JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2018, 69, 6, 875-887
www.jpp.krakow.pl | DOI: 10.26402/jpp.2018.6.12

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EFFECTS OF CONTROLLED PHYSICAL ACTIVITY ON IMMUNE CELL PHENOTYPE IN PERIPHERAL BLOOD IN PREHYPERTENSION - STUDIES IN PRECLINICAL MODEL AND RANDOMISED CROSSOVER STUDY

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Hypertension (HT) is a global public health issue. There are many behavioural risk factors including unhealthy diet, tobacco use and alcohol consumption as well physical inactivity that contribute to the development of high blood pressure (BP) and its complications. Favourable effect of regular physical activity on treatment or prevention of hypertension by improvement of endothelial function is widely accepted however little is known about its relationship with immune system. Thus, the aim of this study was to assess the role of moderate regular physical activity on immune cell phenotype. T cell and monocyte subsets were characterised in 31 subjects with prehypertension (130 – 139 mmHg systolic and 85 – 89 mmHg diastolic blood pressure) who participated in moderate training (3 times/week) on cyclometers for 3 months in crossover study design. Complementary study was performed in murine model of Ang II-induced hypertension and ten-week-old animals were trained on a treadmill (5 times/week, 1 hour) for 2 weeks before and 1.5 weeks after minipumps implantation. In the context of elevated blood pressure regular physical activity had modest influence on immune cell phenotype. Both in human study and murine model we did not observe effects of applied exercise that can explain the mechanism of BP reduction after short-term regular training. Twelve-weeks regular training did not affect the activation status of T lymphocytes measured as expression of CD69, CD25 and CCR5 in human study. Physical activity resulted in higher expression of adhesion molecule CD11c on CD16+ monocytes (especially CD14 high) without any changes in leukocytes subpopulation counts. Similar results were observed in murine model of hypertension after the training. However the training caused significant decrease of CCR5 and CD25 expressions (measured as a mean fluorescence intensity) on CD8+ T cells infiltrating perivascular adipose tissue. Our studies show modest regulatory influence of moderate training on inflammatory markers in prehypertensive subjects and murine model of Ang II induced hypertension.

Key words: *physical activity, hypertension, blood pressure, T lymphocyte, monocytes, activation markers, moderate training, cardiovascular diseases*

INTRODUCTION

Hypertension (HT) is a global public health issue. HT accounts for more than 50% of deaths caused by cardiovascular diseases (CVD), which is approximately 9.4 million deaths every year (1). There are many factors that contribute to the development of high blood pressure and its complications. Among them are behavioural risk factors, such as unhealthy diet, tobacco use, harmful use of alcohol and physical inactivity (1). In 2013 the European Society of Hypertension and the European Society of Cardiology, defined new classification of blood pressure (2). Hypertension is defined as ≥ 140 mmHg of systolic and ≥ 90 mmHg of diastolic blood pressure and divided into 3 grades of severity. The state that precedes hypertension is prehypertension (or high normal blood pressure). It is defined as 130 – 139 mmHg of systolic and/or 85 – 89 mmHg of diastolic blood pressure. Although prehypertension is not a disease category, it is a predictor of individuals at high risk of developing hypertension (3). In those individuals early intervention by adoption of healthy lifestyle could reduce blood pressure (BP), decrease the rate of progression of BP to hypertensive levels with age or prevent HT entirely.

Favourable effects of exercise on BP reduction have been well characterized in recent years (4-6). Physical activity (PA) is a key factor in non-pharmacological intervention in hypertensive and prehypertensive individuals and few mechanisms of its action are considered. PA leads to increased activity of endothelial nitric oxide synthase (eNOS) (7), which is a source of nitric oxide (NO)

in endothelium (8). NO is the main vasodilator in blood vessels (9). Our previous study also confirmed that aerobic exercise improves nitric oxide-dependent endothelial function of the vessels and can initiate regression of atherosclerosis in adults with high normal blood pressure (10). PA decreases concentration of asymmetric dimethyloarginine (ADMA) (11), which is competitive inhibitor of NOS and its level is enhanced in hypertensive individuals (12). Regular exercise leads to increased expression of *Mas* receptor, which in turns improves relaxant effect of angiotensin (1-7) (13). Aerobic training influences metabolic parameters, which have impact on BP. It decreases level of triglycerides and low-density lipoprotein (LDL) and improves insulin sensitivity in tissues (14). Beneficial effect of PA on circulation and CVD is multidirectional and complex (15).

Although direct effect of physical training in hypertensive patients is well documented, little is known about its relationship with immune system. The role of immune system in pathogenesis of hypertension has been studied since the 60' of the last century. It was shown that immunosuppression caused reduction of BP (16). Studies on murine model of angiotensin II (AngII)-induced hypertension provided evidences about crucial role of T cells in development of hypertension (16). Apart from T cells (17, 18), B cells and antibodies that they produce (19, 20) as well as monocytes (21-25), NK cells (26) and NKT (27) cells are involved in mechanism of pathogenesis of hypertension and other CVD (28). This is also important in the context of development of novel CVD biomarkers (29).

Beneficial effect of regular physical activity on treatment or prevention of hypertension by improvement of endothelial function is widely accepted. However, based on studies performed so far it is difficult to define if immune system is involved in this mechanism. Immunomodulatory role of exercise would make it particularly important in the light of recent results of CANTOS trial (30). Targeting IL1β and its signalling may be the future of cardiovascular prevention (31, 32). Thus, the aim of this study was to assess the role of moderate regular physical activity in regulation of inflammatory markers in prehypertensive individuals. Complementary study was performed in murine model of AngII-induced hypertension.

MATERIALS AND METHODS

Study participants

Thirty-one adults (8 female and 23 male; mean ± SD: age 44.3 ± 5.57 years; body mass 83.19 ± 15.22 kg; body height 1.72 ± 0.09 m; body mass index 27.79 ± 3.38 kg m^{-2}) with prehypertension were recruited for this study. Inclusion criteria consisted of a mean screening BP of 130 – 139 mmHg systolic and/or 85 – 89 mmHg diastolic and physical activity at a low level (inadequate and sedentary), which was examined using the International Physical Activity Questionnaire, which is a well-validated measure of physical activity (33). Exclusion criteria included significant CVD, chronic or acute inflammation diseases, antibiotic therapy during 1 month or anti-inflammatory drugs during 2 months prior to the study, medication with antihypertensive drugs, regular physical activity.

Human study design

Before joining the study, all participants underwent verification of level of physical activity and BP level with 24 hour ABPM (ambulatory blood pressure monitoring) (SpaceLabs 90217 Ultralite). The subjects underwent also a standard medical evaluation, ECG and blood tests (blood count, glucose, C-reactive protein) to eliminate medical contraindications to exercise. Then, they were divided into two groups in crossover study design. First group of volunteers (26 persons) participated in controlled, moderate intensity aerobic training for 3 months and after 10 months interval they started 3 months of control period, without supervised exercise. Second group of volunteers (5 persons) at the beginning participated in control period and after that in aerobic training.

All subjects gave their informed consent prior to their inclusion in the study. The study complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects. The study protocols received approval from the Bioethics Committee (Approval No. 71/KBL/OIL/2010).

