

# Hemodialysis Affects Phenotype and Proliferation of CD4-Positive T Lymphocytes

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**Abstract** CD4<sup>+</sup> T lymphocytes of patients with chronic kidney disease (CKD) are characterized by reduced levels of crucial surface antigens and changes in the cell cycle parameters. Recombinant human erythropoietin (rhEPO) normalizes their altered phenotype and proliferative capacity. Mechanisms leading to the deficient responses of T lymphocytes are still not clear but it is postulated that immunological changes are deepened by hemodialysis (HD). Study of activation parameters of CD4<sup>+</sup> T lymphocytes in hemodialyzed and predialysis CKD patients could bring insight into this problem. Two groups of patients, treated conservatively (predialysis, PD) and hemodialyzed (HD), as well as healthy controls, were included into the study; neither had received rhEPO. Proportions of main CD4<sup>+</sup>CD28<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>, CD4<sup>+</sup>CD69<sup>+</sup>, CD4<sup>+</sup>CD95<sup>+</sup>, and CD4<sup>+</sup>HLA-DR<sup>+</sup> lymphocyte subpopulations and proliferation kinetic parameters were measured with flow cytometry, both *ex vivo* and *in vitro*. No differences were seen in the proportions of main CD4<sup>+</sup> lymphocyte subpopulations (CD4<sup>+</sup>CD28<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>, CD4<sup>+</sup>HLA-DR<sup>+</sup>, CD4<sup>+</sup>CD69<sup>+</sup>, CD4<sup>+</sup>CD95<sup>+</sup>) between all examined groups *ex vivo*. CD4<sup>+</sup> T lymphocytes of HD patients exhibited significantly decreased expression of costimulatory molecule CD28 and activation markers CD25 and CD69 after stimulation *in vitro* when compared with

PD patients and healthy controls. HD patients showed also decreased percentage of CD4<sup>+</sup>CD28<sup>+</sup> lymphocytes proliferating *in vitro*; these cells presented decreased numbers of finished divisions after 72 h of stimulation *in vitro* and had longer G0→G1 time when compared to healthy controls. CD4<sup>+</sup> T lymphocytes of PD patients and healthy controls were characterized by similar cell cycle parameters. Our study shows that repeated hemodialysis procedure influences phenotype and proliferation parameters of CD4<sup>+</sup> T lymphocytes.

**Keywords** CD4<sup>+</sup> T lymphocytes · antigens · proliferation · hemodialysis

## Introduction

Chronic kidney disease (CKD) is characterized by slow loss of kidney function over a period of years. The goal of CKD treatment is to delay progression of the disease, but when the patient reaches stage 5 of CKD (end-stage renal disease—ESRD), renal replacement therapy (dialysis or kidney transplantation) is necessary [1]. Apart from uremia, electrolyte disturbances, hypertension, and anemia, CKD patients also present inappropriate parameters of nonspecific and specific immunity. The most frequent findings include abnormally high activity of monocytes based on production of proinflammatory cytokines: tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1, and IL-6 [2, 3]. It is also reported that CKD patients present decreased percentages of peripheral T lymphocytes, both CD4<sup>+</sup> and CD8<sup>+</sup> cells, and B lymphocytes in the blood [4, 5]. Furthermore, decreased IL-2 and interferon gamma production as an answer to stimulation are observed in CKD patients [6, 7]. A detailed study revealed that stimulated CD4<sup>+</sup> T lymphocytes of CKD

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patients present decreased expression of antigens involved in T lymphocyte activation: major co-stimulatory CD28 antigen and early activation antigen CD69 [7]. The decreased levels of these antigens, especially CD28, could be one of the reasons for deficient reactions of T lymphocytes. CD28 regulates IL-2 production on the transcriptional level [8], and its lower expression explains reduced levels of IL-2 in response to mitogenic stimulation in CKD patients. Also, fewer CD4<sup>+</sup>CD28<sup>+</sup> lymphocytes of CKD patients proliferate in vitro, and these cells require more time to enter the first phase (G1) of the cell cycle [7]. CD4<sup>+</sup> T lymphocytes are involved in both cell-mediated and humoral immune responses; consequently, their changed phenotype and disturbances in proliferation kinetics lead to impaired immunity observed as decreased immunoglobulin production by B lymphocytes resulting in poor response of CKD patients to vaccination and increased susceptibility to infections [9].

There is still little known about the mechanisms that cause immunological deficiency in CKD, but it seems that these changes are deepened mainly by hemodialysis (HD) procedures. At least, all the aforementioned disturbances in cellular and humoral immunity were described in HD patients. Dialytic membrane incompatibility, bacterial contamination of dialyzer, and vascular access are the main reasons for inflammatory response in CKD patients. Direct contact of peripheral blood mononuclear cells (PBMC) with artificial membrane initiates abnormal production of proinflammatory cytokines by monocytes [10]. Furthermore, the HD procedure induces also apoptosis of T lymphocytes, which leads to T cell lymphopenia observed in patients [11]. However, it is not clear if changes in phenotype, cytokine production, and proliferation of T lymphocytes result only from repeated hemodialysis procedure or are connected with CKD progression, since there are no detailed studies comparing these parameters in hemodialyzed and predialysis CKD patients. Most of the articles focus only on numbers or proportions of lymphocytes in the blood of patients. Meanwhile, surface antigens of lymphocytes and their proliferation kinetics are the key parameters crucial for cell-mediated and humoral responses. Therefore, we have chosen for our study the molecules crucial for the activity of CD4<sup>+</sup> T lymphocytes (CD28 antigen—co-stimulatory molecule important for antigen presentation;

activation markers—CD69 (early), CD25, CD95 (middle), HLA-DR (late)) and analyzed their expression along with proliferation parameters of T CD4<sup>+</sup> lymphocytes in hemodialyzed and predialysis patients.

## Patients and Methods

**Patients** The study groups consisted of 25 CKD patients (mean age 60.32±17.92 years) and 14 healthy individuals (mean age 53.14±11.55 years). The 15 patients underwent 5-h sessions of hemodialysis three times a week. The average time of hemodialysis was 1.18±0.57 months with the shortest time of 2 weeks and the longest of 2 months. Ninety-five percent of examined CKD patients were CMV seropositive but none of them presented symptoms of an active infectious process during the study. Ten patients were treated conservatively (predialysis patients). The study has been approved by the Ethical Committee of the Medical University of Gdańsk. The main features of patients and controls are presented in Table I. Mean hemoglobin was significantly decreased in all groups of patients with CKD when compared to healthy control. All patients had stage 5 of CKD (ESRD) (Table I).

**Stainings and Flow Cytometry Analysis of Antigens of CD4<sup>+</sup> Lymphocytes** Thirty milliliters of venous peripheral blood was collected in tubes containing EDTA as the anti-coagulant from each HD patient before the HD session and from patients treated conservatively and healthy volunteers. Five milliliters of blood was designed for morphology. Samples of 50 µl per tube blood were transferred for staining with monoclonal antibodies ex vivo and red blood cell lysis. Blood cells were stained with FITC-conjugated anti-CD3, RPE-Cy5-conjugated anti-CD4 (DAKO, Denmark), PE-conjugated anti-CD28, PE-conjugated anti-CD69 (BD-Pharmigen, USA), PE-conjugated anti-CD25 (DAKO, Denmark), PE-conjugated anti-CD95, and PE-conjugated anti-HLA-DR (BD-Pharmigen) antibodies and incubated for 30 min at 4°C.

