased, and the CD14, CCR5, CCR2, and CXCR4 expressions changed in different subsets. Only IL-1β mRNA increased in monocytes. Finally, a redistribution of B and T cell subsets was observed, suggesting the migration of mature cells to tissue. WBC seems to induce changes in both innate and adaptive branches of the immune system, hormones, and metabolic status in non-professional male athletes, suggesting a beneficial involvement of WBC in tissue repair.

Keywords Cryotherapy · Inflammation · Innate immunity · Wounds and injuries · Cytokines

Introduction

Skeletal muscle undergoes continuous repair as a result of its contractile activity. The early phase of muscle regeneration is characterized by the migration/infiltration of innate immune cells, including monocytes and the activation of resident muscle stem cells, which are essential for efficient muscle regeneration. Thus, the inflammation plays a crucial role in tissue repair, and there is a cross-talk between skeletal muscle and the immune system [1]. Regular exercise leads to changes in circulation, metabolism, and in the immune system, with an anti-inflammatory effect, while a mechanical overload associated with intense physical effort is associated with more pronounced muscle necrosis and inflammation [2]. Whole-body

✉ Milena Nasi
milena.nasi@unimore.it

[1] Department of Surgery, Medicine, Dentistry and Morphological Sciences, University of Modena and Reggio Emilia, Modena, Italy

[2] Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy

[3] Department of Medical and Surgical Sciences for Children and Adults, University of Modena and Reggio Emilia, Modena, Italy

[4] Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy

[5] Department of Public Healthcare, Sports Medicine Service, Azienda USL of Modena, Modena, Italy

[6] Department of Laboratory Medicine and Pathology, Azienda USL of Modena, Modena, Italy

[7] National Institute for Cardiovascular Research – INRC, Bologna, Italy

[8] Poliambulatorio San Tommaso, Vignola, Modena, Italy

[9] Department of Quality of Life, "Alma Mater Studiorum", Bologna, Italy

[10] "La Fratellanza 1874" Not-for-profit sport Association, Modena, Italy

cryotherapy (WBC) is the short exposure (few minutes) to dry air at cryogenic temperatures and has recently been applied for the muscle recovery after injury to counteract the inflammatory response due to overload syndrome [2, 3]. Due to its anti-inflammatory effects, WBC start to be used also for pathologies in which the modulation of systemic inflammation could exert beneficial effects, as in the case of rheumatoid arthritis [4, 5], fibromyalgia [6, 7], or ankylosing spondylitis [8, 9]. Moreover, WBC is used with increasing frequency as a method of well-being [10]. Recent reports suggest that one or more WBC sessions can induce acute hormonal, anti-inflammatory, perceptual, and physiological responses [11]. However, most studies investigating the effects of WBC on athletes have evaluated a small panel of both hematological and hormonal parameters and very few cytokines [3]. Further, dedicated studies have not analyzed possible changes in monocytes: cells that migrate to tissue as major drivers of inflammation and tissue repair. In this study, we investigated changes induced by WBC treatment on a variety of immunological parameters and inflammatory markers during the training period of non-professional male cyclists and runners. We analyzed a large panel of cytokines, hematological and hormonal parameters, as well as circulating mitochondrial DNA, a molecule released by necrotic cells able to trigger inflammation acting as a damage-associated molecular pattern. Moreover, to better understand the influence of WBC on innate and acquired immunity, we studied the phenotype of monocytes, T and B lymphocytes, as well as the expression of inflammatory genes in monocytes.

Materials and methods

Subjects

Twenty volunteer non-professional athletes were recruited by the Sports Medicine Unit of Modena for consecutive WBC sessions, proposed over a 3-day schedule. WBC consists of brief exposure (3 min) to extremely cold air (− 190 °C) inside a cylindrical chamber (Cryomed Manufacture s.r.o., Nové Zámky, Slovakia) in which the subject's head and hands remain outside and not subjected to the cold stimulus. Subjects have always worn intimate clothing. Cyclists ($n = 10$, mean age ± SD: 44 ± 5 years) and middle-distance runners ($n = 10$, 38 ± 12 years old) were recruited from dedicated sports clubs. Subjects with known injuries or inflammatory diseases were excluded. During the study, the athletes' training programs were maintained from previous weeks: cyclists trained three times a week (average 30 km/ride) and runners trained once a day (average 60 min/run). For both cyclists and runners, training sessions were in the evenings. WBC sessions (as well as blood and urine collection) were conducted at lunchtime. Regarding the training, cyclists have no requirement for speed; as non-professional athletes, they ride at constant speed for an average of 30 km per day, three times a week. The training of runners was scheduled for alternating a day of 20-min run with an easy warm-up plus aerobic repeats and a day of 40/70 min of endurance running. Regardless of the training day or type, at least 16 h passed between training and blood collection/WBC treatment, avoiding the interference of acute, transient effects of exercise on the immune system. This study was conducted in agreement with ethical recommendations of the Declaration of Helsinki, and all experiments were approved by the Ethics Committee of Area Vasta Nord Emilia Romagna (protocol number 88/2018/SPER/AUSLMO). All the participants gave written informed consent. Volunteers were involved in the reporting and dissemination of our research.

Blood and urine samples collection

Subjects underwent two sessions of capillary blood and urine samples collection: before the initial WBC session (day 1) and immediately following the third and final WBC session (day 3). For each sample collection, 40 ml of venous blood was collected. Hematology, clinical chemistry, and hormonal parameters were assessed at the BLU Laboratory (NOCSAE, Baggiovara, Modena, certification #ISO90012015) according to routine hospital protocol. Immunological analyses were performed in the "Laboratory of Pathology and Immunology" of the University of Modena and Reggio Emilia. Urine samples collected in sterile containers were analyzed by the BLU Laboratory. The study's workflow is summarized in Fig. 1.

Plasma and monocytes isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood using a standard procedure of density-gradient centrifugation. A minimum of 2 million CD14+ cells was isolated from 20 million PBMCs through well-standardized immunomagnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany). Plasma was separated from blood and further centrifuged to remove and discard platelets (at 4 °C, 2850 g for 15 min). The supernatant was collected, aliquoted, and stored at − 80 °C.

Quantification of plasmatic soluble factors

Soluble factors were analyzed in plasma samples by magnetic bead-based multiplex assay for the Luminex® platform (RandD System, Minneapolis, MN, USA). In particular, TNF-α, IL-6, IL-1β, IL-10, IL-12p70, IL-2, IL-8 (CXCL8), and VEGF-A were quantified using a "Luminex Performance Human High Sensitivity Cytokine Magnetic Panel A" while CCL2, CCL19, CXCL13, TNFSF6, IL-1α, IL-1ra, and IL-18 using a "Human Magnetic Luminex Assay."

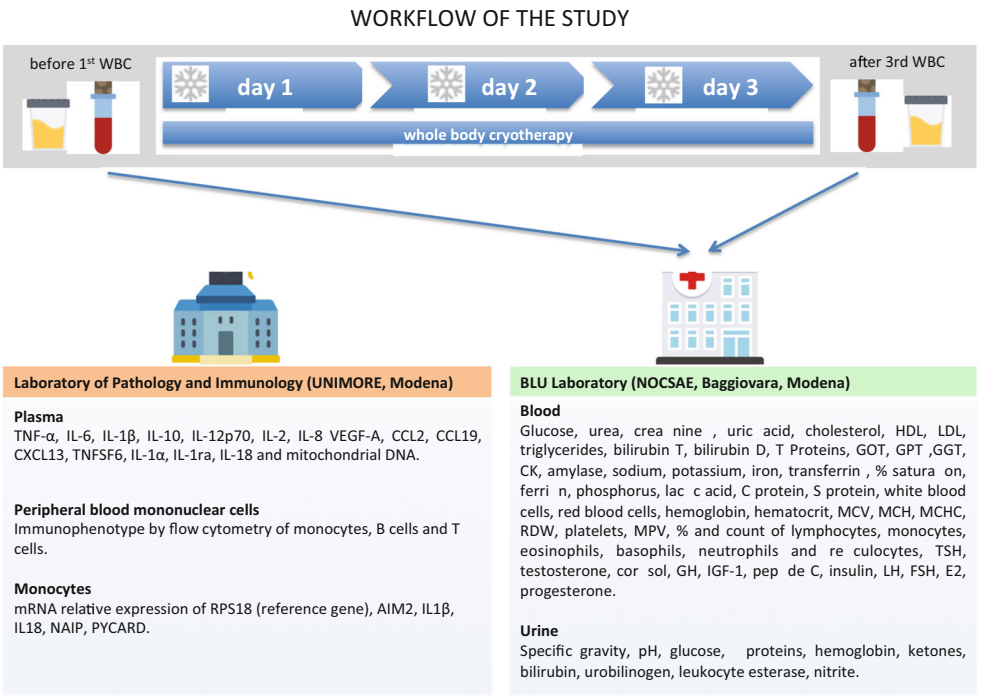

Fig. 1 The study's workflow. Free icons used in the figure are made by "https://www.flaticon.com/authors/smashicons"

DNA extraction from plasma samples and quantification of circulating mtDNA

Total DNA was extracted from plasma samples using a QIAmp DNA Minikit, Qiagen (Alameda, CA, USA), following manufacturer instructions. A droplet digital (dd)PCR assay was used to quantify circulating mtDNA. One microliter of DNA was added to a 20 uL final volume mixture containing 10 uL of 2x ddPCR Supermix for Probes, 1 uL of ddPCR assay for ND2 (UniqueAssayID, dHsaCPE5043508), 1 uL of ddPCR assay for EIF2C1 (UniqueAssayID, dHsaCP2500349), and 7 uL of nuclease-free water (all reagents from Bio-Rad, Hercules, CA, USA). Droplet generation and reading were performed on a Bio-Rad QX200 ddPCR droplet system [12]. Circulating mtDNA content was expressed as the number of copies per milliliter of plasma.