Training procedure

Before training, subjects underwent electrocardiographic exercise stress test restricted to symptoms, performed on cycle ergometer to check their health and to determine the pulse peak. Then the subjects participated in the 3 months moderate intensity supervised aerobic (endurance) training program on a cycle ergometer CRG 200, working in Beta AsTER Rehabilitation System (Aspel, Poland). Training included 3 sessions per week (Mondays, Wednesdays and Fridays). The intensity of exercise was 40 – 65% of heart rate reserve (HRR) (34). Each training unit consisted of warm up (5 – 10 minutes of omni-directional movements, stretching, and cycling with a slow increase in load to 50% of training intensity), main exercise (cycling with a range of training pulse) and cool down exercises (5 – 10 minutes of cycling with low load and then stretching). Duration of the main exercise was 30 minutes in the 1st week, 35 minutes in the 2nd week and 40 minutes in the following weeks. The intensity of the main part of the training unit was increased during the training program: the training range in the first 3 weeks was 40 – 50% HRR, in the 4th week 45 – 55% HRR, in the 5th week 50 – 60% HRR and in the following weeks 55 – 65% HRR. The subjects had a pedalling rate at which they felt most comfortable (60 – 80 revolutions per minute) and the training system automatically dosed the load according to the training heart rate. Subjects tolerated intensity of exercise well and their subjective fatigue during exercise ranged from 12 to 13 points in a Borg's 6 – 20 rating, which is well correlated with the heart rate (35). The training was always conducted at regular times of day, in the afternoon, in an air-conditioned room with a temperature of 18 – 20ºC.

Analysis of leukocytes activation in human blood

Blood samples were collected (BD Vacutainer with EDTA collection tube) before and after control and training periods. Samples were immediately transferred on ice to the laboratory and peripheral blood mononuclear cells (PBMC) were isolated using density gradient separation media (LSM 1077, PAA). After isolation, cells were resuspended in Phosphate-Buffered Saline (PBS, Gibco) supplemented with 1% Fetal Bovine Serum (FBS, Gibco).

PBMC were stained for flow cytometry analysis with FACSCantoII and FACSVerse (BD Biosciences). For evaluation of surface markers of T cells and monocytes following monoclonal antibodies (BD Biosciences) were used: anti-CD3 PerCP (Clone SK7), anti-CD4 APC (Clone SK3), anti-CD8 APC-H7 (Clone SK1), anti-CD195 PE-Cy7 (clone 2D7/CCR5), anti-CD25 PE (cloneM-A251), anti-CD69 FITC (clone FN50), anti-CD16 PE (clone 3G8), anti-CD14 APC-H7 (clone MΦ P9), anti-HLA-DR PE-Cy7 (L243), anti-CD11c APC (clone B-LY6), anti-CD11b/Mac-1-Pacific Blue (clone ICRF44). Cells were stained for 20 minutes in the dark on ice and after washes cells

were resuspended in PBS/1%FBS. Data were analysed with use of FlowJo v10 software.

Lymphocytes were gated according to forward scatter (FSC) and side scatter (SSC) signals from PBMC and T cells were gated according to CD3 expression. Percentages of CD4 and CD8 positive subpopulations were assessed. In T cells and their subsets the expressions of surface activation markers were then assessed. Fluorescence Minus One (FMO) controls were used to determine the positivity of evaluated antigens.

Monocytes were gated according to FSC and SSC signals. Subsequently, cells were gated in an HLA-DR/CD14 plot to exclude HLA-DR-negative Natural Killer cells. Finally, we analyzed cells for CD14 and CD16 expression, which allowed for discrimination of major monocyte subpopulations: CD14++CD16−, CD14++CD16+ and CD14+CD16++(36).

Murine model of AngII-induced hypertension and study design

In 13 male C57BL/6J mice (obtained from Jackson Laboratory) osmotic minipumps (Model 2002; Alzet) were placed subcutaneously with a solution of the angiotensin II and dosing speed of 490 ng/min/kg. Seven animals were subjected to physical training on treadmill for rodents (Exer 3/6, Columbus Instruments). On the treadmill 6 mice (one lane for each) can exercise on single belt construction with dividing walls over the tread surface. At the end of each lane there is an electrical stimulus assembly composed of three or six shock grids, each with individual on/off switches. In our study, electrical stimulus was used only at the beginning of the training period before minipumps implantation.

Animals without training were divided into following groups: angiotensin II infused mice (n = 6) and angiotensin II infused mice subjected to exercise training (n = 7).

Ten-week-old animals were trained on a treadmill 5 times a week for 1 hour for 2 weeks before and 1.5 weeks after minipumps implantation. All animals underwent non-invasive blood pressure measurements by tail-cuff plethysmography (Visitech BP 2000 BP Analysis System) following a one-week period of training before surgery. At the end of the study following samples were collected: blood, perivascular (pv), mesenteric (mes) and visceral (v) adipose tissues (AT) and analysed with flow cytometry.

The Institutional Animal Care and Use Committees at Jagiellonian University approved the protocols used in the study (Approval No. 100/2013, 159/2013, 40/2015).

Analysis of leukocytes activation in murine tissues

PBMC were isolated from the blood with cell density gradient (LSM 1077, PAA). Adipose tissues were subjected to enzymatic digestion using collagenase type XI (125 U/ml), collagenase type IS (450 U/ml), and hyaluronidase IV-S (60 U/ml) dissolved in PBS containing calcium and magnesium for 20 min at 37ºC, with regular agitation. The digested tissue was then passed through a 70 µm sterile cell strainer (Falcon; BD Biosciences) to yield a single-cell suspension. Cells were resuspended in PBS/1% FBS buffer and stained for flow cytometry. For evaluation of surface markers of T cells from AT following antibodies (BD Biosciences, unless otherwise stated) were used: anti-CD3e APC (Clone 145-2C11), anti-CD4 APC-H7 (Clone GK1.5), anti-CD8a PerCP (Clone 53-6.7), anti-CD45 V450 (Clone 30-F11), anti-CD69 FITC (Clone H1.2F3), anti-CD25 PE-Cy7 (Clone PC61) and anti-CCR5 PE (Clone HM-CCR5 (7A4), eBioscience). Monocytes were analysed with use of anti-CD45 V450, anti-Ly6C PE (Clone AL-21), anti-CD43 FITC (Clone S7), anti-CD11b APC-Cy7 (Clone M1/70), anti-F4/80 APC (Clone BM8, eBioscience), anti-Ly6G PE-Cy7 (Clone 1A8) and anti-NK1.1 PerCP (Clone PK136, BioLegend). Cells were stained for 20 minutes in the dark on ice and after washes were resuspended in PBS/1% FBS. Dead cells were eliminated from the analysis using Zombie Aqua V510 dye (BioLegend). Cells were analysed with use of FACSVerse (BD Biosciences) and data were analysed with FlowJo v10.

Statistical analysis

For human study, statistical analysis was performed with use of IBM SPSS Statistic 24.0 and two-way repeated measures ANOVA was used. In this analysis the most important results that we focused on, is the interaction between activity periods (control versus training) and time points. The significance for activity alone shows that the level of the parameter tested is different when comparing training and control period, but does not change during the period. FDR (False Recovery Rate) correction was used to address multiple. For animal model, t test was performed with use of GraphPad Prism 7.05. For each variable mean value and standard deviation (SD) were calculated and presented on the graphs.

RESULTS

Effect of physical activity on T lymphocytes subpopulations in prehypertensive subjects.

Firstly we wanted to determine how physical activity influences the main T lymphocyte subpopulations in prehypertensive subjects. Using flow cytometry we did not observe any significant differences in the distribution of the main T lymphocyte subsets in the blood between groups (trained and control subjects). There were no differences in the percentages of either CD4+ or CD8+ T lymphocytes (*Fig. 1A*).

Although, regular training did not influence distribution of main subpopulations of T cells, we decided to investigate the role of exercise on activation markers expressed on T cells. First we evaluated percentage distribution of cells expressing receptor for chemokine RANTES - CCR5 and its density (MFI, mean fluorescence intensity) (*Fig. 1B* and *1C*). No significant interaction between activity and time points was observed, however there was higher level of CD8+CCR5+ cells in training period in comparison to the control period ($P < 0.05$). The same observation was noticed during analysis of male samples alone (*Fig. 2B*). Next, we estimated percentage of cells expressing interleukin-2 receptor – CD25 and its MFI on T cell subsets (*Fig. 1D* and *1E*). We did not observed significant interaction between activity periods and time points was observed, however higher level of CD4+CD25+ cells ($P < 0.05$) and tendency of increased level in CD8+CD25+ population ($P = 0.08$) in training period in comparison to the control period was noticed. Both of these observations were significant when analysing male group only ($P < 0.05$) (*Fig. 2D*). We also decided to assess the percentage of cells expressing early activation marker CD69 and its MFI on T cell subsets (*Fig. 1F* and *1G*). No significant interaction between activity periods and time points was noted, however there was tendency of lower CD4+CD69+ cells number in training period ($P = 0.08$).

