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Application of an intermediate concentration of cyclophosphamide does not specifically deplete regulatory T cells in a mouse experimental model

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Abstract: Cyclophosphamide (CP) is a cytostatic, widely used to treat different carcinomas and autoimmune diseases. It is commonly used in experimental designs modeling immunosuppression in laboratory animals, with different approaches for CP treatment but without a consensus on the dose, timing, and route of administration. We aimed to establish if treatment with CP in C57BL/6 mice depletes regulatory T cells (Tregs). Tregs are a crucial component of the immune system that helps maintain immune tolerance and prevent excessive immune reactions. They are significant in autoimmune diseases, allergies, and immune-related therapies. CP was applied intraperitoneally (i.p.) twice in a 5-day interval in doses of 100 mg/kg. Monitoring of Treg prevalence in peripheral blood after each treatment and in the spleen after the second treatment with CP revealed a drop in the number of Tregs after two doses of CP because of the decreased number of total lymphocytes but not as a specific response of the Tregs. The prevalence of Tregs in peripheral blood after CP treatment mirrored the change in Treg number in the spleen. CP treatment induced a decrease in the number of CD3+ cells in the spleen while increasing their proportion, indicating that CP affected the B lymphocyte population rather than T cells. Our results suggest that CP treatment cannot be used as a specific Treg-depleting agent in the C57BL/6 animal model.

Keywords: cyclophosphamide, immunosuppression, regulatory T cells (Tregs), lymphocytes, mouse experimental model

INTRODUCTION

Cyclophosphamide (CP) is a commonly used cytostatic in the medical treatment of metastatic carcinomas, such as multiple myeloma, lymphoma, small cell lung carcinoma, and breast cancer [1-3], but also in some autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis) [4,5]. It has been shown that the clinical application of CP often causes immunosuppression [6], which is helpful in treating autoimmune disorders. At the same time, it has also been widely used for its ability to deplete regulatory T cells (Tregs) in the treatment of malignant diseases [7]. CP also exhibits other immune-modifying effects, such as the inhibition of T-cell proliferation [7]. Although the influence of CP on T cells was mostly focused on Tregs, since these cells are among the main components of the immune system responsible for tumor progression, and their prevalence among CD4+ T cells in patients' blood is usually decreased after the treatment [8], it has been shown that short-term treatments with CP have a substantial impact on both CD4+ and CD8+ central and effector memory T-cell subpopulations [9].

Regarding its potential in treating different diseases, the effect of CP on the immune system has been explored in several studies in animal experimental model systems, primarily in mice. It was shown that CP applied in different doses induces strong leukopenia and lymphopenia and that cells are affected by such treatment in a dose-dependent manner [10]. The frequent use of CP in experimental studies, especially in models of immunodeficiency, confirmed the effect of CP on peripheral blood cells but also emphasized that CP has a critical impact on the spleen, including the reduction of both spleen weight and splenocyte proliferation [9-11]. Studies have shown that CP efficiently induces Treg depletion even after the application of a single dose, subsequently enabling an enhanced tumor-specific immune response in rats [12,13]. Despite these results

regarding the efficiency of a single CP dose, it appears that the effect of CP on the decrease of CD4+ CD25+ Foxp3+ T-cell subset largely depends on the applied dose, the timing, and the number of doses, since those parameters differ among studies. Doses ranging from 20 to 200 mg/kg were tested for the potential to deplete splenic CD4+ CD25+ Foxp3+ Tregs [14] and showed that low doses (20 mg/kg) induce selective depletion of Tregs, while high doses (200 mg/kg) decrease both effector and regulatory T-cell numbers to a high degree (around 90%). Such low doses of CP appeared to be effective in cancer treatments, and their effectiveness relies on the ATP levels expressed in Tregs [15]. On the other hand, in a few studies, larger doses (mostly 100-150 mg/kg) were applied one or more times to induce immunosuppression [10,16,17].