Immunophenotyping of monocytes

Three million cryopreserved PBMCs were thawed and stained with Aqua Live Dead Probe (Thermo Fisher Scientific) and the fluorochrome-conjugated monoclonal antibodies anti-CD16 AF488, anti-CD14 APC, anti-HLA-DR PE-Cy7, anti-CCR2 BV605, anti-CXCR4 PE, and anti-CCR5 BV421 (from BioLegend, San Diego, USA). Different subsets of monocytes were identified by an Attune Nxt flow cytometer (Thermo Fisher Scientific), and data were analyzed by FlowJo 9.9.6 (Ashland, OR, USA) according to recent guidelines [13]. To identify the three main monocytes subsets (classical, non-classical, and intermediate), we applied a previously described sequential gating strategy [14].

Phenotype of B and T cells

Up to 3 million PBMC were stained with the DuraClone IM B or T panel (ref. B53318 and B53328, respectively; Beckman Coulter, BC, FL, USA), and cells were acquired with the Cytoflex LX (Beckman Coulter). For phenotypic analysis of B lymphocytes, a gating strategy was employed according to the manufacturer's instructions. The T cell panel was enriched by the fluorochrome-conjugated mAbs Promokine 840 (for determining viable cells), CD127 BV650, CD25 BV785, CD95 BUV 395, and HLA-DR BUV661 for regulatory T (Treg) cells and activated T cells identification.

Relative quantification of mRNA expression

Cryopreserved monocytes were thawed, and RNA was extracted (QuickRNA miniPrep kit from Zymo Research, Irvine, CA, USA) and reverse transcribed (iScript cDNA synthesis kit from Bio-Rad, Hercules, CA, USA). The CFX96 Touch Detection System (Bio-Rad) was used to quantify mRNA with SYBR Green chemistry. Seven genes involved in inflammasome activation were detected using pre-validated Prime PCR Assay (Bio-Rad) as previously described [10]: RPS18 was the reference gene, AIM2, IL1β, IL18, NAIP, NLRP3, and PYCARD. Relative expression of mRNA was calculated through ΔΔ-cycle method referred to pre-WBC treatment data [15].

Statistical analysis

Quantitative variables were compared between pre- and post-WBC by the Wilcoxon matched-pairs signed-rank test or by two-way ANOVA and Sidak's multiple comparisons test. Correlations between clinical and molecular data were explored with linear regression analysis. p values < 0.05 were considered statistically significant. All data reported in tables are expressed as mean and standard deviation. All data shown in column graphs represented the mean and the standard error of the mean (SEM). Statistical analyses were performed using Prism 6.0 (GraphPad Software Inc., La Jolla, USA).

Results

Blood, plasma, and serological determinations

We summarized in Table 1 the results of blood, plasma, and serological determinations, which resulted in increased or decreased after WBC treatment. Among the 24 blood parameters evaluated by analytical chemistry, cyclists showed a decrease of blood glucose and an increase of urea, and runners experienced a slight decrease of creatinine and an increase of T proteins and phosphorus. In all cases, the laboratory test results were within normal ranges. No appreciable variations in urine tests were registered (specific gravity, pH, glucose, proteins, hemoglobin, ketones, bilirubin, urobilinogen, leukocyte esterase, nitrite; data not shown). Twenty-four blood count determinations were performed, and only protein C and S (two vitamin K-dependent plasma proteins that work together as a natural anticoagulant system) decreased significantly in cyclists. No blood count changes were observed for runners. In all cases, laboratory test results were within normal ranges. Eleven hormones were quantified, and analysis revealed an increase of insulin-like growth factor (IGF)-1 and follicle-stimulating hormone (FSH) in cyclists and only a slight decrease in insulin in runners. In all cases, laboratory test results were within normal ranges. From a total of fourteen soluble factors quantified, an increase of CXCL8 and CCL2 in both cyclists and runners and IL-18 and IL-1ra increase in cyclists were registered. Circulating mtDNA was similar before and after WBC in both groups, but it is lower in runners ($p = 0.029$ and $p = 0.023$, before and after WBC, respectively).

Table 1 Blood, plasma, and serological determinations

	Cyclists			Runners		
	Before	After	p value	Before	After	p value
Blood analytical chemistry						
Glucose (mg/dL)	91.00 ± 6.09	82.90 ± 5.09	*0.0117*	80.60 ± 12.66	83.70 ± 10.87	0.4375
Urea (mg/dL)	35.20 ± 10.76	42.00 ± 10.53	*0.0117*	35.40 ± 6.15	33.60 ± 5.54	0.4746
Creatinine (mg/dL)	0.86 ± 0.12	0.86 ± 0.10	0.8164	0.90 ± 0.10	0.86 ± 0.09	*0.0430*
HDL (mg/dl)	66.50 ± 10.27	68.90 ± 10.77	*0.0391*	66.80 ± 13.38	67.90 ± 12.85	0.4824
T proteins (g/dL)	7.49 ± 0.54	7.60 ± 0.48	0.1563	7.49 ± 0.35	7.88 ± 0.57	*0.0410*
Phosphorus (mg/dl)	3.55 ± 0.55	3.50 ± 0.37	0.7109	3.27 ± 0.35	3.58 ± 0.36	*0.0020*
Blood count determinations						
C protein (%)	116.30 ± 21.47	99.90 ± 21.80	*0.0098*	101.20 ± 17.31	95.91 ± 17.36	0.3076
S protein (%)	98.90 ± 19.40	84.60 ± 16.93	*0.0137*	102.40 ± 14.40	98.27 ± 16.63	0.4746
Hormone analysis						
IGF-1 (ng/ml)	194.20 ± 30.21	221.80 ± 29.97	*0.0059*	242.00 ± 66.74	243.70 ± 69.56	0.8311
Insulin (microIU/ml)	4.50 ± 3.36	3.74 ± 1.80	0.9219	4.58 ± 2.50	3.24 ± 1.60	*0.0488*
FSH (mIU/ml)	4.63 ± 4.86	5.19 ± 5.67	*0.0195*	3.83 ± 1.96	3.92 ± 1.97	0.6523
Cytokines and chemokines						
IL-18 (pg/ml)	415.20 ± 178.00	442.20 ± 172.20	*0.014*	315.17 ± 87.04	321.9 ± 87.56	0.193
IL-1RA (pg/ml)	409.6 ± 140.2	467.3 ± 161.4	*0.002*	375.3 ± 125.6	394.1 ± 197.00	0.625
CXCL8 (IL-8) (pg/ml)	2.54 ± 0.70	3.09 ± 0.90	*0.020*	2.54 ± 1.07	3.26 ± 2.03	*0.008*
CCL2 (pg/ml)	173.80 ± 55.15	198.90 ± 58.39	*0.020*	155.1 ± 26.97	193.8 ± 53.18	*0.027*
Circulating mtDNA						
mtDNA copies/ml	2.978e+06 ± 1.208e+06	3.844e+06 ± 1.326e+06	0.250	1.641e+06 ± 1.510e+06	2.398e+06 ± 1.712e+06	0.3236

Blood analytical chemistry, blood count determinations, hormone and cytokines, and chemokines measured before and after a 3 one-day of WBC treatment. Values are expressed as mean and standard deviation. p values are calculated by Wilcoxon matched-pairs signed-rank test. p values < 0.05 are in italics. *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *T* total, *D* direct, *GOT* glutamic oxaloacetic transaminase, *GPT* glutamic pyruvic transaminase, *GGT* gamma-glutamyl transpeptidase, *CK* creatine kinase, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *RDW* red cell distribution width, *MPV* mean platelet volume, *TSH* thyroid-stimulating hormone, *GH* growth hormone, *IGF-1* insulin-like growth factor, *LH* luteinizing hormone, *FSH* follicle-stimulating hormone, *E2* estradiol. Data are reported as mean and standard deviation

Phenotypic analysis of monocytes

To identify the main monocytes subsets (classical, non-classical, and intermediate), we applied a gating strategy described in Fig. 2. Figure 3 shows the results of monocyte flow cytometry analysis. In particular, we reported only the subsets where a significant change was observed before and after WBC. The percentage of total monocytes did not change. Interestingly, cyclists showed a decrease in intermediate and non-classical monocytes, and runners showed a decrease in non-classical monocytes only. In total monocytes, decreased expressions were observed in CD14, CXCR4, CCR5, and CCR2 for cyclists and in CCR5 and CXCR4 for runners. Within the classical subset, CCR2, CCR5, and CXCR4 expression decreased for cyclists (observed as a trend only in runners). The CCR5 expression in the intermediate subset decreased only for cyclists. No changes were observed for either group in the CCR5, CCR2, and CXCR4 non-classical monocytes expression.