Effect of regular training on monocyte subpopulations and adhesion molecule in prehypertension

In monocytes, the main subpopulation was the classical monocytes, characterized by the high expression of CD14 and lack of expression of CD16 (CD14highCD16-). No significant interaction between activity periods and time points was observed. Also intermediate monocytes (CD14highCD16+) and non-classical monocytes, characterized by low expression of CD14 and expression of CD16 (CD14dimCD16+) did not

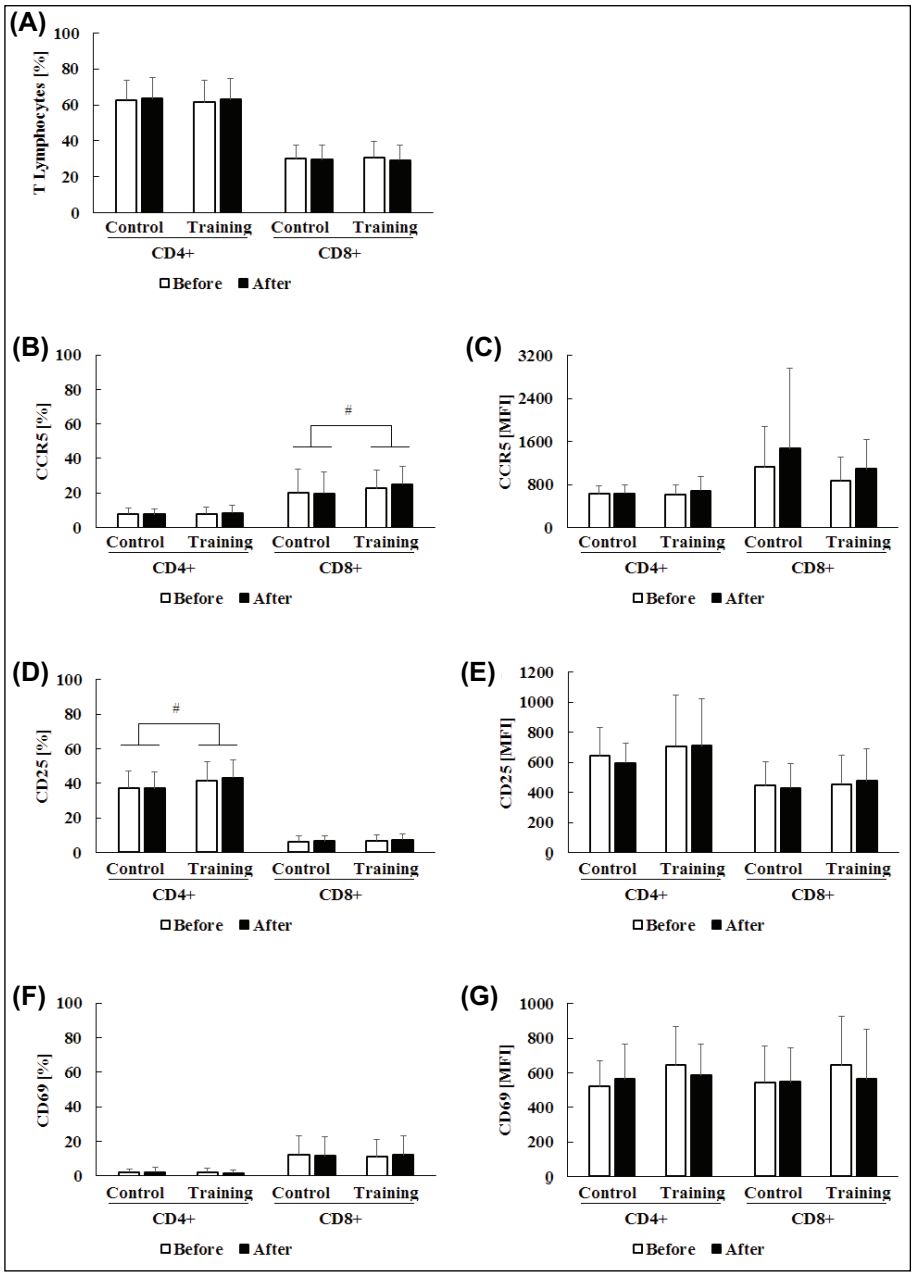

Fig. 1. Main T lymphocyte subsets and expression of activation markers on T cells in prehypertensive subjects after training. Percentage distribution of T cell subsets (CD4+ and CD8+) (A) (n = 31) and percentage and MFI (mean fluorescence intensity) of activation markers: CCR5 (B, C), CD25 (D, E) and CD69 (F, G) were assessed by flow cytometry in pre-hypertensive subjects before and after control and training period (n = 28 (D-G) and n = 31(B, C)), # $P < 0.05$ for comparison between control and training periods.

Table 1. Distribution of leukocytes subsets in peripheral blood mononuclear cells and adipose tissue in Ang II dependent hypertension.

	Leukocytes	**T lymphocytes**	**CD4+ T cells**	**CD8+ T cells**
Peripheral blood (% mean ± SD)				
Control AngII	96.68 ± 0.92	23.3 ± 5.36	53.4 ± 2.14	40.44 ± 3.76
Training AngII	96.01 ± 0.86	28.99 ± 8.09	51.14 ± 1.21	43.46 ± 1.67
Perivascular adipose tissue (cells/mg of tissue mean ± SD)				
Control AngII	2277.73 ± 745.97	344.96 ± 60.45	170.56 ± 38.69	87.14 ± 18.56
Training AngII	1805.23 ± 346.13	330.35 ± 31.63	166.05 ± 18.79	98.24 ± 7.16
Mesenteric adipose tissue (cells/mg of tissue mean ± SD)				
Control AngII	2479.57 ± 805.47	384.28 ± 152.81	204.51 ± 75.12	80.01 ± 48.51
Training AngII	1617.69 ± 1091.04	197.42 ± 191.6	91.12 ± 92.18	42.32 ± 47.14
Visceral adipose tissue (cells/mg of tissue mean ± SD)				
Control AngII	926.83 ± 373.84	218.01 ± 91.7	98.64 ± 37.7	28.14 ± 14.94
Training AngII	983.26 ± 160.28	229.17 ± 47.67	94.47 ± 8.45	32.59 ± 6.69

indicate any significant effect of regular physical activity on monocytes (*Fig. 3A*).

Finally, we evaluated expression of adhesion molecules, CD11b and CD11c, on monocytes surface, as they are involved in