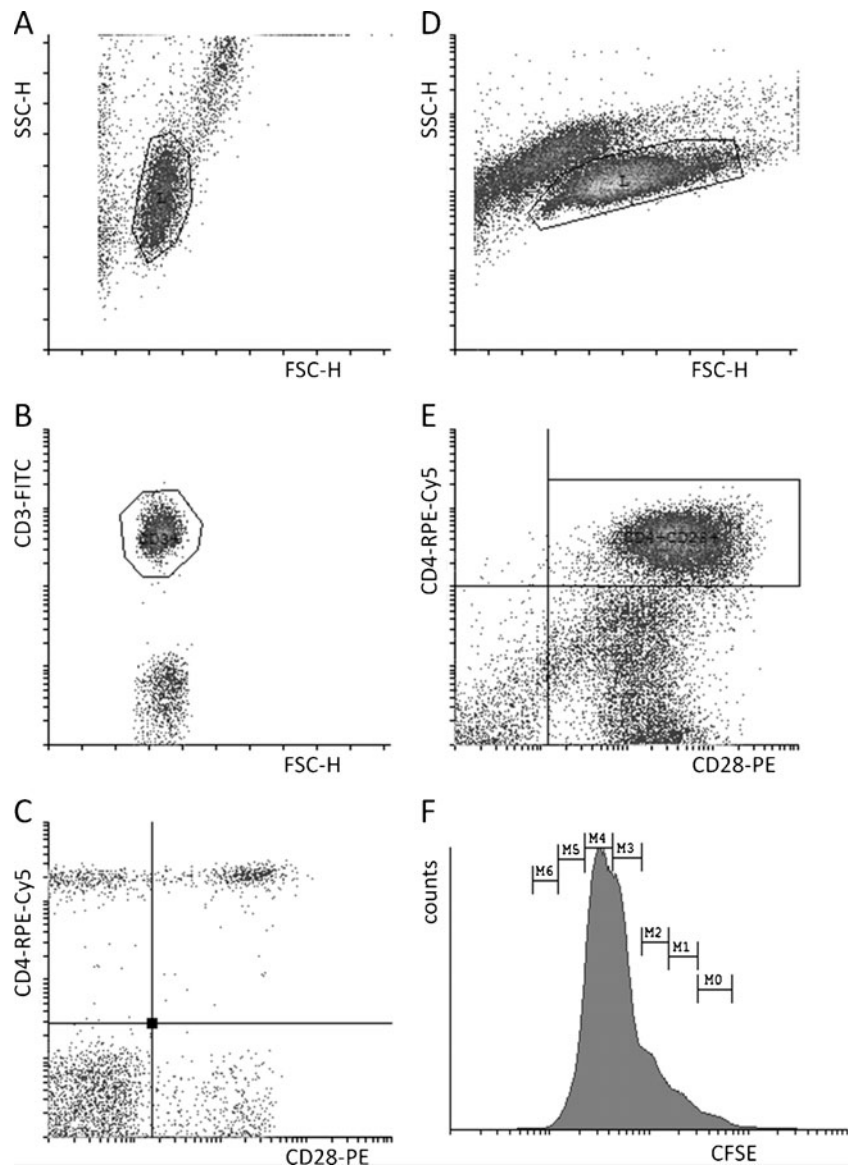
After antibody staining, red blood cells were lysed with buffer containing 0.8% NH<sub>4</sub>Cl and 0.1% KHCO<sub>3</sub>. Finally, cells were resuspended in 200 µl of PBS buffer

**Table I** Basic characteristics of patients and controls

	Predialysis patients	Hemodialyzed patients	Control
Number	10	15	14
Age (years)	57.66±19.28	62.15±17.46	53.14±11.55
Sex (M/F)	7/3	11/4	9/5
GFR (min/ml/1.73 m <sup>2</sup> )	13.10±5.53	10.68±3.27	>60
Hemoglobin (g/dl)	10.51±1.33*	9.86±1.03*	14.04±0.73

\**p*<0.05 vs. control group, *t* test for independent groups

**Fig. 1** Flow cytometric analysis of subpopulations and proliferation of T lymphocytes ex vivo and in vitro. Whole blood cells were stained with monoclonal antibodies against CD3, CD4, and CD28 antigens. First, T lymphocytes were selected on the basis of their forward and side scatter characteristics (a) and CD3 expression (b). Finally, CD3<sup>+</sup> T lymphocytes were visualized in dot-plot showing CD4 and CD28 (or other antigen) on the x- and y-axes, respectively (c). Meanwhile, proliferating T lymphocytes (stained with CFSE as described in “Patients and Methods”) were also selected on the basis of their forward and side scatter characteristics (d). Next, the subpopulation of lymphocytes of interest (for example CD4<sup>+</sup>CD28<sup>+</sup> cells) was gated (e) and their divisions were shown in histogram (f)