Expression of inflammasome genes

The only gene expression of the main components involved in inflammasome activation that significantly increased during the study period was the IL-1β for all athletes, and this change was significantly higher for cyclists ($p < 0.05$, Sidak's multiple comparisons test; Fig. 4).

Phenotype of B and T cells

For phenotypic analysis of B lymphocytes, a gating strategy was employed according to the manufacturer's instructions (Fig. 5). In cyclists, no changes were observed in the percentage of B cells (CD19+) on total lymphocytes, but, within them, the percentage of CD21low CD38low B cells increased significantly, while, marginal zone, class unswitched, and switched memory B cells decreased after treatment. In runners, we observed no significant variations in B cell subsets, despite exhibiting a similar trend for other cell subsets identified in cyclists (Fig. 6). Figure 7 shows a representative gating strategy for T cell identification. In cyclists, the percentage of CD4+ T cells increased and CD8+ decreased after treatment. Analysis of the T cell differentiation showed no significant changes in CD4+ T cell subsets. In CD8+ EMRA decreased and an increase in naïve T cells were observed.

Exhausted CD4+ and CD8+ T cells decreased as well as Treg cells among CD4+ (Figs. 8 and 9). The percentage of CD4+ and CD8+ T cells did not change in runners. Among CD4+ T cells, they showed a similar trend to that of cyclists for the decrease of exhausted cells. Additionally, naïve decreased and central memory cells increased. Among CD8+ T cells, treatment does not seem to affect cell distribution (Figs. 8 and 9). Finally, we did not find any significant correlations between laboratory and immunological parameters (data not shown).

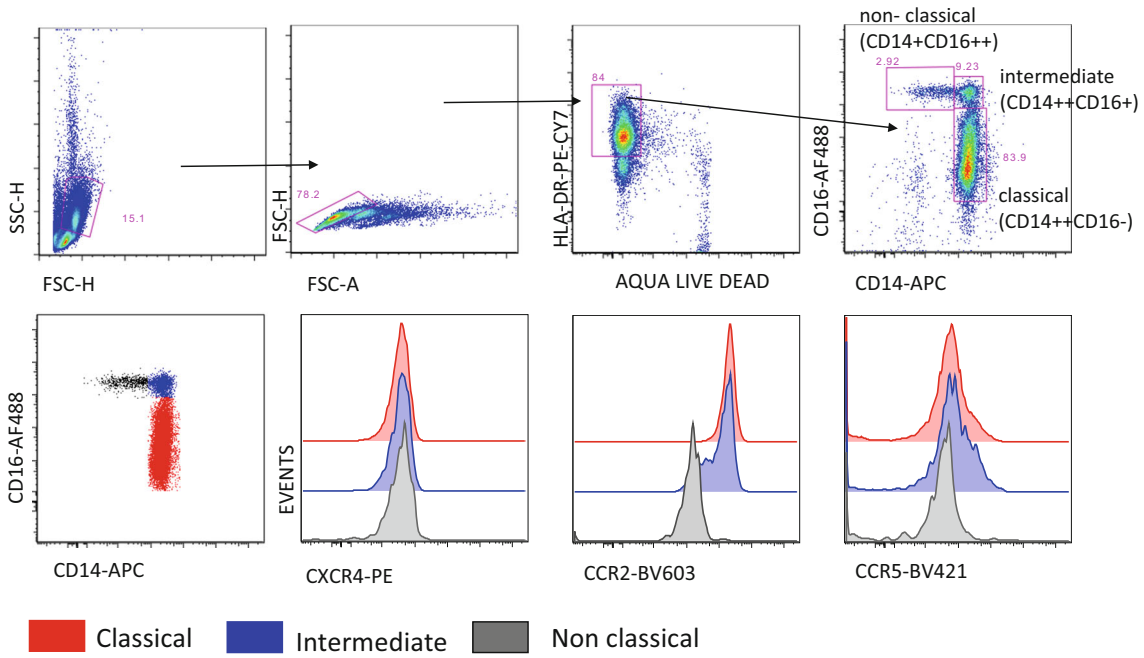

Fig. 2 Gating strategy of monocytes. Monocytes were identified according to physical parameters, i.e., forward scatter-height (FSC-H) and side scatter-height (SSC-H). Then, we excluded cell doublets from the analysis, and we identified live monocytes that express HLA-DR. Finally, we recognized monocyte subpopulations on the basis of CD14 and CD16 expression: classical (CD14++, CD16-), intermediate (CD14++, CD16+), and non-classical (CD14+, CD16+) monocytes. The median fluorescence intensity (MFI) value for the three membrane receptors CCR2, CCR5, and CXCR4 was evaluated in the different monocyte subsets

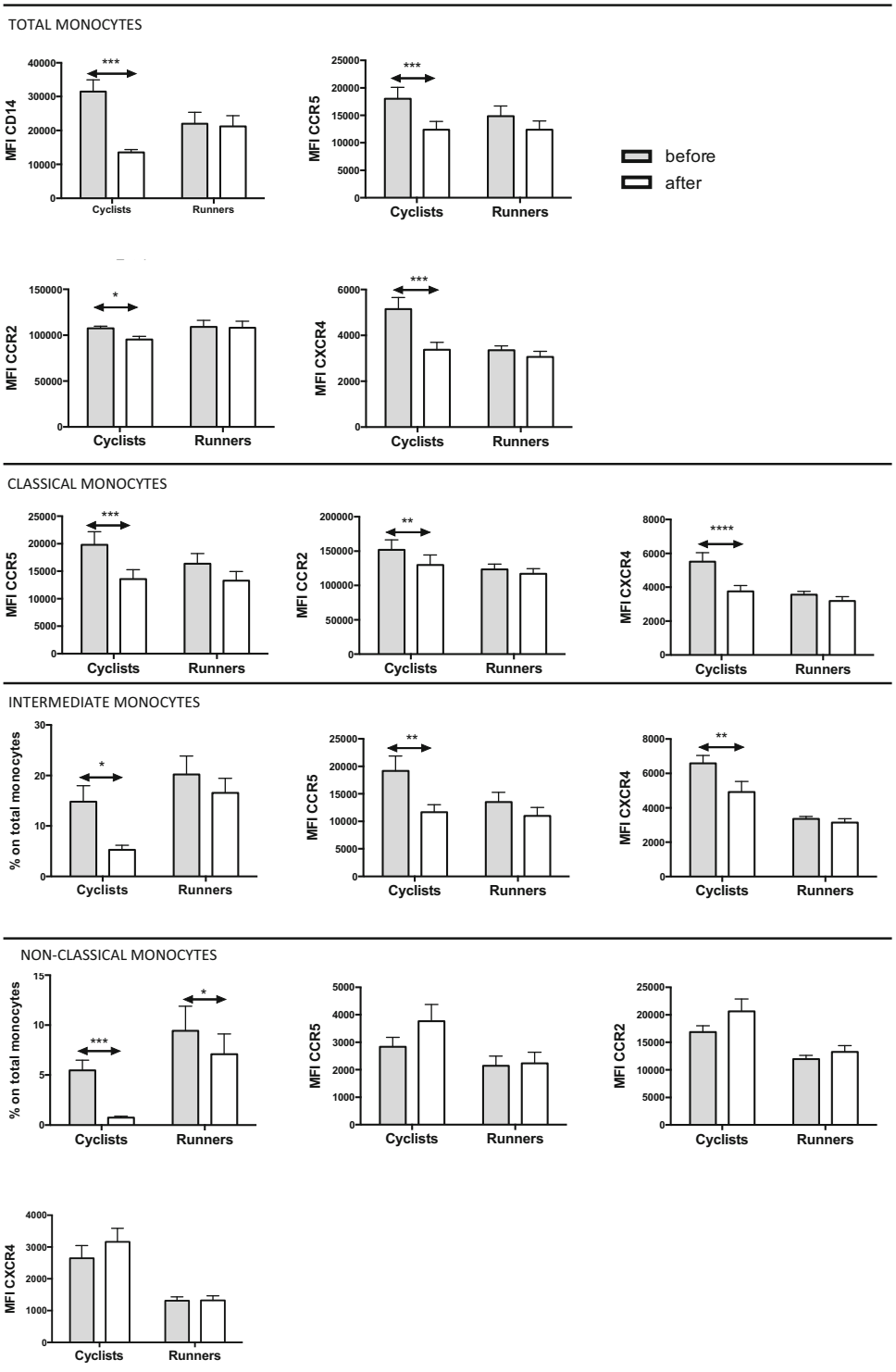

Fig. 3 Phenotypic analysis of monocytes. Mean fluorescence intensity (MFI) of CD14, CXCR4, CCR5, and CCR2 on total monocytes from non-professional male cyclists and runners before and after a 3 one-a-day sessions of WBC (rows 1 and 2). Mean fluorescence intensity of CXCR4, CCR5, and CCR2 on classical monocytes (row 3). Percentage of intermediate monocytes and their expression of CCR5 and CXCR4 (row 4). Percentage of non-classical monocytes and their expression of CCR5, CCR2, and CXCR4 (rows 5 and 6). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Column graphs represented the mean + SEM (standard error of the mean)

Discussion

The current study suggests that WBC treatment in non-professional male cyclists and runners acts on the innate branch of the immune system and tissue repair mechanisms. The greatest benefit seems to be gained by cyclists compared to runners, probably due to the more intense training, causing more muscle damage, soreness, and systemic inflammation, in runners compared to cyclists [16, 17]. Previous studies have only included very few hematological parameters [18], and the current study highlights for the first time the effect of WBC on innate and adaptive immune systems.