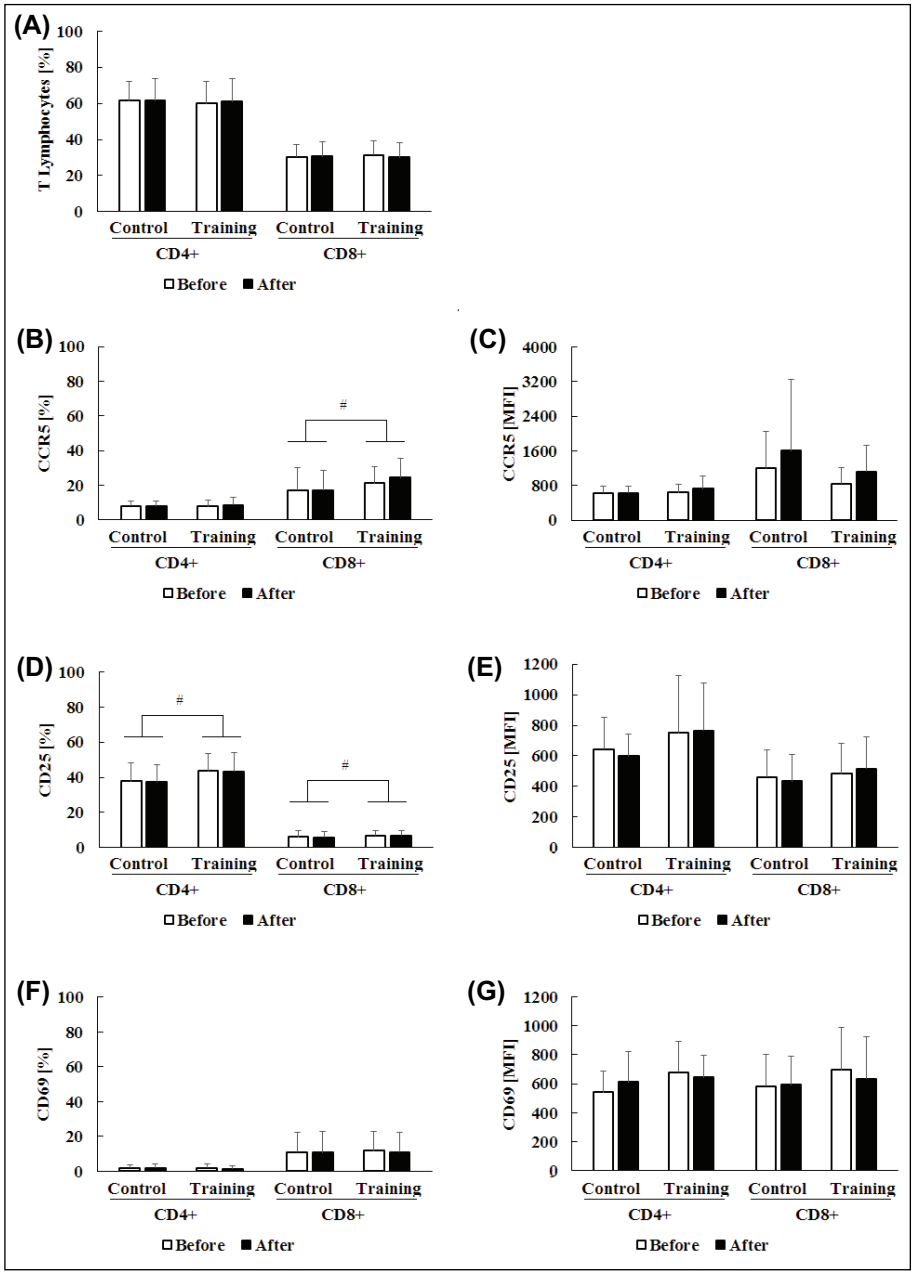

Fig. 2. Main T lymphocyte subsets and expression of activation markers on T cells in male prehypertensive subjects after training. Percentage distribution of T cell subsets (CD4+ and CD8+) (A) (n = 23) and percentage and MFI (mean fluorescence intensity) of activation markers: CCR5 (B, C), CD25 (D, E) and CD69 (F, G) were assessed by flow cytometry in pre-hypertensive subjects before and after control and training period (n = 21 (D-G) and n = 23(B, C)), # $P < 0.05$ for comparison between control and training periods.

adhesion of monocytes to vascular wall and atherogenesis. We did not notice any significant interaction between activity periods and time points in mean fluorescence intensity of CD11b and CD11c (*Fig. 3B*, *3C*) on CD14highCD16- was observed. No significant interaction between control versus training periods and time points for CD11b integrin on surface of CD14highCD16+ monocytes was found (*Fig. 3B*). Significant interaction between activity periods and time points was noticed in amount of CD11c on CD14highCD16+ monocytes surface ($P < 0.05$) (*Fig. 3C*). No significant interaction between control versus training periods and time points for CD11b on CD14dimCD16+ monocytes was observed, however tendency of interaction was noticed in MFI of CD11c ($P = 0.06$) (*Fig. 3B* and *3C*).

Higher level of mean fluorescence intensity of CD11b and CD11c (respectively) on monocytes surface: CD14highCD16- ($P < 0.01$; $P = 0.01$), CD14highCD16+ ($P < 0.01$, $P < 0.01$), CD14dimCD16+ ($P < 0.05$, $P < 0.05$) in training period in comparison to the control was observed (*Fig. 3B* and *3C*). The same observation was found when analysing male group only (*Fig. 4B*, *4C*).

Significant interaction for control versus training periods and time points was noticed for CD14highCD16- ($P < 0.01$), CD14highCD16+ in male group, ($P < 0.01$) and CD14dimCD16+ ($P < 0.01$) (*Fig. 4C*).

Effect of regular training on T cells infiltration into perivascular adipose tissue in Ang II dependent hypertension

To complete our knowledge about influence of physical activity on immune cell phenotype in elevated blood pressure state, we decided to examine leukocytes infiltrating perivascular adipose tissue (pvAT), mesenteric adipose tissue (mesAT) and visceral adipose tissue (vAT) in murine model of hypertension. Leukocytes, T cell and their subsets (CD4+, CD8+) were evaluated from peripheral blood mononuclear cells (PBMC) and from different adipose tissue (AT) compartments from all

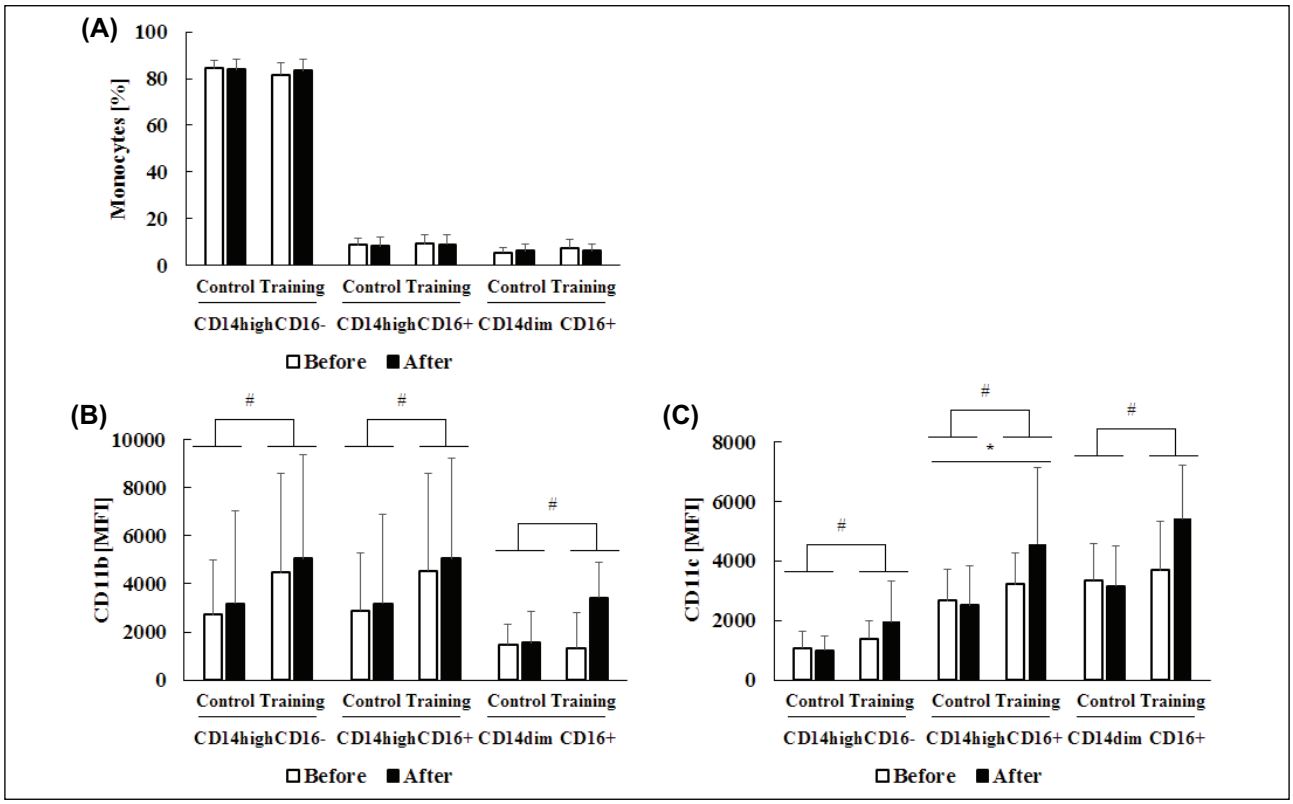

Fig. 3. Main monocytes subsets and expression of adhesion molecules on monocyte in prehypertensive subjects after training. Percentage distribution of monocyte subsets (CD14highCD16-, CD14highCD16+, CD14dimCD16+) (A) (n = 31) and expression of CD11b (B) and CD11c (C) on monocyte subsets were assessed by flow cytometry in pre-hypertensive subjects before and after control and training period (n = 25 (B) and n = 31 (C)), * $P < 0.05$ for interaction between time and activity periods, # $P < 0.05$ for comparison between control and training periods.