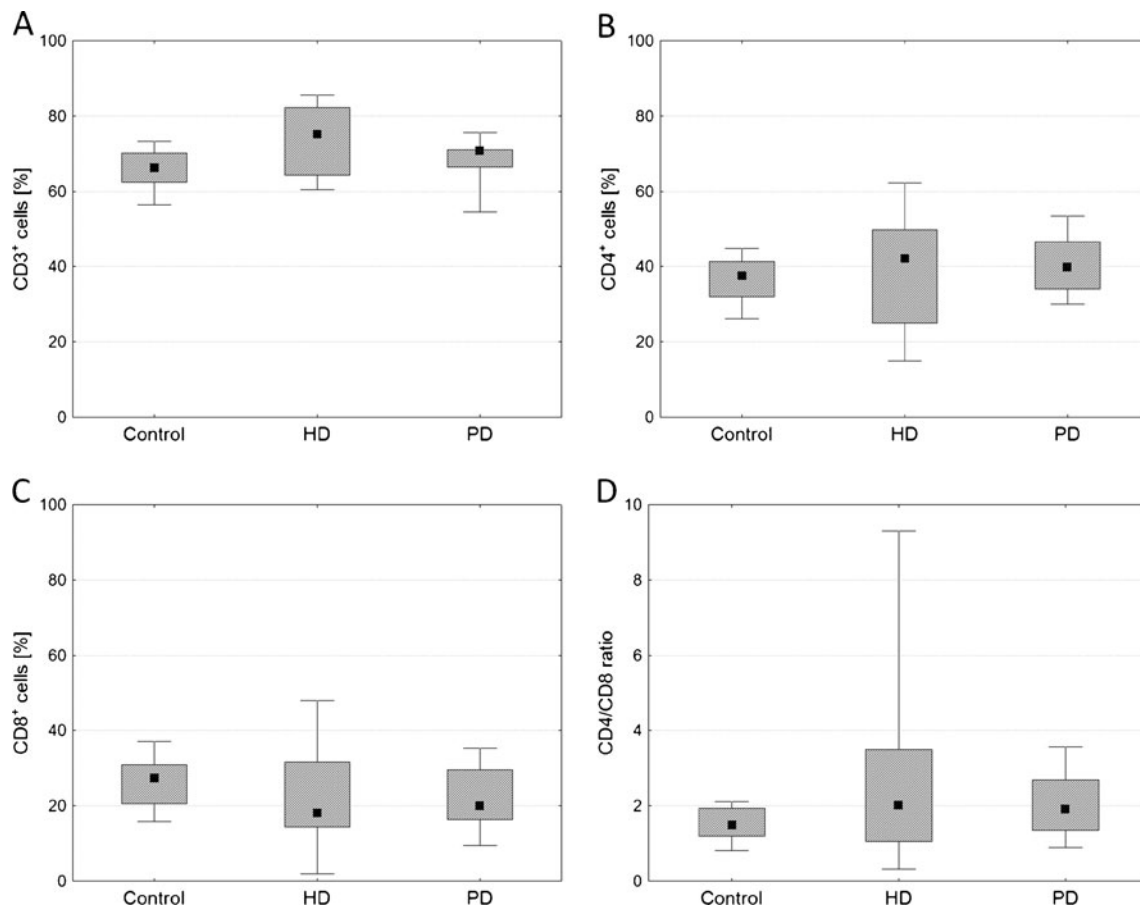


for final flow cytometric analysis. Twenty milliliters of venous blood was designed for PBMC isolation by centrifugation on Histopaque™ gradient (Sigma Chemical Co., USA).

**PBMC In Vitro Stimulation and Dividing Cell Tracking** The dividing cell tracking method used to examine differences in proliferation rate and the percentage of dividing cells were based on Hasbold's method [12] and modified using the method of Witkowski et al. [13]. The advantage of this method allows to stimulate the peripheral blood mononuclear cells without their purification before and to analyze the different subpopulations of dividing cells by staining with different monoclonal antibodies and using flow cytometry. The comparison between different subpopulations from one person is very valuable because the

stimulation conditions are the same; therefore, the observed differences in kinetics are dependent on cell type, not on different stimulation conditions.

Twelve million of PBMC were stained with 3  $\mu$ M CFSE (Sigma Chemical Co.) for 15 min in the dark at 37°C and washed one time with complete medium supplemented with 0.5% FBS and two times with complete medium. Then cells were stimulated with immobilized anti-CD3 antibody (250 ng per 2 million cells in 2 ml of complete medium) and incubated for 5 days at 37°C, 5% CO<sub>2</sub>. The same conditions were applied to the unstimulated PBMC as negative control. Stimulated and unstimulated cells were collected after 72 and 120 h and stained with RPE-Cy5-conjugated anti-CD4 (DAKO), PE-conjugated anti-CD28, PE-conjugated anti-CD69 (BD-Pharmigen), and PE-conjugated anti-CD25 (DAKO) antibodies for 30 min at

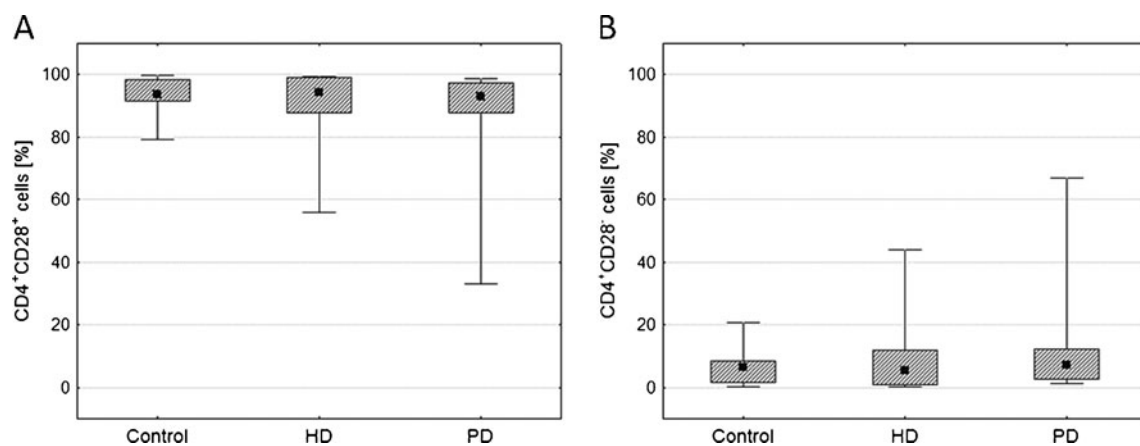


**Fig. 2** Comparison of populations of main T lymphocytes ex vivo. *Graphs* show percentages of main populations: CD3<sup>+</sup> (a), CD4<sup>+</sup> (b), and CD8<sup>+</sup> (c) as well as CD4/CD8 ratio (d) in all examined groups—healthy controls (*Control*), hemodialyzed patients (*HD*), and predialysis

patients (*PD*). *Midpoints* of figures present medians, *boxes* present 25–75%, and *whiskers* outside present the minimum and maximum of all the data, Kruskal–Wallis test

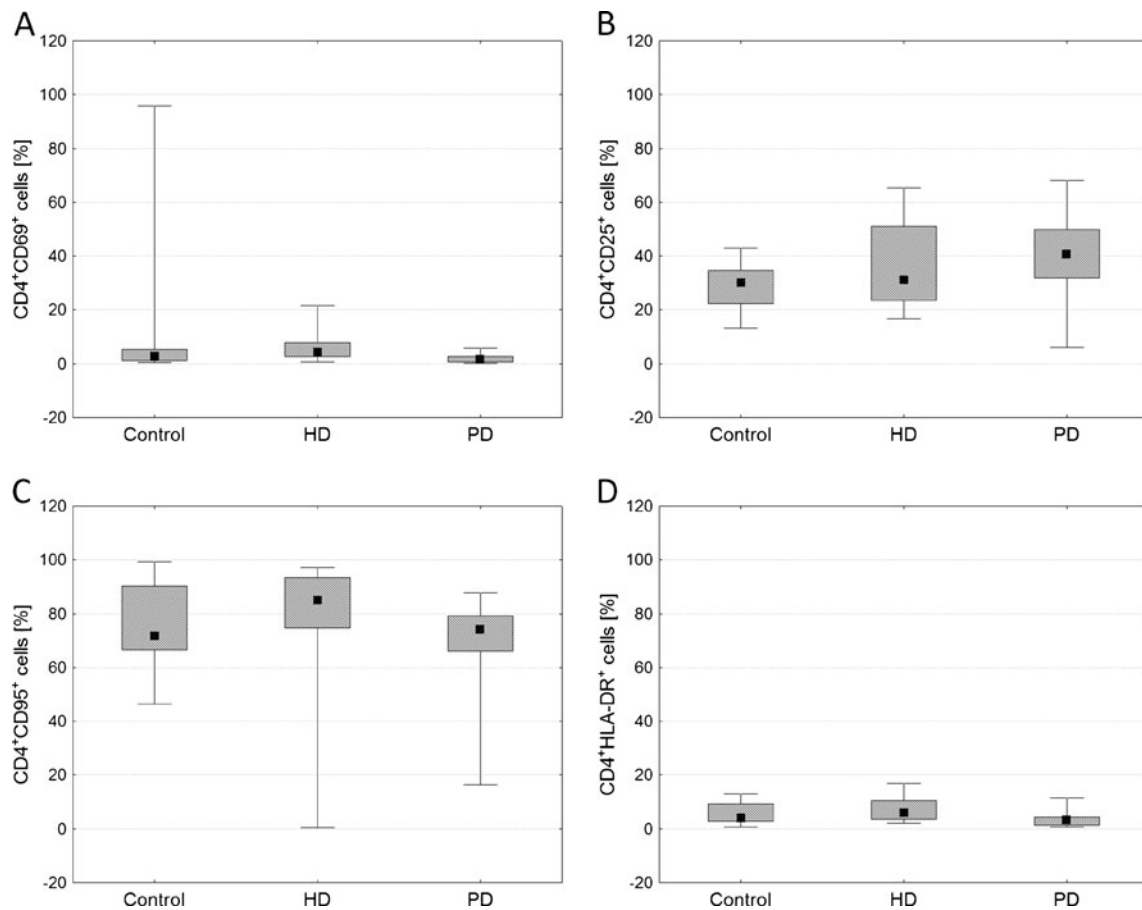
4°C. After washing, cells were resuspended in 200  $\mu$ l of PBS buffer for flow cytometric analysis performed directly after sample preparation.