According to that reported by previous studies, we found an overall beneficial effect of WBC on lipid, glucose, and

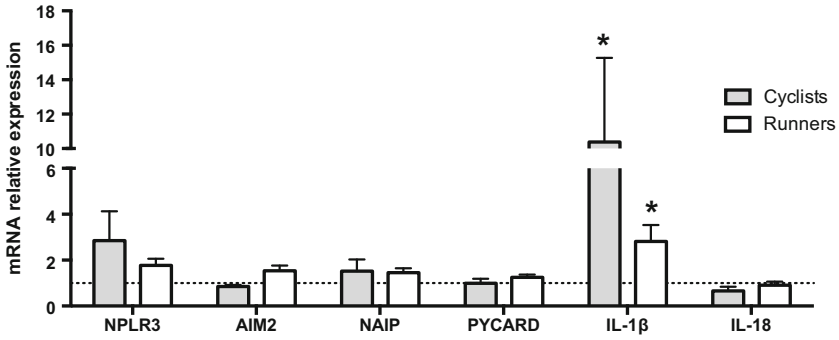

Fig. 4 mRNA expression of inflammasome genes. mRNA expression of AIM2, NLRP3, NAIP, PYCARD, IL-1β, and IL-18 after WBC relative to pre-WBC expression (posed equal to 1, dotted line) in monocytes of cyclists and runners. Column graphs represented the mean + SEM

protein metabolism. Previous studies have cited the importance of at least 10 WBC sessions before lipid improvements, but in this study, an increase of HDL in cyclists was observed after only 3 WBC sessions [19–21]. WBC intensifies the metabolism of proteins (as evident by the increase of urea concentration) and glucose (non-shivering thermogenesis during cold exposure in brown adipocytes) [22, 23]. WBC also acts on skeletal muscle contraction and fatigue (observed through the increase in phosphorous) [24, 25]. Acute temperature decline has been proven to favor a coagulation state [26]; a significant decrease was observed in C and S proteins [27]. As other hematological and clinical chemistry parameters have not been included in longer studies, the lack of changes observed in this short study period cannot be compared.

Among many hormones included in this study, we observed an increase in IGF-1 and FSH in cyclists and a decrease in insulin levels in runners. IGF-1 is an anabolic growth factor, important for muscle repair and remodeling, and its effect on exercise is currently inconclusive and has never been reported in WBC studies [28–30]. Contrary to our results, FSH was found reduced in male subjects during winter, and the authors suggested that it was due to seasonal effects, not only cold exposure [31]. Insulin has also been reported to decrease in some cold conditions, such as a shower at 10 °C after strenuous exercise, or swimming in 6.8 °C water [32, 33].

We observed an increase of IL-18 in athletes, which plays a key role in enhancing the cytotoxic activity of CD8+ T cells and NK cells, as well as the production of TNF and INF-γ [34]. Currently, no comparative data are available from the literature. IL-18 has been reported to increase after regular physical activity in skeletal muscle [35, 36] but not in plasma [37, 38]. Thus, the effects observed in this study suggest that

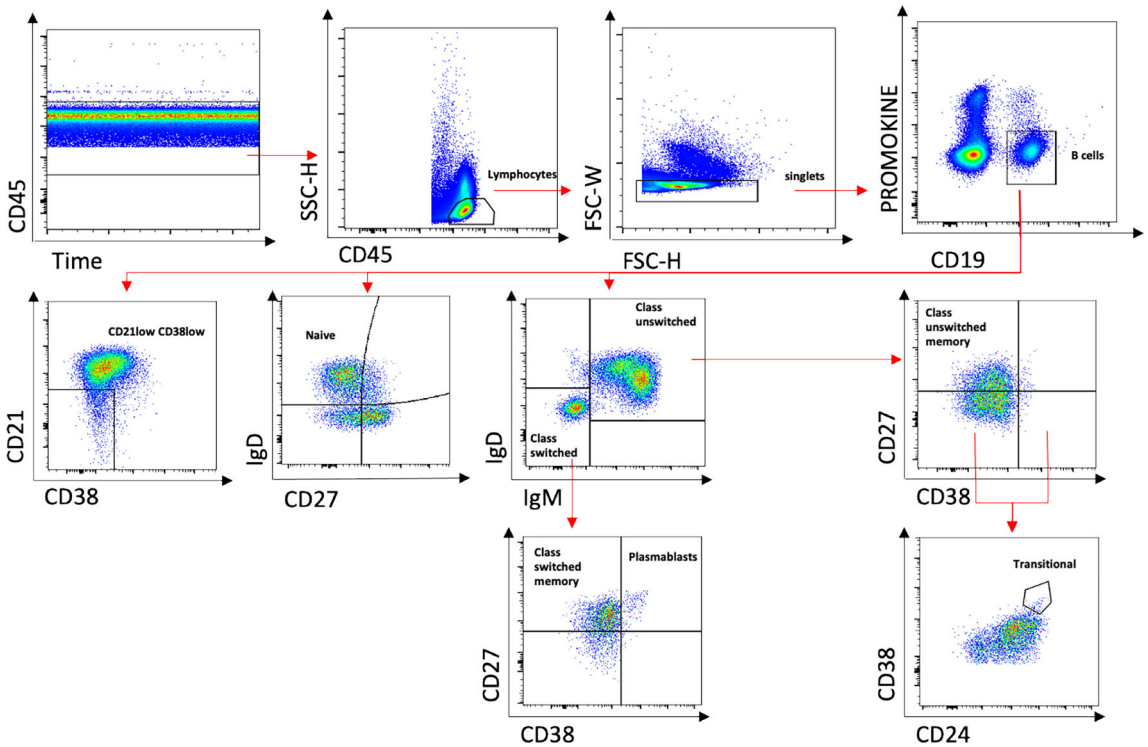

Fig. 5 Gating strategy of B cells. Gating strategy used to identify CD19, naïve, CD21low CD38low, marginal zone, class switched memory, class unswitched memory, and transitional and plamasblast B cells in athletes before and after WBC treatment. A first gate was set in CD45+ cells and time, then on $CD45^{+}$ cells and SSC-H, on physical parameters (FSC-H vs FSC-W) to eliminate doublets, then on CD19+ that identify B cells