Table 2. Expression of inflammatory markers on CD4+ and CD8+ T cells in Ang II dependent hypertension.

	CD4+ T cells			CD8+ T cells		
	CCR5	**CD25**	**CD69**	**CCR5**	**CD25**	**CD69**
Peripheral blood (% mean ± SD)						
Control AngII	3.95 ± 0.92	8.17 ± 2.51	8.51 ± 6.28	2.23 ± 1.05	1.08 ± 0.57	3.29 ± 0.88
Training AngII	3.85 ± 1.32	6.6 ± 0.92	6.49 ± 3.15	1.76 ± 0.77	1.29 ± 0.63	3.05 ± 1.26
Perivascular adipose tissue (cells/mg of tissue mean ± SD)						
Control AngII	54.4 ± 22.77	63.39 ± 22.91	43.96 ± 17.05	16.02 ± 8.80	10.44 ± 5.32	17.55 ± 5.99
Training AngII	40.8 ± 15.8	42.94 ± 15.75	50.56 ± 24.19	17.56 ± 8.36	8.92 ± 2.47	21.48 ± 13.36
Mesenteric adipose tissue (cells/mg of tissue mean ± SD)						
Control AngII	39.26 ± 14.49	43.95 ± 11.9	43.51 ± 10.52	14.14 ± 9.97	3.8 ± 2.27	7.47 ± 4.18
Training AngII	25.18 ± 23.36	26.67 ± 24.31	29.62 ± 35.17	9.03 ± 12.26	3.34 ± 3.53	9.02 ± 12.90
Visceral adipose tissue (cells/mg of tissue mean ± SD)						
Control AngII	26.02 ± 12.91	31.77 ± 12.81	26.21 ± 9.42	4.16 ± 2.39	1.92 ± 1.0	5.49 ± 4.14
Training AngII	21.69 ± 12.80	32.37 ± 14.88	31.67 ± 7.32	3.44 ± 2.24	1.66 ± 1.04	6.64 ± 2.56

examined groups (*Table 1*). Analysis of individual T cell subsets revealed no significant difference between groups.

Role of physical activity in regulation of inflammatory markers on T cells in Ang II dependent hypertension

In the next step the expression of activation markers: CCR5, CD25 and CD69 on T cell subsets (CD4+, CD8+) isolated from peripheral blood and different compartments of adipose tissue was measured using flow cytometry. No significant difference was observed between groups in CD4+ and CD8+ T cells (*Table 2*).

Expression of receptor for chemokine RANTES - CCR5 on CD4+ T cells in PBMC and different adipose tissue compartments indicated no significant difference between control and training groups (*Fig. 5A-5D*). No significant difference between groups was observed in expression of CCR5 on CD8+ T cells surface in PBMC (*Fig. 5A*), mesenteric adipose tissue (*Fig. 5C*) and visceral adipose tissue (*Fig. 5D*). Training caused significant decrease of CCR5 expression on CD8+ T cells infiltrating perivascular adipose tissue (*Fig. 5B*) ($P < 0.05$). Next, the expression of receptor for interleukin 2 - CD25, was evaluated on CD4+ T cells in PBMC, perivascular, mesenteric and visceral adipose tissue compartments. No significant difference between control and training groups was observed (*Fig. 6A-6D*). No significant difference between groups was noticed in expression of CD25 on CD8+ T cells in PBMC (*Fig.*

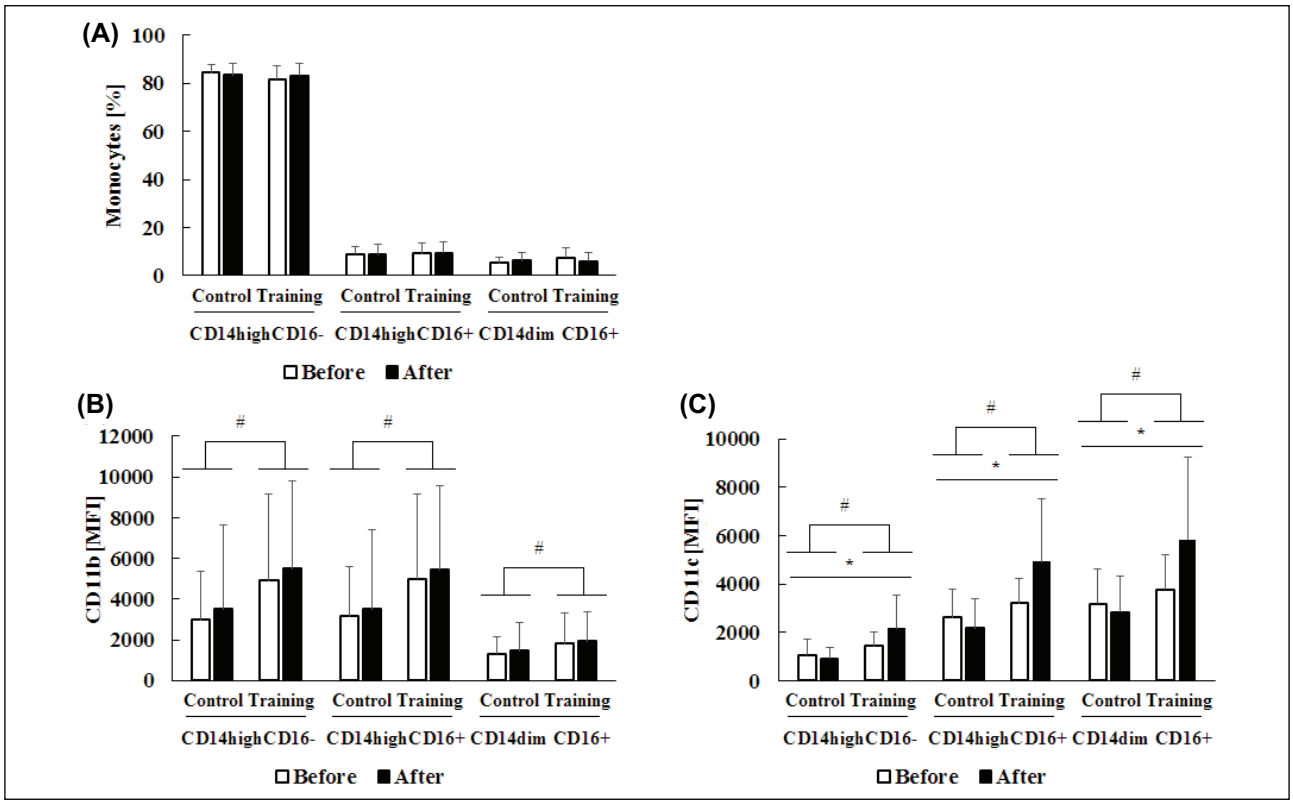

Fig. 4. Main monocytes subsets and expression of adhesion molecules on monocyte in male prehypertensive subjects after training. Percentage distribution of monocyte subsetss (CD14highCD16-, CD14highCD16+, CD14dimCD16+) (A) (n = 23) and expression of CD11b (B) and CD11c (C) on monocyte subsets were assessed by flow cytometry in pre-hypertensive subjects before and after control and training period (n = 21 (B) and n = 23 (C)), * P < 0.05 for interaction between time and activity periods, # P < 0.05 for comparison between control and training periods.