**Analysis and Statistics** Quantitative fluorescence analysis was performed on FACScan (Becton Dickinson, USA). Expression of surface antigens measured as mean fluores-



**Fig. 3** Comparison of CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD28<sup>-</sup> of subpopulations of T lymphocytes ex vivo. *Graphs* demonstrate the percentages of CD4<sup>+</sup>CD28<sup>+</sup> (a) and CD4<sup>+</sup>CD28<sup>-</sup> (b) cells in all examined groups.

*Midpoints* of figures present medians, *boxes* present 25–75%, and *whiskers* outside present the minimum and maximum of all the data, Kruskal–Wallis test



**Fig. 4** Comparison of subpopulations of main active CD4<sup>+</sup> T lymphocytes ex vivo. *Graphs* demonstrate the percentages of CD4<sup>+</sup>CD69<sup>+</sup> (a), CD4<sup>+</sup>CD25<sup>+</sup> (b), CD4<sup>+</sup>CD95<sup>+</sup> (c), and CD4<sup>+</sup>HLA-

DR<sup>+</sup> (d) cells in all examined groups. *Midpoints* of figures present medians, *boxes* present 25–75%, and *whiskers* outside present the minimum and maximum of all the data, Kruskal–Wallis test

cence intensity (MFI) and the percentage of CD4<sup>+</sup> T lymphocytes were analyzed with the Cyflogic, version 1.2.1 (© Perttu Terho and © CyFlo Ltd).

Gating strategy: Ex vivo, T lymphocytes were selected on the basis of their forward and side scatter characteristics and CD3 expression (Fig. 1). Isotype control (MultiMix™ Triple-Colour Control Reagent, DAKO) was used to determine the positive subpopulation of CD4<sup>+</sup> T lymphocytes expressing CD28, CD69, CD25, CD95, or HLA-DR antigen. The percentage of positive T cells was calculated as the percentage of CD4<sup>+</sup> T lymphocytes.

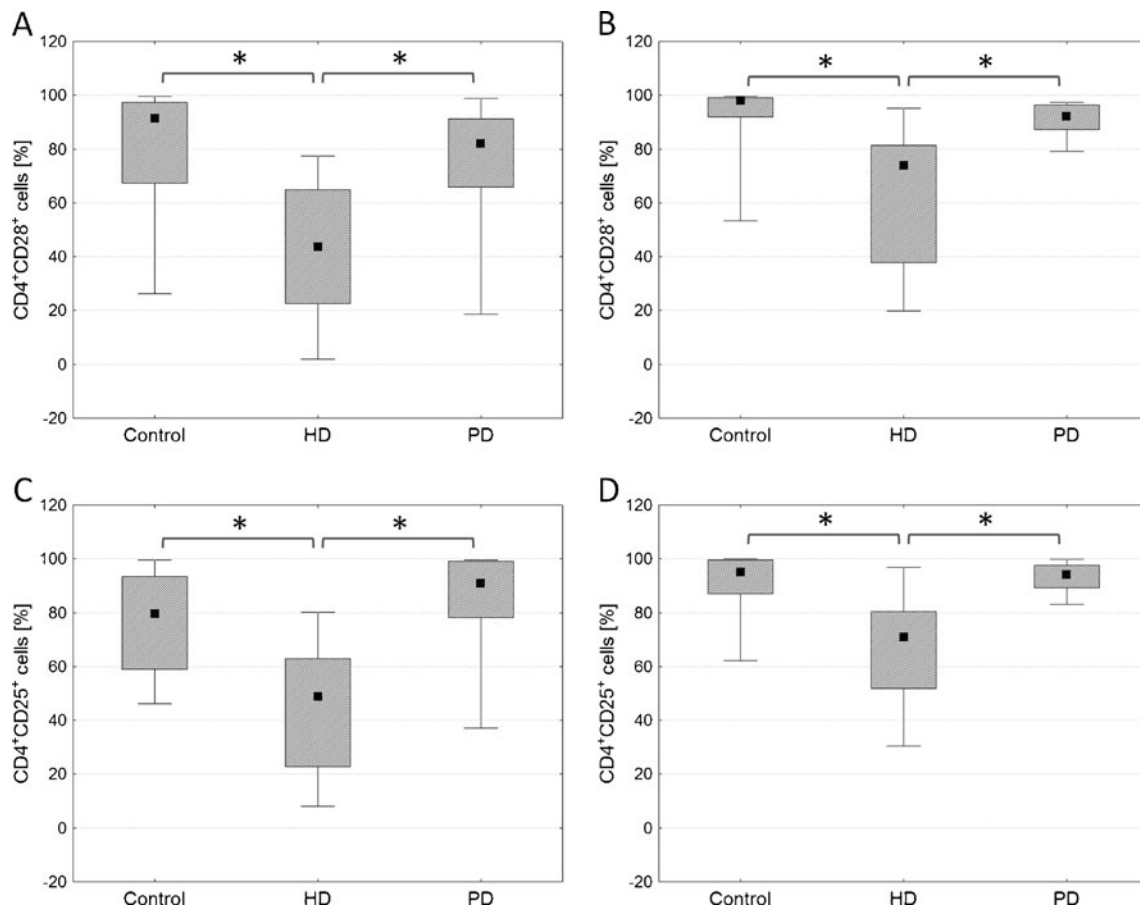
Proliferating T lymphocytes were also selected on the basis of their forward and side scatter characteristics (Fig. 1d). Next, the population of lymphocytes of interest was gated (Fig. 1e) and their divisions were shown in histogram (Fig. 1f). Calculation of cell cycle kinetics (G0→G1 time, cell cycle duration, number of divisions per one cell, and number of finished divisions) of CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD28<sup>-</sup> cells was performed according to the Witkowski protocol [13].