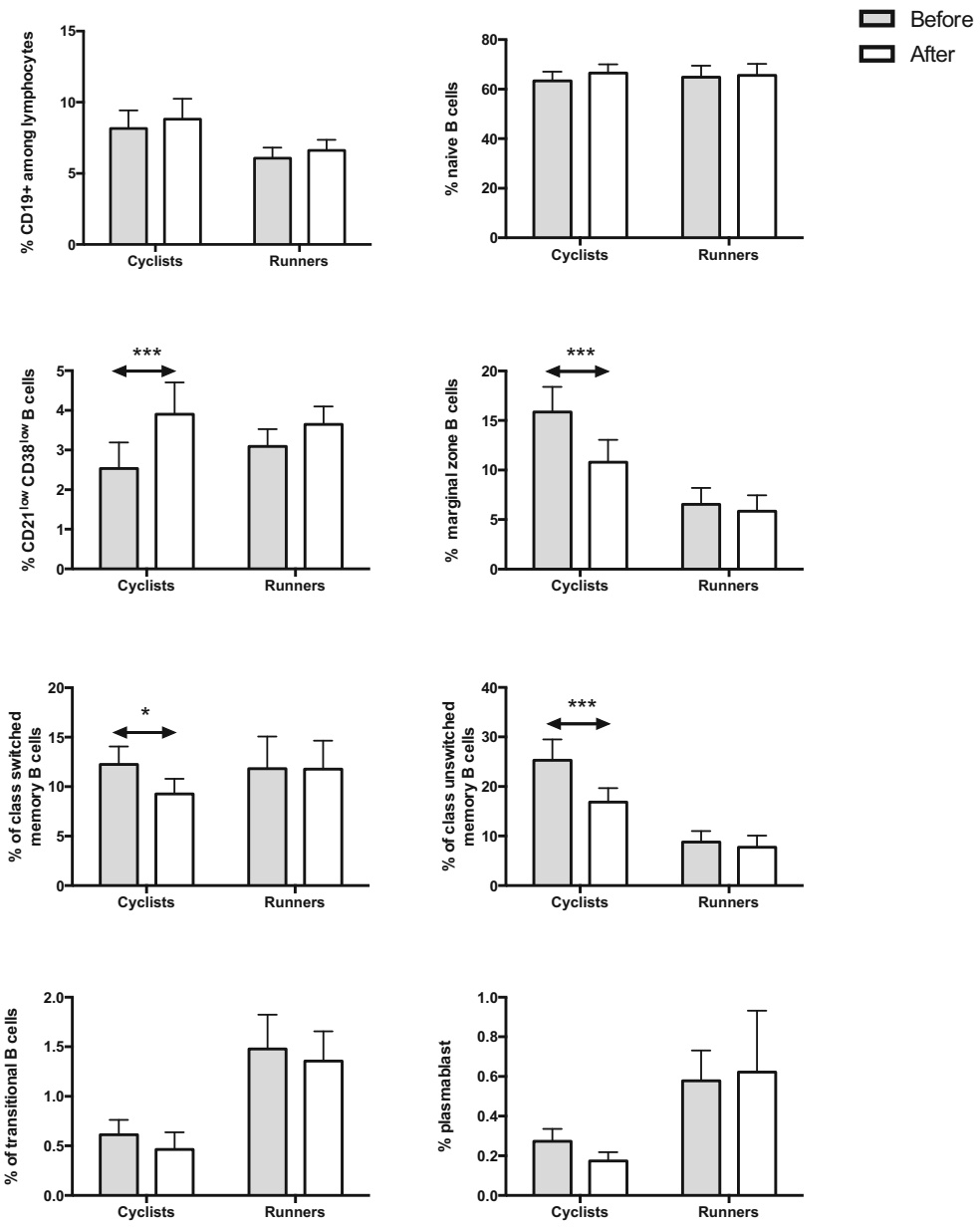

Fig. 6 Distribution of main B cell subsets in athletes. Percentage of CD19, naïve, CD21low CD38low, class switched memory, class unswitched memory, and transitional and plamasblast B cells in athletes before and after WBC treatment. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Column graphs represented the mean + SEM

WBC potentially enhances immunosurveillance. IL-1RA exerts an anti-inflammatory action through the competitive inhibition of the IL-1 receptor [39]. According to what has already been observed in runners [40], we found an increase of this cytokine in all athletes. CXCL8 (IL-8) is a potent neutrophil attractant [41], and until now the effects of WBC on this chemokine have only been studied by Banfi et al. on professional rugby players; they reported a decrease in CXCL8 levels [42]. The current study found an increase of CXCL8 levels in both groups. This difference could be due to training protocols, the number and timing of WBC sessions, as well as the different levels of the sport activity. CCL2, also known as MCP-1 (monocyte chemoattractant protein-1), acts as a potent monocyte attractant [43]. Thus, WBC could favor the recruitment of monocytes into the tissue for tissue repair. The downregulation of CD14 was observed on circulating monocytes; downregulation produces a differentiation into endothelial-like cells that adhere to the endothelium and promote vascular repair [44]. WBC treatment induces a general decrease in chemokine receptors predominantly in cyclists, except for of non-classical monocytes CCR2 and CCR5 and CXCR4, whose expressions tend to increase after WBC.

Non-classical and intermediate monocytes decreased after WBC, which may be due to their redistribution into the surrounding tissue. The higher expression of CCR2 and their ligand CCL2 suggests the promotion of the proliferation stages of tissue repair, reported in soft tissue injury [45, 46]. CCR5 binds several chemokines and therefore monocytes that

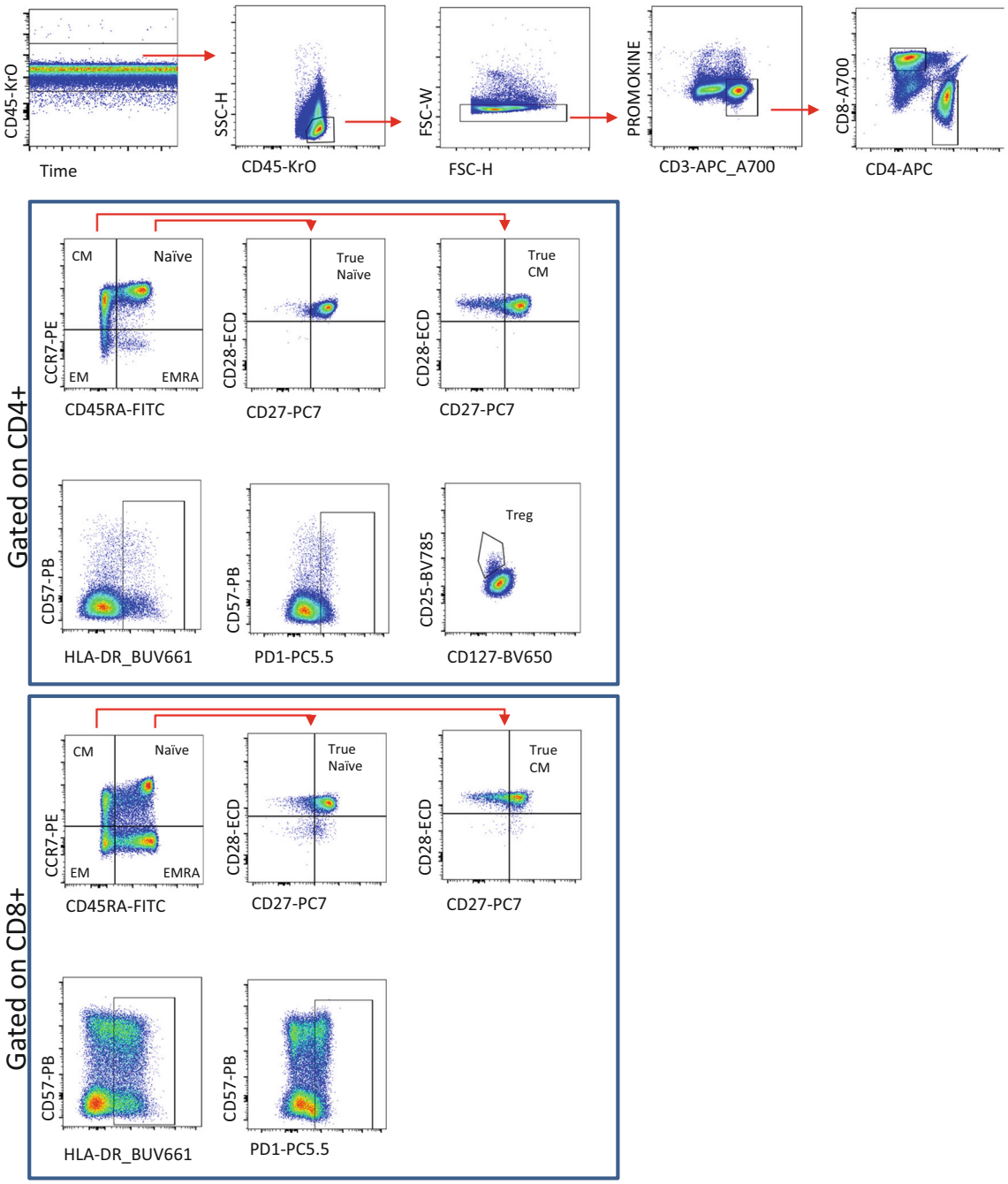

Fig. 7 Gating strategy of T cells. Sequential gating strategy applied to identify different subsets of CD4+ and CD8+ T cells. First time-gated cells were selected and then total lymphocytes were identified using SSC-H and CD45. Singlets were selected based on FSC-H and FSC-W parameters, whereas live T cells were gated using Promokine probe and CD3. These cells were then further used to identify CD4 or CD8 lymphocytes with the same gating strategy. T cells were identified on the basis of their differentiation status in naïve (CCR7+ CD45RA+), central memory (CCR7+ CD45RA-), effector memory (CCR7- CD45RA-), and effector memory RA+(CCR7- CD45RA+). Note that true naïve and CM were also identified as CD27+ CD28+. Moreover, activated (HLA-DR+) or exhausted (PD1+) T cells were gated on total CD4+ or CD8+ cells. Finally, we identified T regulatory cells (Treg) on CD4+ T cells only, as CD127- CD25bright

express this receptor are involved in all stages of wound healing [46]. Finally, the decrease of CXCR4 in some subsets could be consistent with their differentiation process; blood monocytes are not fully differentiated cells, and when they start to differentiate, they express high levels of CXCR4 followed by a downregulation in macrophages in tissue [46]. Thus, WBC through mechanisms as yet unknown seems to promote monocyte differentiation.