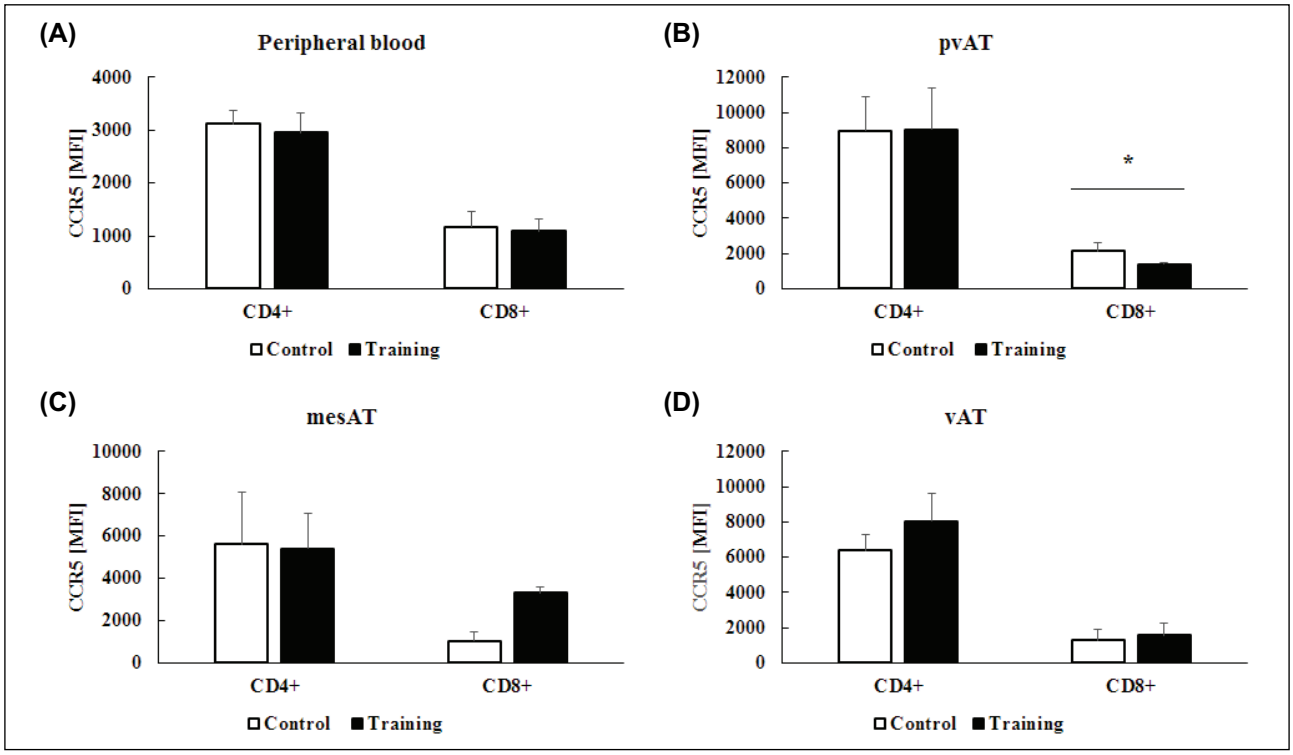

Fig. 5. Expression of CCR5 on CD4+ and CD8+ T lymphocytes in Ang II dependent hypertension. Mean fluorescence intensity (MFI) of CCR5 was assessed on T cell subsets (CD4+, CD8+) isolated from peripheral blood- PBMC (A) and pvAT (B), mesAT (C) and vAT (D). * P < 0.05.

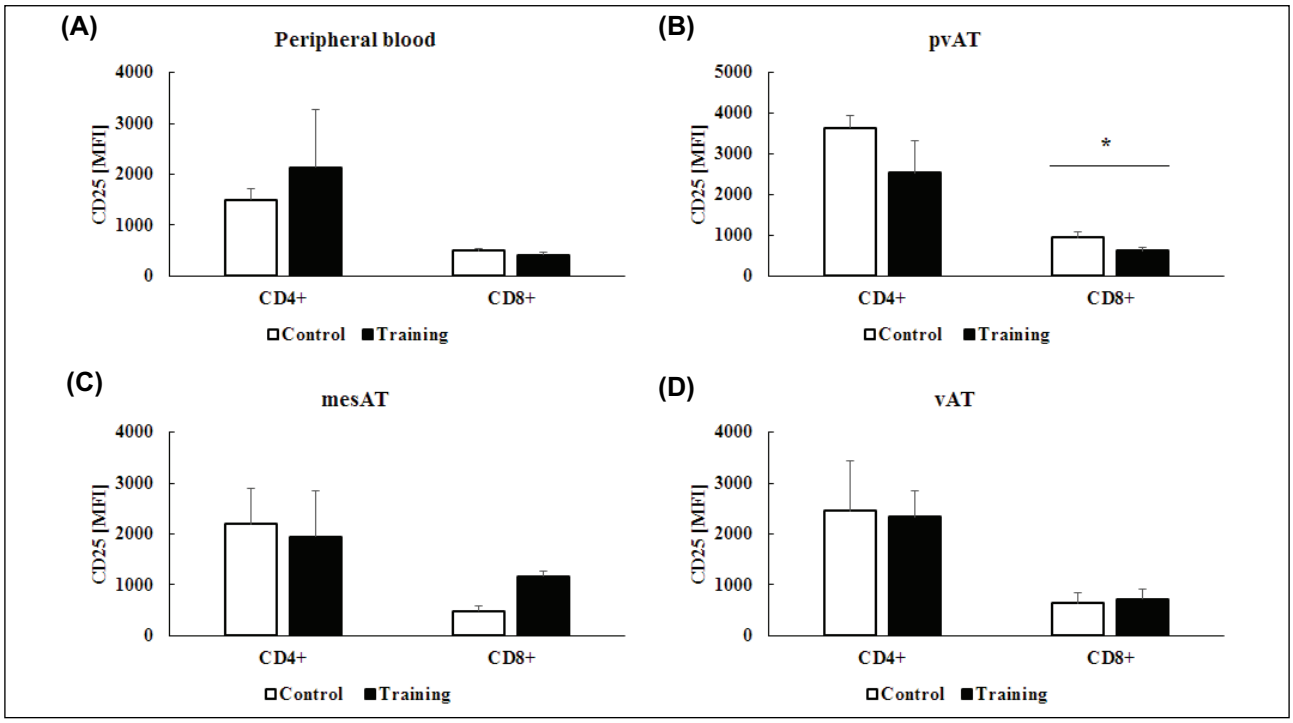

Fig. 6. Expression of late activation marker CD25 on T lymphocyte in Ang II dependent hypertension. Mean fluorescence intensity (MFI) of CD25 was assessed on T cell subsets (CD4+, CD8+) isolated from peripheral blood- PBMC (A) and pvAT (B), mesAT (C) and vAT (D). * $P < 0.05$.