Statistical analysis was done using the computer program Statistica, version 8 (StatSoft, Poland). The significance tests were chosen according to the data distribution (as indicated in the “Results”). Due to irregularly distributed variances, appropriate nonparametric tests were applied. The level of significance in all was  $p \leq 0.05$ .

## Results

*Composition of Subpopulations of Main T Lymphocytes Ex Vivo* No changes were seen in the percentage of T lymphocytes (CD3<sup>+</sup> cells) between PD patients, HD patients, and healthy controls ex vivo (Fig. 2a). Also, we did not observe any differences in proportions of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes as well as in CD4/CD8 ratio in all examined groups (Fig. 2b–d). There was no difference in the proportions of CD4<sup>+</sup>CD28<sup>+</sup>/CD4<sup>+</sup>CD28<sup>-</sup> lymphocytes in all examined groups (Fig. 3). The percentages of main active





**Fig. 5** Proportions of CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T lymphocytes *in vitro*. *Graphs* present the percentages of CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells after 72 h (a, c) and 120 h (b, d) of stimulation with anti-CD3

antibody in examined groups. *Midpoints* of figures present medians, *boxes* present 25–75%, and *whiskers* outside present the minimum and maximum of all the data, \**p*<0.05, Kruskal–Wallis test

CD4<sup>+</sup> T lymphocyte subpopulations, that is, CD4<sup>+</sup>CD25<sup>+</sup>, CD4<sup>+</sup>HLA-DR<sup>+</sup>, CD4<sup>+</sup>CD69<sup>+</sup>, or CD4<sup>+</sup>CD95<sup>+</sup> cells, were also at similar level in patients and healthy controls (Fig. 4).

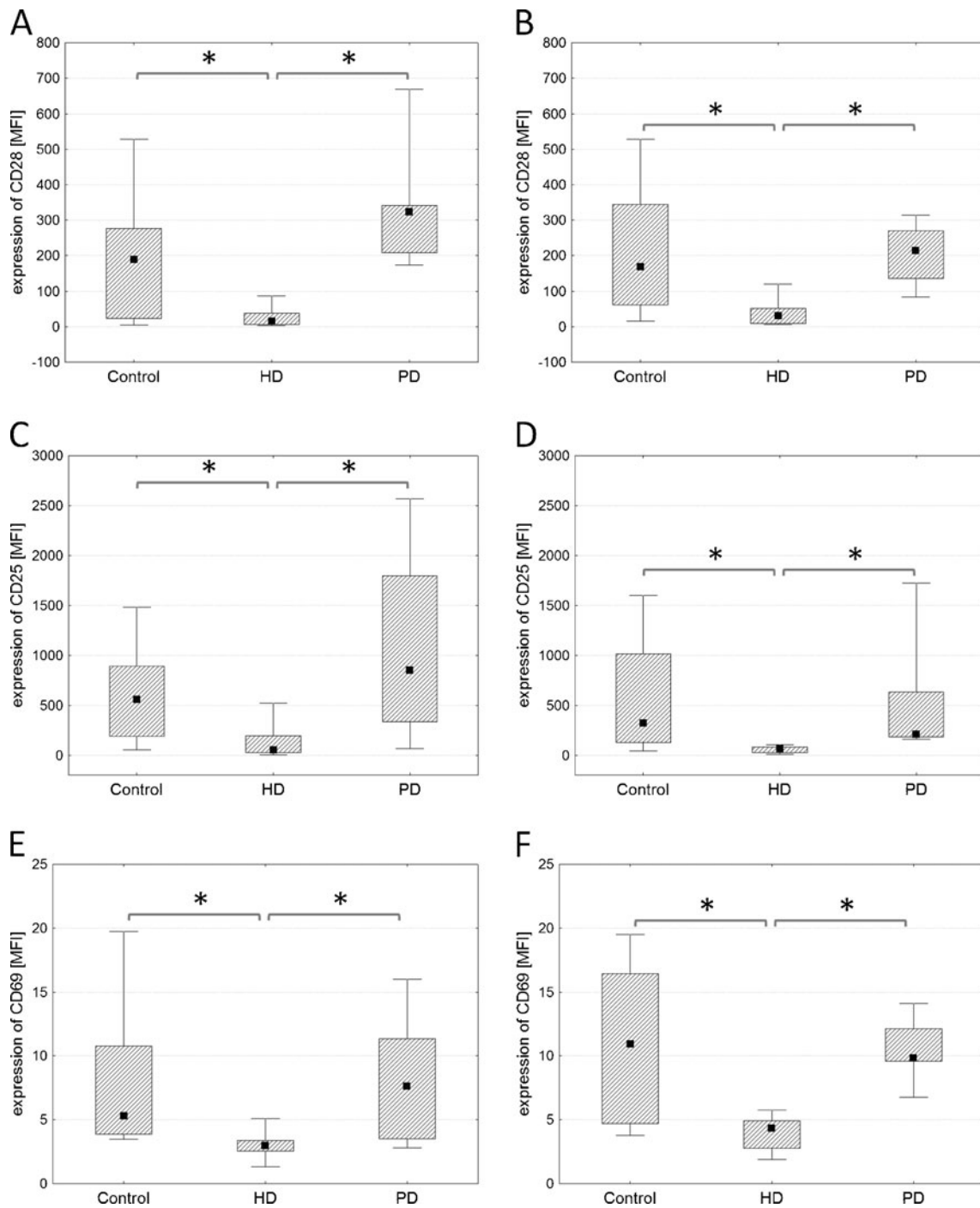
*In Vitro Studies of Subpopulations of Main CD4<sup>+</sup> T Lymphocytes and Their Antigens* The percentages of CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T lymphocytes of HD patients were significantly decreased after 72 and 120 h of stimulation with anti-CD3 antibody when compared to predialysis patients and healthy controls (Fig. 5).

The major findings of the study were related to changes in the expression of CD28, CD25, and CD69 antigens on CD4<sup>+</sup> T lymphocytes measured as MFI. Stimulated CD4<sup>+</sup> T lymphocytes of HD patients presented decreased expression of co-stimulatory molecule CD28 when compared to predialysis patients and healthy controls (Fig. 6a, b). The expression of middle activation antigen CD25 (Fig. 6c, d) and early activation antigen CD69 (Fig. 6e, f) was also lower in HD patients as compared to the other two examined groups. The group of HD patients was characterized both by a lower percentage of cells with co-stimulatory and

activation antigens and by a reduced level of these antigens on a single cell. In contrast to HD patients, CD4<sup>+</sup> T lymphocytes obtained from PD patients presented levels of CD28, CD25, and CD69 similar to those observed in healthy controls.

*In Vitro Studies of the Proliferation of CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD28<sup>+</sup> T Lymphocytes* HD patients showed a significantly decreased percentage of CD4<sup>+</sup>CD28<sup>+</sup> lymphocytes proliferating after stimulation with the anti-CD3 antibody (Fig. 7a, b) when compared to PD patients and healthy controls. The duration of cell cycle of CD4<sup>+</sup>CD28<sup>+</sup> cells was similar in all examined groups (Fig. 7c), while the calculated period of time required by these cells to enter the G1 phase (the G0→G1 time) was longer in HD patients compared to healthy controls (Fig. 7d).