B cells contribute to tissue regeneration, and mature B cells have previously been shown to improve healing in a diabetic mouse model [47, 48]. Thus, the decrease of mature B cells in blood circulation could be ascribed to the redistribution to

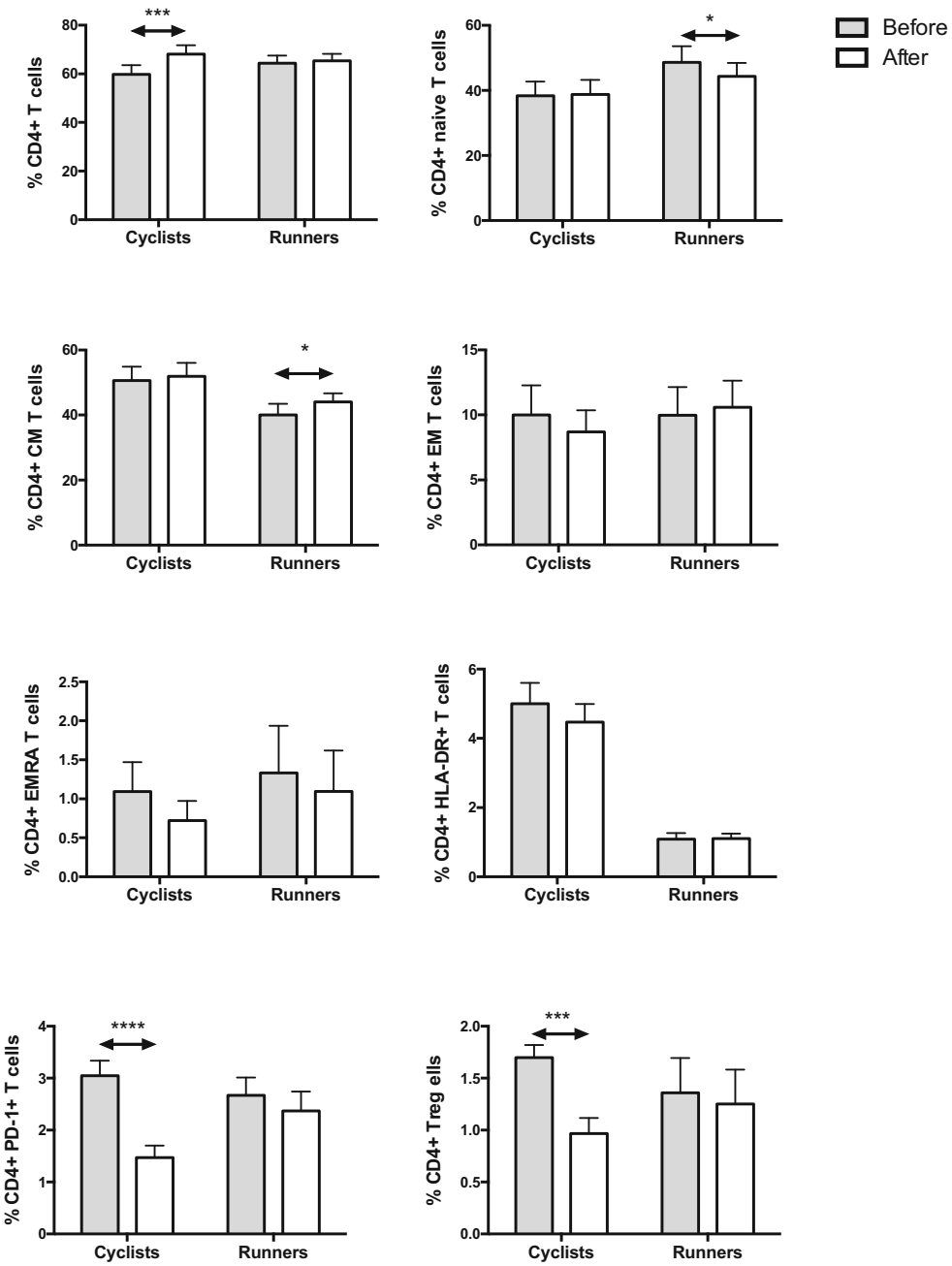

Fig. 8 Distribution of CD4+ T cell subsets in athletes. Percentage of CD4+ T cells among leukocytes and percentage of naïve, central memory, effector memory (EM), and terminally differentiated EM (EMRA), HLA-DR+, PD1+, and Treg cells among CD4+ T cells in athletes before and after WBC treatment. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Column graphs represented the mean + SEM

tissue, favoring its regeneration. The increase of naïve and the simultaneous decrease of effector or exhausted T cells could counteract the effect of inflammaging in which we observed progressive exhaustion of adaptive immunity [49]. Finally, the redistribution of Treg cells (probably into the tissue) could have a role in muscle regeneration and healing, previously observed in skeletal muscle Treg [50].

MtDNA probably derives from damaged cells due to several types of injuries and is associated with different physiopathological conditions [12, 51, 52]. Among them, we previously reported that regular exercise induces a decrease of circulating mtDNA [53], while intense and strenuous exercise was found to have the opposite [54]. Thus, WBC does not seem to have any additional effect on circulating mtDNA.

Among the inflammasome genes analyzed, only IL-1β increased significantly. IL-1β secretion required two signals. The first, via Toll-like receptor engagement, causes the upregulation of pro-IL-1β mRNA transcription, and the second activates the inflammasome assembly that cleaves pro-IL-1β in the active form [55]. Considering that we did not observe an increase of circulating IL-1β after WBC and that inflammasome-independent mechanisms of pro-IL-1β activation exist, we conclude that WBC probably does not influence inflammasome activation [56].

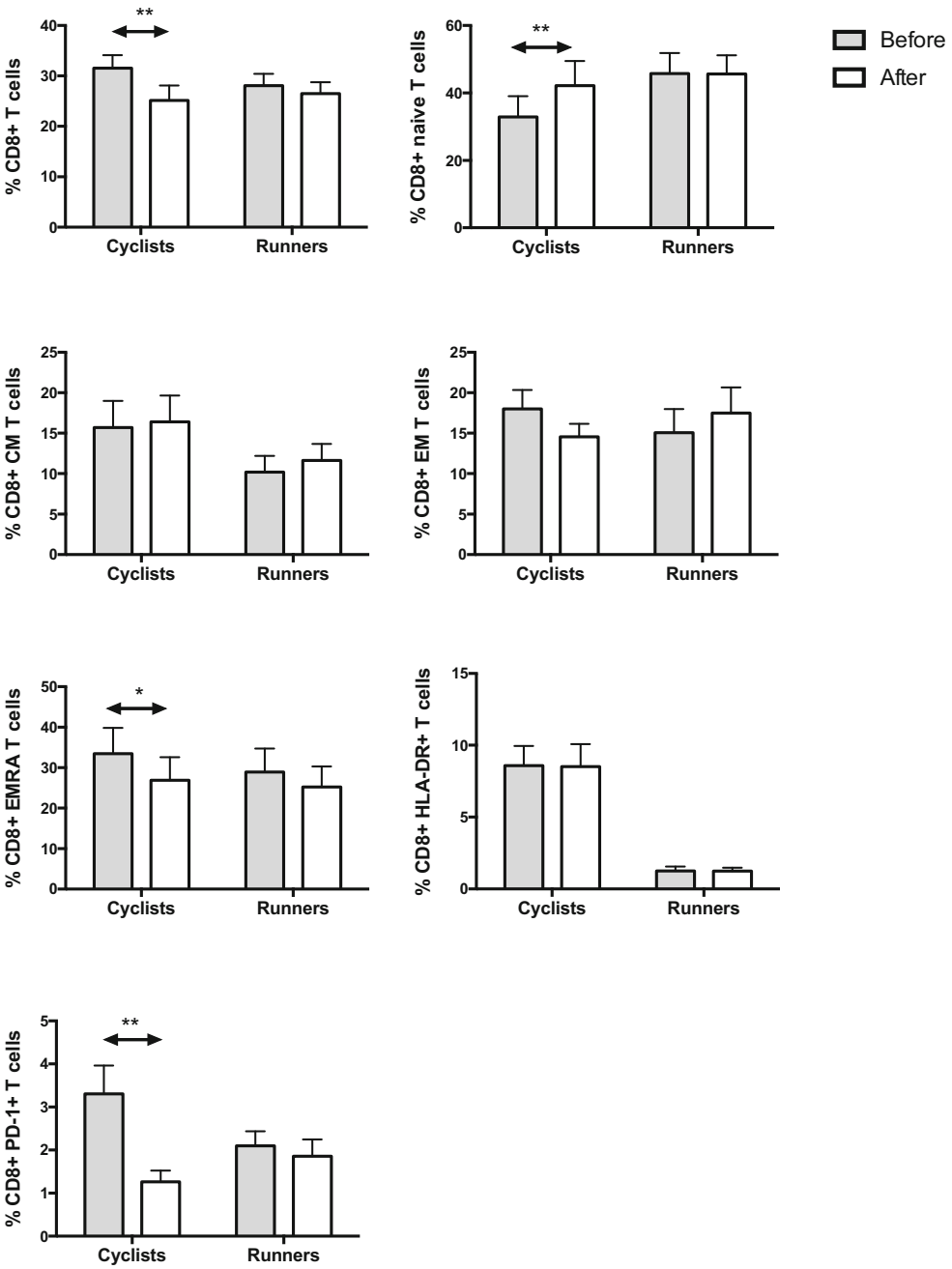

Fig. 9 Distribution of CD8+ T cell subsets in athletes. Percentage of CD8+ T cells among leukocytes and percentage of naïve, central memory, effector memory (EM), and terminally differentiated EM (EMRA), HLA-DR+, and PD1+ cells among CD8+ T cells in athletes before and after WBC treatment. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Column graphs represented the mean + SEM

The obtained results should be interpreted with caution as this study includes a small cohort, without control subjects, over a short interventional period. However, a homogeneous group of non-professional athletes, following the same weekly protocol of consistent training, was assembled, and all subjects completed all 3 WBC sessions.