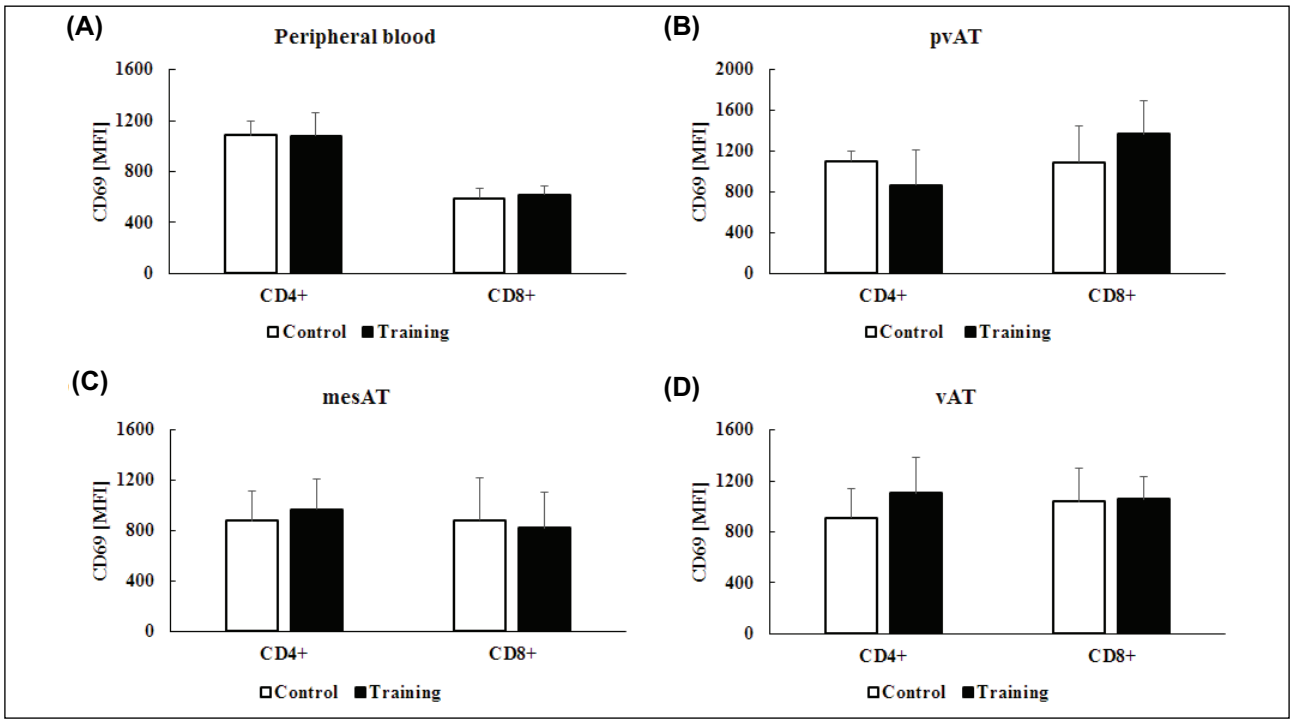

Fig. 7. Expression of early activation marker CD69 on T lymphocyte in Ang II dependent hypertension. Mean fluorescence intensity (MFI) of CD69 was assessed on T cell subsets (CD4+, CD8+) isolated from peripheral blood- PBMC (A) and pvAT (B), mesAT (C) and vAT (D).

6A), mesenteric adipose tissue (*Fig. 6C*) and visceral adipose tissue (*Fig. 6D*). Training caused significant decrease of CD25 expression on CD8+ T cells infiltrating perivascular adipose tissue (*Fig. 6B*) ($P < 0.05$). Finally, the expression level of CD69 was checked on CD4+ T and CD8+ T cells. We did not observe any statistically significant changes in the expression of CD69 on T cell subsets in PBMC and infiltrating different adipose tissue compartments between control and training groups (*Fig. 7A-7D*).

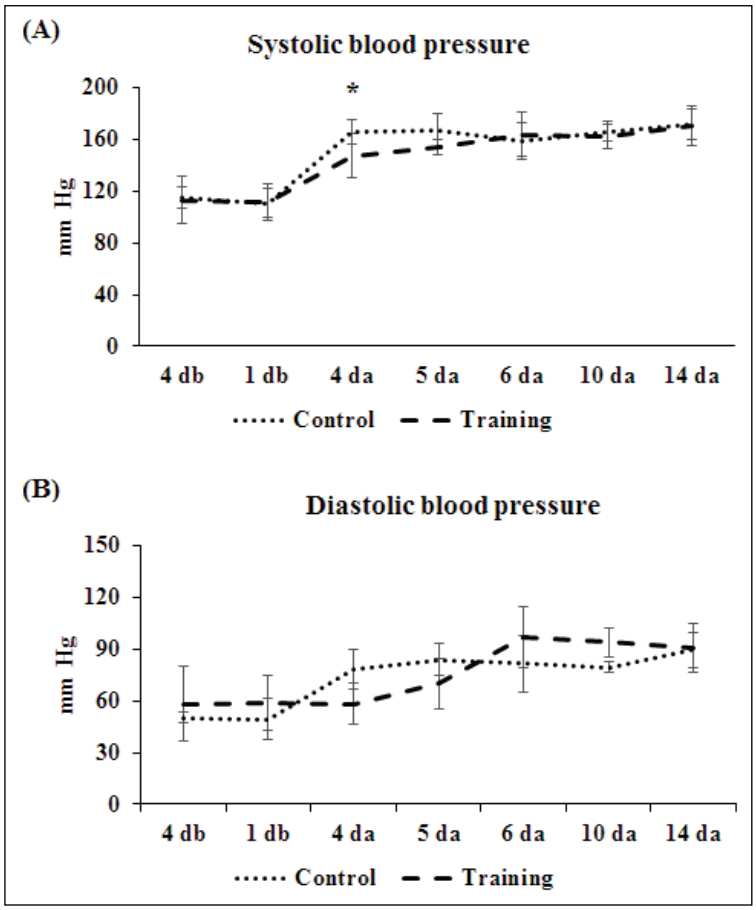

Fig. 8. Systolic and diastolic blood pressure in Ang II dependent hypertension. Systolic and diastolic blood pressure was measured 4 and 1 day before (db) and 4, 5, 6, 10, 14 days after (da) implantation of osmotic minipump with angiotensin II. * $P < 0.05$.

Effects of regular training on monocytes distribution in hypertensive mice

Finally, to complete our observation about the role of regular training in regulation of immune cell phenotype we analysed monocyte distribution. The percentage of monocyte subsets (control versus training) was 77.50 ± 6.03% versus 83.17 ± 5.16% of classical, 12.92 ± 3.34% versus 9.12 ± 2.45% of intermediate and 7.23 ± 2.18% verus 6.28 ± 2.54% of non-classical monocytes. No significant difference was observed between groups (data not shown).

Effect of regular training on blood pressure of hypertensive mice

We also analysed effect of regular training on blood pressure of hypertensive mice. Blood pressure was measured before (db, days before) and after (da, days after) implantation of minipump. There was no significant difference between groups before the surgery for systolic (*Fig. 8A*) and diastolic (*Fig. 8B*) blood pressure. Four-days after minipump implantation, systolic blood pressure was higher in control group than in training animals ($P < 0.05$). No significant difference was noticed for later time points and for diastolic blood pressure after the surgery (*Fig. 8A-8B*).

DISCUSSION

Hypertension is a common disorder. Undetected or uncontrolled high blood pressure can lead to many complications including heart attack, stroke, heart failure and organ damage. In 2018 the European Society of Hypertension and the European Society of Cardiology defined new management of arterial hypertension (37). New concepts include a wider use of home blood pressure monitoring to confirm diagnosis and single pill treatment as an initial therapy in most patients. Also simplified treatment algorithms, comprising ACE inhibitor or ARB, combined with calcium channel blocker and/or diuretic combination, were proposed. New target blood pressure ranges were established (the aim 140/90 mm Hg, then proceed 130/80 mm Hg but not lower than 120/70 mm Hg). Detecting of poor adherence to drug therapy as a major cause of poor blood pressure control has been highlighted as well (37). Physical activity is an important modulator of hypertensive target organ damage (10) and vascular remodelling which appears to be immune mediated (38).

Given the fact that T cells and other components of immune system have an impact on development of vascular disease and hypertension (16, 39) and that physical activity has beneficial effect in reduction of BP (40), the hypothesis of direct effect of exercise on immunity which in turns leads to decrease of BP seems reasonable.