The general number of divisions of proliferating CD4<sup>+</sup>CD28<sup>+</sup> T lymphocytes did not vary between all examined groups (Fig. 8a), but the number of divisions per one cell and the number of finished divisions were significantly reduced in HD patients when compared with healthy controls after 72 h of stimulation (Fig. 8b, c).



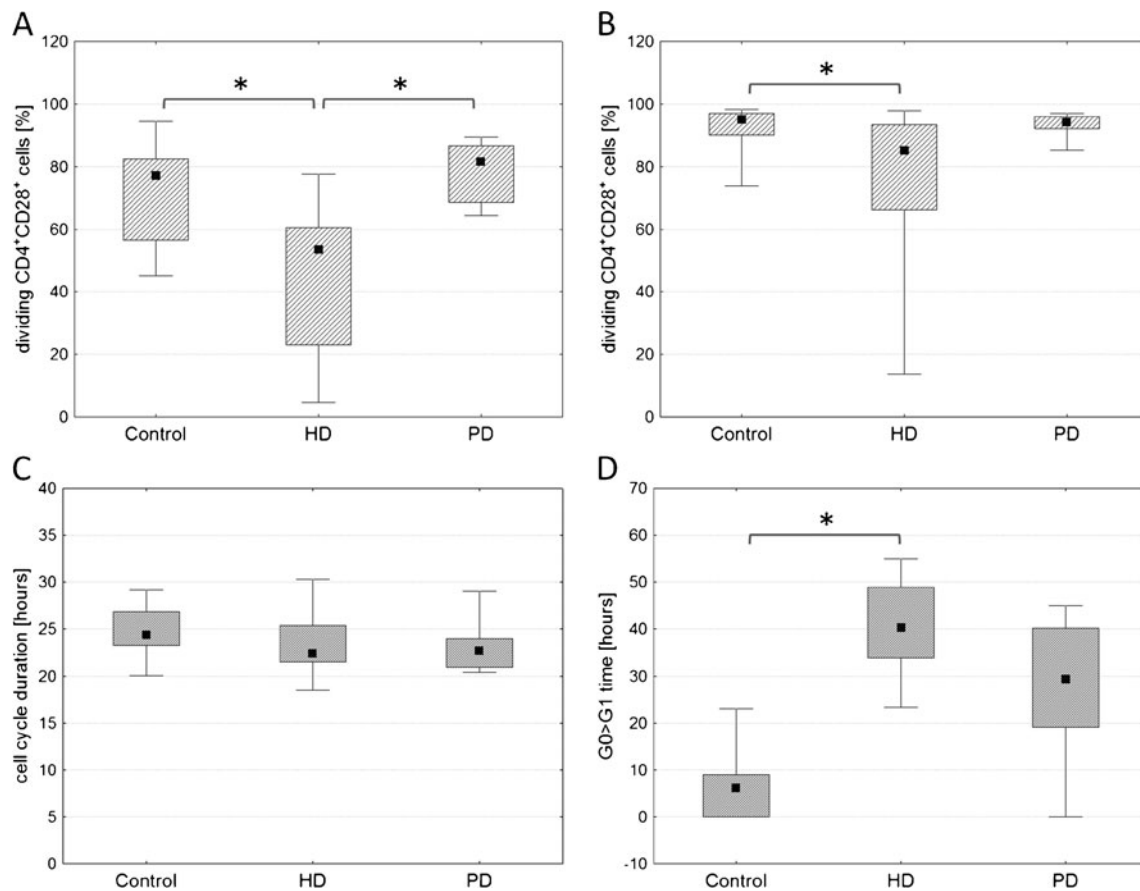
**Fig. 6** Changes in the expression of CD28, CD69, and CD25 antigens on CD4<sup>+</sup> T lymphocytes in vitro. *Graphs* show the expression of CD28, CD69, and CD25 antigens on CD4<sup>+</sup> T lymphocytes after 72 h (a, c, e) and 120 h (b, d, f) of stimulation

with anti-CD3 antibody in all examined groups. *Midpoints* of figures present medians, *boxes* present 25–75%, and *whiskers* outside present the minimum and maximum of all the data, \**p*<0.05, Kruskal–Wallis test. *MFI* mean fluorescence intensity

Moreover, a decreased number of divisions per one cell was positively correlated with a decreased percentage of proliferating CD4<sup>+</sup>CD28<sup>+</sup> lymphocytes (Fig. 8d) and negatively correlated with longer G0→G1 time (Fig. 8e) in that group of patients. Also, a decreased number of

finished divisions was negatively correlated with longer G0→G1 time (Fig. 8f).

No changes were seen in the percentage of proliferating CD4<sup>+</sup>CD28<sup>-</sup> T lymphocytes between HD and PD patients and healthy controls (Fig. 9a). Also, the general number of



**Fig. 7** Changes in main cell cycle parameters of CD4<sup>+</sup>CD28<sup>+</sup> T lymphocytes. *Graphs* present the percentage of proliferating CD4<sup>+</sup>CD28<sup>+</sup> cells after 72 h (a) and 120 h (b) of stimulation with anti-CD3 antibody, cell cycle duration (c), and time between phase G0

and G1 (d) in examined groups. *Midpoints* of figures present medians, *boxes* present 25–75%, and *whiskers* outside present the minimum and maximum of all the data, \**p*<0.05, Kruskal–Wallis test

divisions or number of divisions per one cell did not vary between all examined groups (Fig. 9b, c).