Conclusions

In non-professional athletes (cyclists and runners), WBC seems to induce beneficial immunological and metabolic responses and may be protagonist in promoting a process of tissue repair. However, further studies are required to confirm these promising results and should consider the selected parameters identified by this study.

Acknowledgments Elena Bianchini received a 2019 post-doctoral fellow from Fondazione Umberto Veronesi and Sara De Biasi a Marylou Ingram Scholar from the International Society for Advancement of Cytometry (ISAC).

Authors' contributions MN, AC, TT, and GS contributed to the conception and the design of the work. MN, AC, TT, GS, TP, JC, and MP contributed to the revision and the final approval of the manuscript.

MN and EB drafted the work. DLT and MN contributed to the statistical analysis. AC, MN, JC, and AVM contributed to the interpretation of data. DLT, SDB, and EB performed the cytofluorimetric analysis. MM and ADG provided to the processing and storage of biological samples. AP and LG performed the molecular analysis. PM, OS, FT, and EG enrolled the subjects for the study. LR and RDA are responsible for all the hematology, clinical chemistry, and hormonal assays.

Funding This work was supported by the Italian Ministry of Health, Section of Prevention of addictions, doping and mental health (reference number Project 2017-1 "Effects of whole body cryotherapy on inflammatory mechanisms and on the hormonal profile of athletes").

Data availability Deidentified participant data and protocols are available upon reasonable request to the corresponding author.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval The study complied with the Declaration of Helsinki and was approved by the Local Ethical Committee (Area Vasta Nord Emilia Romagna #88/2018/SPER/AUSLMO) and each subject provided written, informed consent.

References

1. Londhe P, Guttridge DC. Inflammation induced loss of skeletal muscle. Bone. 2015;80:131–42. https://doi.org/10.1016/j.bone.2015.03.015.
2. Peake JM, Neubauer O, Della Gatta PA, Nosaka K. Muscle damage and inflammation during recovery from exercise. J Appl Physiol (1985). 2017;122(3):559–70. https://doi.org/10.1152/japplphysiol.00971.2016.
3. Patel K, Bakshi N, Freehill MT, Awan TM. Whole-body cryotherapy in sports medicine. Curr Sports Med Rep. 2019;18(4):136–40. https://doi.org/10.1249/JSR.0000000000000584.
4. Sadura-Sieklucka T, Soltysiuk B, Karlicka A, Sokolowska B, Kontny E, Ksiezopolska-Orlowska K. Effects of whole body cryotherapy in patients with rheumatoid arthritis considering immune parameters. Reumatologia. 2019;57(6):320–5. https://doi.org/10.5114/reum.2019.90825.
5. Hirvonen H, Kautiainen H, Moilanen E, Mikkelsson M, Leirisalo-Repo M. The effect of cryotherapy on total antioxidative capacity in patients with active seropositive rheumatoid arthritis. Rheumatol Int. 2017;37(9):1481–7. https://doi.org/10.1007/s00296-017-3771-9.
6. Rivera J, Tercero MJ, Salas JS, Gimeno JH, Alejo JS. The effect of cryotherapy on fibromyalgia: a randomised clinical trial carried out in a cryosauna cabin. Rheumatol Int. 2018;38(12):2243–50. https://doi.org/10.1007/s00296-018-4176-0.
7. Vitenet M, Tubez F, Marreiro A, Polidori G, Taiar R, Legrand F, et al. Effect of whole body cryotherapy interventions on health-related quality of life in fibromyalgia patients: a randomized controlled trial. Complement Ther Med. 2018;36:6–8. https://doi.org/10.1016/j.ctim.2017.10.011.
8. Stanek A, Cholewka A, Wielkoszynski T, Romuk E, Sieron A. Decreased oxidative stress in male patients with active phase ankylosing spondylitis who underwent whole-body cryotherapy in closed cryochamber. Oxidative Med Cell Longev. 2018;2018:7365490–9. https://doi.org/10.1155/2018/7365490.
9. Stanek A, Cholewka A, Gadula J, Drzazga Z, Sieron A, Sieron-Stoltny K. Can whole-body cryotherapy with subsequent kinesiotherapy procedures in closed type cryogenic chamber improve BASDAI, BASFI, and some spine mobility parameters and decrease pain intensity in patients with ankylosing spondylitis? Biomed Res Int. 2015;2015:404259–11. https://doi.org/10.1155/2015/404259.
10. Stanek A, Sieron-Stoltny K, Romuk E, Cholewka A, Wielkoszynski T, Cieslar G, et al. Whole-body cryostimulation as an effective method of reducing oxidative stress in healthy men. Adv Clin Exp Med. 2016;25(6):1281–91. https://doi.org/10.17219/acem/65980.
11. Partridge EM, Cooke J, McKune A, Pyne DB. Whole-body cryotherapy: potential to enhance athlete preparation for competition? Front Physiol. 2019;10:1007. https://doi.org/10.3389/fphys.2019.01007.
12. Nasi M, Bianchini E, De Biasi S, Gibellini L, Neroni A, Mattioli M, et al. Increased plasma levels of mitochondrial DNA and pro-inflammatory cytokines in patients with progressive multiple sclerosis. J Neuroimmunol. 2019;338:577107. https://doi.org/10.1016/j.jneuroim.2019.577107.
13. Cossarizza A, Chang HD, Radbruch A, Acs A, Adam D, Adam-Klages S, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). Eur J Immunol. 2019;49(10):1457–973. https://doi.org/10.1002/eji.201970107.
14. Nasi M, Pecorini S, De Biasi S, Bianchini E, Digaetano M, Neroni A, et al. Altered expression of PYCARD, interleukin 1beta, interleukin 18, and NAIP in successfully treated HIV-positive patients with a low ratio of CD4+ to CD8+ T cells. J Infect Dis. 2019;219(11):1743–8. https://doi.org/10.1093/infdis/jiy730.
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001;25(4):402–8. https://doi.org/10.1006/meth.2001.1262.
16. Millet G. Physiological differences between cycling and running. Rev Med Suisse. 2009;5(212):1564–7.
17. Nieman DC, Luo B, Dreau D, Henson DA, Shanely RA, Dew D, et al. Immune and inflammation responses to a 3-day period of intensified running versus cycling. Brain Behav Immun. 2014;39:180–5. https://doi.org/10.1016/j.bbi.2013.09.004.
18. Lombardi G, Ziemann E, Banfi G. Whole-body cryotherapy in athletes: from therapy to stimulation. An updated review of the literature. Front Physiol. 2017;8:258. https://doi.org/10.3389/fphys.2017.00258.
19. Lubkowska A, Szygula Z, Klimek AJ, Torii M. Do sessions of cryostimulation have influence on white blood cell count, level of IL6 and total oxidative and antioxidative status in healthy men? Eur J Appl Physiol. 2010;109(1):67–72. https://doi.org/10.1007/s00421-009-1207-2.
20. Wiecek M, Szymura J, Sproull J, Szygula Z. Decreased blood asprosin in hyperglycemic menopausal women as a result of whole-body cryotherapy regardless of metabolic syndrome. J Clin Med. 2019;8(9). https://doi.org/10.3390/jcm8091428.
21. Stanek A, Romuk E, Wielkoszynski T, Bartus S, Cieslar G, Cholewka A. Decreased lipid profile and oxidative stress in healthy subjects who underwent whole-body cryotherapy in closed cryochamber with subsequent kinesiotherapy. Oxidative Med Cell Longev. 2019;2019:7524878–10. https://doi.org/10.1155/2019/7524878.