The mechanistic aspects of exercise in hypertensive human subjects and experimental animal models have been studied before (41, 42). The authors have focused on the mechanism of hyperkalemia resulting from skeletal muscle activity in patients with arterial hypertension and normal renal function subjected to the therapy with ACE inhibitors and statins (41). Interestingly, Kumral *et al.* have shown that regular exercise alleviates experimental renovascular hypertension causing cardiac and endothelial dysfunction due to oxidative injury in rats implicating the involvement of NO in the beneficial mode of action of exercise (42).

In the present study we show that regular physical activity has modest influence on immunity in the context of elevated

blood pressure. Both in human study and murine model we did not observe effects of applied exercise that can explain the mechanism of BP reduction after short-term training. In samples from prehypertensive subjects no significant changes were observed in main subpopulations of T cells and monocytes. Regular training lasting for 12 weeks did not affect the activation status of T lymphocytes measured as expression of activation markers CD69, CD25 and CCR5. Physical activity resulted in higher expression of adhesion molecule, CD11c on CD16+ monocytes (especially CD14high), however without any changes in leukocyte subpopulation counts. Similar results were obtained from murine model of hypertension after the training in peripheral blood mononuclear cells (PBMC) and cells infiltrating adipose tissues. Decreased expression of receptor for chemokine RANTES, CCR5 and receptor for IL-2, CD25 on CD8+ T cell infiltrating perivascular adipose tissue was not accompanied by the change in lymphocyte subpopulation counts.

Although the effect of regular activity in context of hypertension is not well characterised, there are many reports about role of exercise in older population, in which the prevalence of hypertension is the highest. Nieman *et al.* described an increase of NK cells activity and improvement of T cells function in women, who participated in sport competition during minimum 5 years prior to the study in comparison to the ones with sedentary lifestyle (43). In a group of men who were jogging for about 17 years in comparison to those who did not exercise regularly, neither changes in activation of NK cells nor the number of cells in subpopulations of leukocytes were observed. However, physical activity resulted in higher proliferation of T cells and production of interleukin 2 (IL-2), IL-4 and interferon-γ (44). It was also shown, that regular training caused improved response of T cells expressing CD25 to stimulation with anti-CD3 antibodies (45). The above evidences were collected from cross-sectional study and refer to long-term physical activity. Application of regular training did not improve immunity function in women with sedentary life style (43). Similar effect was observed after introduction of 6 months of regular aerobic training with relaxation exercises. No changes in percentage of leukocytes populations were observed. Physical activity did not influence distribution of main subpopulations of T cells (46). Resistance training lasting 8 – 12 weeks did not cause any changes in T lymphocytes subpopulations and cytokines production (47, 48). In animal models of aging it was shown that physical training has protective effects on immunity parameters that weaken with age. Beneficial influence on macrophages, lymphocytes and T cells (49) as well as proliferation of T cells in response to the mitogens was shown (50).

Our study does not provide evidence about influence of exercise on activation of T cells. In patients with chronic kidney disease, in whom hypertension is likely to occur, 6 months of regular training did not change percentage of CD69 T cells, however it caused a decrease of its expression on CD4+ and CD8+ after stimulation of staphylococcal enterotoxin B (51). In prehypertensive subjects we did not observe changes in percentage and expression of CD69 and CD25 after 12 weeks of training in comparison to the control group. In our study the percentage of cells expressing CD25 was higher in CD4+ T cells in comparison to CD8+. Also the mean fluorescence intensity of CD25 was higher in CD4+ cells in comparison to CD8+ cells. It is important according to the fact that CD4+CD25+ cells expressing FoxP3 are small population of CD4+ T cells with regulatory function (Treg). Their protective function was observed in mice with pulmonary hypertension caused by increased production of pro-inflammatory cytokines (52). In pre-eclampsia, decreased level of Treg with concomitant elevated level of pro-inflammatory cytokines was observed (53). In elderly women, who lead healthy life style, increased expression of CD25 on T cells was noticed in comparison to those with sedentary life style (45). This observation is based on long-term activity, thus it is difficult to compare with our results.

Apart from activation of T cells, their migration into inflammatory site is also important (30). Chemokines play the main role in this process. RANTES (Regulated on Activation Normal T Cell Expressed and Secreted) chemokine and its receptor CCR5 are involved in pathogenesis of hypertension (54). In diabetic SHR rats, anti-cholesterol drug administration reduced blood pressure and expression of CCR5, what indicates the link between hypertension and RANTES/CCR5 (55). It was also shown, that CCR5 expression is elevated in patients with pulmonary hypertension and that activation of CCR5 on smooth muscle cells of pulmonary artery and macrophages is necessary in the development of this disease (56, 57). In our study we did not observe effect of regular training on percentage of T cells expressing CCR5 nor the level of its expression in main subpopulations of T cells from prehypertensive subjects. However we noticed the moderate effect of physical activity on T cells infiltrating adipose tissue in murine model of hypertension after the training. We observed that training caused a statistically significant decrease of CCR5 expression measured as mean fluorescence intensity on CD8+ T cell infiltrating perivascular adipose tissue. It is interesting because in our previous study we found that CCR5/RANTES axis is involved in T cell homing and vascular dysfunction independently of blood pressure regulation (54). Additionally, CCR5+ cells exhibited particularly high production of INF-γ, which had direct effect on endothelial function (54).

It is intriguing since in another study it was shown that exercise resulted in reduced expression of RANTES and CCR5 in adipose tissue of obese people (58). It might be caused by the occurrence of pathophysiology factors connected with obesity but not with hypertension itself (59-62).

Finally, we characterized the effect of regular training on monocytes subpopulations. Although anaerobic exercises were previously shown to increase percentage of CD14+ (63) and non-classical monocytes (64), we did not observe any changes in this subpopulation in pre-hypertensive subjects nor in murine model of hypertension after regular training. Possible explanation of these, might be that anaerobic and aerobic exercise have different effect on immunity (65). It was shown that moderate aerobic physical activity has anti-inflammatory (2, 66, 67), whereas anaerobic pro-inflammatory effects on immunity (68). In line with our results are those observed in patients with chronic kidney disease, where no changes in monocytes subpopulations after regular training occurred (51). Although no changes in percentage of monocytes subpopulations were observed, physical activity resulted in increased expression of CD11c on CD14highCD16+ and tendency for increase in CD14dimCD16+ monocytes. Analysis of male subjects alone strengthen this observation, as physical activity caused increased expression of CD11 in all subpopulations of monocytes, CD14highCD16-, CD14highCD16+ and CD14dimCD16+. Since CD11c expression is involved in development of pre-eclampsia (69), increase of CD11c expression after physical activity is raising questions about its real role in pathogenesis of hypertension.

There are few limitations in our study, which can influence noticed observation. First of all, the higher number of participants would give us more reliable results. Despite the inclusion and exclusion criteria, the group was still heterogeneous. The higher number of participants would minimize the influence of heterogeneity. Also, more data about the blood cytokine level would help to explain the real role of physical activity in modulation of immune system. In murine model, the best solution to monitor blood pressure would be

telemetry especially diastolic blood pressure is not accurate using tail cuff method. Longer period of training after induction of hypertension, possibly could influence the results observed, however the minipump used in the study is not allowed to be used in long-term studies.

Our studies show modest regulatory influence of moderate training on inflammatory markers in prehypertensive subjects and murine model of hypertension and do not indicate modulation of this mechanism as an important factor in beneficial effect of physical activity on endothelial function and blood pressure decrease.

Acknowledgements: This study was supported by the Polish National Science Centre grants (N N404 179240 and 2013/09/N/NZ4/02211) and the Mobility Plus Program of Polish Ministry of Science and Higher Education (1280/MOB/IV/2015/0 - TM).

Conflict of interests: None declared.

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Received: November 19, 2018
Accepted: December 30, 2018

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