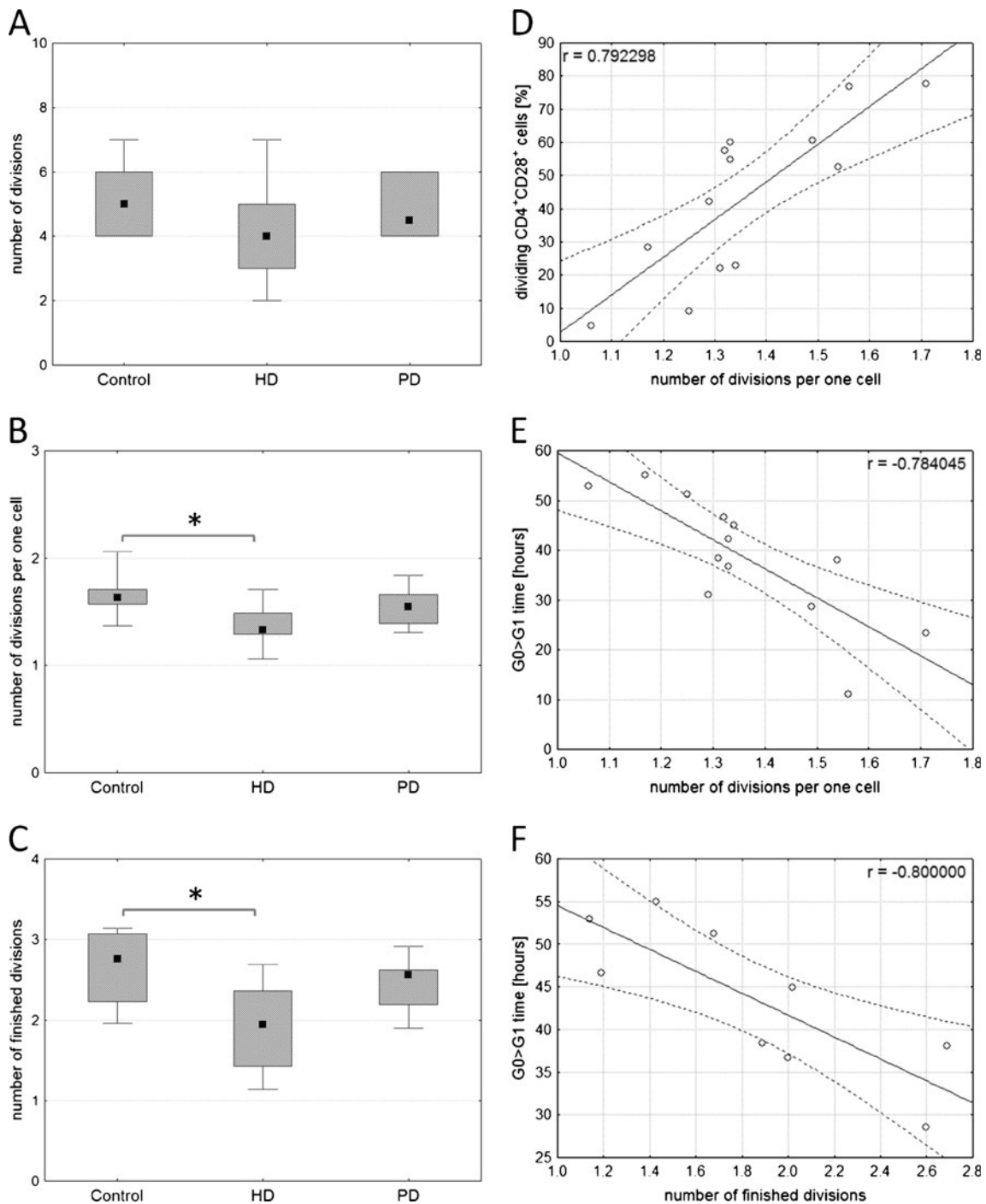
## Discussion

Immunological response mediated by T lymphocytes is known to be weakened in CKD patients. The influence of hemodialysis on immune response is intensively discussed and brings many contradictory information. The majority of the studies show that the numbers of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells are decreased in HD patients and postulate that the HD procedure induces apoptosis of T lymphocytes [11, 14]. Our previous studies showed that HD patients demonstrate disturbances in subpopulations of activated CD4<sup>+</sup> T lymphocytes ex vivo, presented by significantly higher percentages of CD4<sup>+</sup>CD95<sup>+</sup> and CD4<sup>+</sup>HLA-DR<sup>+</sup> and a lower percentage of CD4<sup>+</sup>CD69<sup>+</sup> cells [7]. Also, CD4<sup>+</sup> T lymphocytes of HD patients after stimulation with anti-CD3 antibody exhibit decreased expression of antigens

crucial for their activation and proliferation [7]. The disturbances in phenotype and proliferation of CD4<sup>+</sup> T lymphocytes can be fundamental for deficient cellular and humoral responses in CKD. The question is if these changes depend only on continuous hemodialysis or are strictly connected with CKD progression leading to kidney failure. Therefore, we examined the main parameters of the immune responses of CD4<sup>+</sup> T lymphocytes and compared them in hemodialyzed and predialysis patients and healthy controls.

Studies ex vivo showed that the percentage of CD3<sup>+</sup> lymphocytes and the percentages of their main populations CD4<sup>+</sup> and CD8<sup>+</sup> cells are comparable in all examined groups. These observations are very interesting in the context of the data of Borges and colleagues presenting a significant decrease in these particular populations of lymphocytes [11]. However, these parameters were measured immediately after the HD procedure, while our blood samples were collected from each patient before the HD session. This observation indicates that a single contact of PBMC with the dialytic membrane can induce apoptosis of



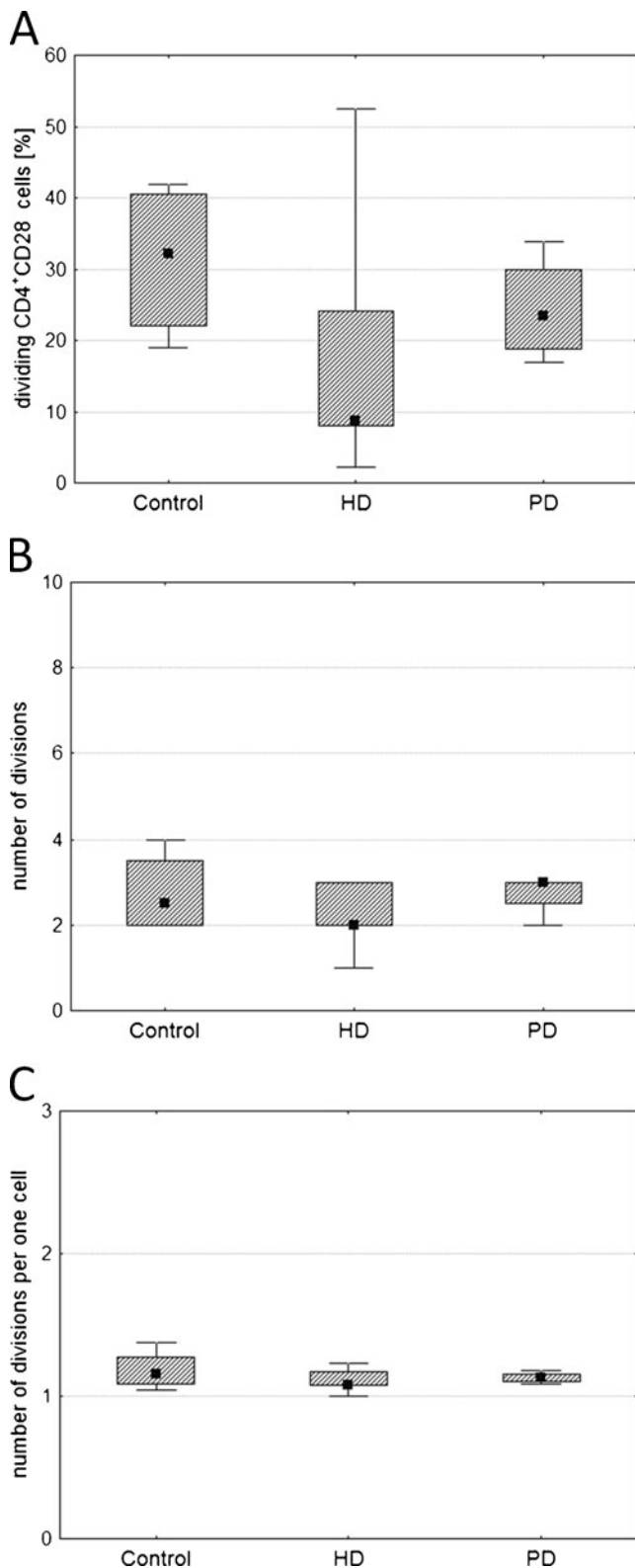


**Fig. 8** Comparison of the number of divisions of CD4<sup>+</sup>CD28<sup>+</sup> T lymphocytes and their correlations with other cell cycle parameters. *Graphs* present the general number of divisions (a), the number of divisions per one cell (b), and the number of finished divisions (c)

after 72 h of stimulation with anti-CD3 antibody in examined groups. *Midpoints* of a–c present medians, *boxes* present 25–75%, and *whiskers* outside present the minimum and maximum of all the data, \* $p < 0.05$ , Kruskal–Wallis test. **d–f** Spearman  $R$  correlation