22. Marlatt KL, Ravussin E. Brown adipose tissue: an update on recent findings. Curr Obes Rep. 2017;6(4):389–96. https://doi.org/10.1007/s13679-017-0283-6.
23. Sutkowy P, Augustynska B, Wozniak A, Rakowski A. Physical exercise combined with whole-body cryotherapy in evaluating the level of lipid peroxidation products and other oxidant stress indicators in kayakers. Oxidative Med Cell Longev. 2014;2014:402631–7. https://doi.org/10.1155/2014/402631.
24. Allen DG, Westerblad H. Role of phosphate and calcium stores in muscle fatigue. J Physiol. 2001;536(Pt 3):657–65. https://doi.org/10.1111/j.1469-7793.2001.t01-1-00657.x.
25. Chen YY, Kao TW, Chou CW, Wu CJ, Yang HF, Lai CH, et al. Exploring the link between serum phosphate levels and low muscle strength, dynapenia, and sarcopenia. Sci Rep. 2018;8(1):3573. https://doi.org/10.1038/s41598-018-21784-1.
26. Cai J, Meng X, Wang C, Chen R, Zhou J, Xu X, et al. The cold effects on circulatory inflammation, thrombosis and vasoconstriction in type 2 diabetic patients. Sci Total Environ. 2016;568:271–7. https://doi.org/10.1016/j.scitotenv.2016.06.030.
27. Dahlback B. Vitamin K-dependent protein S: beyond the protein C pathway. Semin Thromb Hemost. 2018;44(2):176–84. https://doi.org/10.1055/s-0037-1604092.
28. Burgers AM, Biermasz NR, Schoones JW, Pereira AM, Renehan AG, Zwahlen M, et al. Meta-analysis and dose-response metaregression: circulating insulin-like growth factor I (IGF-I) and mortality. J Clin Endocrinol Metab. 2011;96(9):2912–20. https://doi.org/10.1210/jc.2011-1377.
29. Fragala MS, Jajtner AR, Townsend JR, Gonzalez AM, Wells AJ, Oliveira LP, et al. Leukocyte IGF-1 receptor expression during muscle recovery. Med Sci Sports Exerc. 2015;47(1):92–9. https://doi.org/10.1249/MSS.0000000000000392.
30. Gatti R, De Palo EF, Antonelli G, Spinella P. IGF-I/IGFBP system: metabolism outline and physical exercise. J Endocrinol Investig. 2012;35(7):699–707. https://doi.org/10.3275/8456.
31. Demir A, Uslu M, Arslan OE. The effect of seasonal variation on sexual behaviors in males and its correlation with hormone levels: a prospective clinical trial. Cent European J Urol. 2016;69(3):285–9. https://doi.org/10.5173/ceju.2016.793.
32. Hermanussen M, Jensen F, Hirsch N, Friedel K, Kroger B, Lang R, et al. Acute and chronic effects of winter swimming on LH, FSH, prolactin, growth hormone, TSH, cortisol, serum glucose and insulin. Arctic Med Res. 1995;54(1):45–51.
33. Tikuisis P, Ducharme MB, Moroz D, Jacobs I. Physiological responses of exercised-fatigued individuals exposed to wet-cold conditions. J Appl Physiol (1985). 1999;86(4):1319–28. https://doi.org/10.1152/jappl.1999.86.4.1319.
34. Kaplanski G. Interleukin-18: biological properties and role in disease pathogenesis. Immunol Rev. 2018;281(1):138–53. https://doi.org/10.1111/imr.12616.
35. Nieman DC, Wentz LM. The compelling link between physical activity and the body's defense system. J Sport Health Sci. 2019;8(3):201–17. https://doi.org/10.1016/j.jshs.2018.09.009.
36. Peake JM, Della Gatta P, Suzuki K, Nieman DC. Cytokine expression and secretion by skeletal muscle cells: regulatory mechanisms and exercise effects. Exerc Immunol Rev. 2015;21:8–25.
37. Almada C, Cataldo LR, Smalley SV, Diaz E, Serrano A, Hodgson MI, et al. Plasma levels of interleukin-6 and interleukin-18 after an acute physical exercise: relation with post-exercise energy intake in twins. J Physiol Biochem. 2013;69(1):85–95. https://doi.org/10.1007/s13105-012-0191-x.
38. Sahl RE, Andersen PR, Gronbaek K, Morville TH, Rosenkilde M, Rasmusen HK, et al. Repeated excessive exercise attenuates the anti-inflammatory effects of exercise in older men. Front Physiol. 2017;8:407. https://doi.org/10.3389/fphys.2017.00407.
39. Striz I, Brabcova E, Kolesar L, Sekerkova A. Cytokine networking of innate immunity cells: a potential target of therapy. Clin Sci (Lond). 2014;126(9):593–612. https://doi.org/10.1042/CS20130497.
40. Pournot H, Bieuzen F, Louis J, Mounier R, Fillard JR, Barbiche E, et al. Time-course of changes in inflammatory response after whole-body cryotherapy multi exposures following severe exercise. PLoS One. 2011;6(7):e22748. https://doi.org/10.1371/journal.pone.0022748.
41. Boraldi F, Bartolomeo A, De Biasi S, Orlando S, Costa S, Cossarizza A, et al. Innovative flow cytometry allows accurate identification of rare circulating cells involved in endothelial dysfunction. PLoS One. 2016;11(8):e0160153. https://doi.org/10.1371/journal.pone.0160153.
42. Banfi G, Melegati G, Barassi A, d'Eril GM. Effects of the whole-body cryotherapy on NTproBNP, hsCRP and troponin I in athletes. J Sci Med Sport. 2009;12(6):609–10. https://doi.org/10.1016/j.jsams.2008.06.004.
43. Franca CN, Izar MCO, Hortencio MNS, do Amaral JB, Ferreira CES, Tuleta ID, et al. Monocyte subtypes and the CCR2 chemokine receptor in cardiovascular disease. Clin Sci (Lond). 2017;131(12):1215–24. https://doi.org/10.1042/CS20170009.
44. Tso C, Rye KA, Barter P. Phenotypic and functional changes in blood monocytes following adherence to endothelium. PLoS One. 2012;7(5):e37091. https://doi.org/10.1371/journal.pone.0037091.
45. Olingy CE, San Emeterio CL, Ogle ME, Krieger JR, Bruce AC, Pfau DD, et al. Non-classical monocytes are biased progenitors of wound healing macrophages during soft tissue injury. Sci Rep. 2017;7(1):447. https://doi.org/10.1038/s41598-017-00477-1.
46. Ridiandries A, Tan JTM, Bursill CA. The role of chemokines in wound healing. Int J Mol Sci. 2018;19(10). https://doi.org/10.3390/ijms19103217.
47. Hoffman W, Lakkis FG, Chalasani G. B cells, antibodies, and more. Clin J Am Soc Nephrol. 2016;11(1):137–54. https://doi.org/10.2215/CJN.09430915.
48. Sirbulescu RF, Boehm CK, Soon E, Wilks MQ, Ilies I, Yuan H, et al. Mature B cells accelerate wound healing after acute and chronic diabetic skin lesions. Wound Repair Regen. 2017;25(5):774–91. https://doi.org/10.1111/wrr.12584.
49. Simpson RJ, Lowder TW, Spielmann G, Bigley AB, LaVoy EC, Kunz H. Exercise and the aging immune system. Ageing Res Rev. 2012;11(3):404–20. https://doi.org/10.1016/j.arr.2012.03.003.
50. Sharma A, Rudra D. Emerging functions of regulatory T cells in tissue homeostasis. Front Immunol. 2018;9:883. https://doi.org/10.3389/fimmu.2018.00883.
51. Cossarizza A, Pinti M, Nasi M, Gibellini L, Manzini S, Roat E, et al. Increased plasma levels of extracellular mitochondrial DNA during HIV infection: a new role for mitochondrial damage-associated molecular patterns during inflammation. Mitochondrion. 2011;11(5):750–5. https://doi.org/10.1016/j.mito.2011.06.005.
52. Pinti M, Cevenini E, Nasi M, De Biasi S, Salvioli S, Monti D, et al. Circulating mitochondrial DNA increases with age and is a familiar trait: implications for "inflamm-aging". Eur J Immunol. 2014;44(5):1552–62. https://doi.org/10.1002/eji.201343921.
53. Nasi M, Cristani A, Pinti M, Lamberti I, Gibellini L, De Biasi S, et al. Decreased circulating mtDNA levels in professional male volleyball players. Int J Sports Physiol Perform. 2016;11(1):116–21. https://doi.org/10.1123/ijspp.2014-0461.
54. Stawski R, Walczak K, Kosielski P, Meissner P, Budlewski T, Padula G, et al. Repeated bouts of exhaustive exercise increase

circulating cell free nuclear and mitochondrial DNA without development of tolerance in healthy men. PLoS One. 2017;12(5): e0178216. https://doi.org/10.1371/journal.pone.0178216.

55. Guo H, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. Nat Med. 2015;21(7):677–87. https://doi.org/10.1038/nm.3893.

56. Netea MG, van de Veerdonk FL, van der Meer JW, Dinarello CA, Joosten LA. Inflammasome-independent regulation of IL-1-family cytokines. Annu Rev Immunol. 2015;33:49–77. https://doi.org/10.1146/annurev-immunol-032414-112306.