T lymphocytes. There are studies showing increased apoptosis of T lymphocytes of CKD patients, mainly Th1 subpopulation, in comparison to healthy controls [15]. According to Alvarez-Lara et al., Th1 lymphocytes of patients presented decreased expression of anti-apoptotic molecule Bcl-2, which made them more susceptible to

undergo apoptosis, independent of exposure to the dialytic membrane [15]. The mechanisms underlying these results are not known and it is suggested that uremic toxins can act directly on lymphocytes. If this is the case, it is not clear why only Th1 lymphocytes would be more sensitive to these toxins. Anyway, it seems that different results come



**Fig. 9** Comparison of main cell cycle parameters of  $CD4^+CD28^-$  T lymphocytes. *Graphs* present the percentage of proliferating  $CD4^+CD28^-$  cells (**a**), the general number of divisions (**b**), and the number of divisions per one cell (**c**) after 72 h of stimulation with anti-CD3 antibody in examined groups. *Midpoints of a–c* present medians, *boxes* present 25–75%, and *whiskers* outside present the minimum and maximum of all the data, Kruskal–Wallis test

the increase of the number of circulating  $CD4^+CD28^-$  cells in ESRD patients [16, 17], we also examined the proportions of  $CD4^+CD28^+/CD4^+CD28^-$  lymphocytes ex vivo. The percentage of circulating  $CD4^+CD28^+$  lymphocytes in both groups of patients was not decreased in favor of expansion of  $CD4^+CD28^-$  cells as compared to healthy people. No activation-related phenotype of T lymphocytes ex vivo (both early and late) in all examined groups was seen that could indicate that both HD and PD patients did not present an enhanced inflammatory response during the study.

The in vitro experiments showed both significantly lower proportions of  $CD4^+CD28^+$  and  $CD4^+CD25^+$  cells and reduced expression of CD28, CD25, and CD69 antigens only in HD patients, in contrast to PD patients, who had comparable values as the healthy controls. Lower levels of these antigens on  $CD4^+$  T lymphocytes of HD patients strongly indicate that the repeated hemodialysis procedure is the major factor influencing the phenotype of  $CD4^+$  T lymphocytes. These changes appear independently of the patients' immunological status ex vivo, as both HD and PD patients presented the proportions of subpopulations of main  $CD4^+$  T lymphocytes similar to those observed in healthy controls.

Changes in phenotype of  $CD4^+$  T lymphocytes of HD patients consequently lead to changes in their proliferation parameters. HD patients showed lower percentage of proliferating  $CD4^+CD28^+$  lymphocytes when compared to PD patients and healthy controls. There was no difference between cell cycle duration between all examined groups, but  $CD4^+CD28^+$  lymphocytes of HD patients were characterized by longer time between G0 and G1 phases of cell cycle. We already demonstrated that there is a strict connection between the level of co-stimulatory molecule CD28 on  $CD4^+$  T lymphocytes and the time needed for response to stimulation [18]. This observation was described for healthy people [18] as well as in HD patients not receiving rhEPO [7]. Analysis of the cell cycle also revealed that proliferating  $CD4^+CD28^+$  T lymphocytes of HD patients apart from prolonged G0→G1 time are also characterized by reduced numbers of finished divisions and divisions per one cell in 72 h of stimulation. Meanwhile,  $CD4^+$  T lymphocytes of predialysis patients present cell cycle parameters comparable with those of healthy controls.

Changes in cell cycle kinetics did not concern the population of  $CD4^+CD28^-$  T lymphocytes in all examined groups, meaning that the main subpopulations changed by

mainly from various methods of preparing blood samples and experimental models used by the authors.

Since there are articles describing the impact of CMV seropositivity on  $CD4^+$  T lymphocyte subsets, especially

hemodialysis are CD4<sup>+</sup>CD28<sup>+</sup> T cells, which play a central role in acquired immune response.

These observations indicate that repeated hemodialysis procedure causes negative changes in the phenotype of CD4<sup>+</sup> T lymphocytes of CKD patients. We have demonstrated similar results in a previous article paying attention to the immunomodulatory effects of rhEPO treatment in these patients [7]. Our observations showed that rhEPO administration to HD patients positively influences the levels of crucial surface antigens and proliferation kinetics of these cells [7]. Older studies have also shown that rhEPO treatment causes a decline of TNF- $\alpha$  [19]. High levels of TNF- $\alpha$  could be one of the reasons for lower CD28 expression in HD patients and at the same time indirectly affect the proliferation of CD4<sup>+</sup>CD28<sup>+</sup> T lymphocytes, since TNF was shown to directly regulate transcription of the CD28 gene [20] and also influence the CD28 expression on CD4<sup>+</sup> T cells in RA patients treated with anti-TNF [21]. IL-6 is the second proinflammatory cytokine that inhibits differentiation of Th1 lymphocytes [22]. Therefore, one of the depressing effects of repetitive hemodialysis on T lymphocytes could be dependent on the high levels of these proinflammatory cytokines. On the other hand, blood was collected prior to HD session, before monocytes had any contact with the dialytic membrane. Since there is a predominance of Th1 over Th2 lymphocytes in HD patients [23] in opposition to predialysis patients, who mainly exhibit a Th2 response [24], changes in phenotype of CD4<sup>+</sup> T lymphocytes of HD patients could result from cytokine imbalance.

On the other hand, stress-induced premature senescence (SIPS), a process that involves changes in the function and morphology of cells and is detected among others by telomere shortening, could be another explanation of deficient reactions of CD4<sup>+</sup> T lymphocytes of HD patients. Some time ago, Jimenez and colleagues presented the theory of SIPS in CKD [25]. They found the subpopulation of lymphocytes with short telomeres in CKD patients and noticed that the mean telomere length was significantly decreased in mononuclear cells from HD patients treated with cellulosic membranes when compared with non-cellulosic and predialysis patients [25]. Lymphocytes of CKD undergo SIPS and repeated hemodialysis procedure deepens this process. In our study, CD4<sup>+</sup> T lymphocytes of HD patients present lower CD28 and CD25 expression, another sign of proliferative senescence [16, 26, 27].

## Conclusions

For the moment, the mechanisms underlying deficient reactions of CD4<sup>+</sup> T lymphocytes of HD patients remain an open question, and additional studies are needed to

explain these observations. However, disturbances in the activation parameters of T lymphocytes could result from the presence of uremic toxins and proinflammatory cytokines, or be related to their early proliferative senescence, which is deepened by continuous hemodialysis.

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