Several studies in animal models of type 1 diabetes made use of CP as a specific Treg-depleting approach for accelerating the disease [18-21]. Therefore, the presented study intended to investigate whether one or two doses of CP (100 mg/kg), applied intraperitoneally, specifically enable the depletion of CD4+ CD25+ Foxp3+ Tregs. The dose was chosen as an intermediate between 70 mg/kg and 200 mg/kg used in type 1 diabetes acceleration studies. The results indicate that CP, either given in one or two doses, does not explicitly deplete Tregs but rather affects the total numbers of CD3+ and CD4+ T lymphocytes. As an additional finding, the proportion of Tregs in the peripheral blood can be correlated to their proportion in the spleen of CP-treated C57BL6 mice. Therefore, the investigated CP protocol cannot be used for specific *in vivo* Treg depletion during drug discovery research where Tregs occur as a target for a particular drug.

MATERIALS AND METHODS

Ethics statement

The study was performed on C57BL/6 mice (8-week-old males) that were purchased from Charles River Laboratories (Sulzfeld, Germany). The animals were housed at the animal facility at the Institute for Biological Research "Siniša Stanković" according to institutional procedures, in plastic cages and rooms with constant temperature (22±2 °C) and humidity (50±10%), 12:12 h light/dark cycle, with food and water available *ad libitum*. The local ethics committee approved experimental procedures, and all were in accordance with directive 2010/63/EU on the protection of animals used for scientific purposes and approved by the Ministry of Agriculture and Environmental Protection, Republic of Serbia (Decision No. 323-07-05815/2020-05/1).

Experimental design

Cyclophosphamide (Endoxan, Baxter Oncology GmbH, Germany) was reconstituted in 0.9% NaCl and applied i.p. in a 100 mg/kg dose. Four mice were sampled for blood from the retro-orbital plexus immediately before treatment with CP (Day 0) to assess the initial profile of CD4+ lymphocytes/Tregs. The blood sampling was repeated 48 h after the first treatment (CP 1×, 48 h), the second treatment was applied 5 days after the first, and the next blood sampling was done 24 h upon the second treatment (CP 2×, 24 h). Mice treated with CP, as well as the group of four mice i.p.-treated with 0.9% NaCl only (control) at the same time points as the CP-treated mice, were euthanized 48 h after the second treatment, and their spleens were extracted in order to establish the prevalence of Tregs on a systemic level.

Cells

Blood was collected into heparin-containing tubes. Erythrocytes from blood samples were lysed with BD FACS™ lysing buffer (Becton Dickinson, BD Biosciences, USA), and the remaining white blood cells (WBC) were washed in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and an antibiotic/antimycotic solution (all from Capricorn Scientific, Ebsdorfergrund, Hessen, Germany), the complete medium, before counting in Trypan blue dye. The WBC were used for flow cytometric analyses of Tregs.

Splenocytes were isolated from euthanized C57BL/6 mice. The spleens were squeezed with a syringe plunger through a nylon mesh (with 70 μm pores) and washed in a 5% FCS/RPMI medium. The cell pellet was lysed using lysis buffer (0.8% NH_4Cl, pH 7,3) for 5 min, washed once again before resuspension in complete RPMI medium, and counted using Trypan blue dye.

Flow cytometry

Immediately after the isolation of WBC or splenocytes, the cells were stained for flow cytometry. After the cells were washed with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) (Thermo Scientific, USA), they were incubated with antibodies for surface staining (45 min, +4°C). When the biotin-labeled antibody was used, the cells were subsequently incubated with streptavidin FITC (Invitrogen, USA) (30 min, +4°C). After the washing step, the cells were fixed/permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (Invitrogen, eBioscience, USA) according to the manufacturer's instructions, after which intracellular staining of cells was performed (incubation 45 min, +4°C).

The following monoclonal antibodies were used for surface staining: anti-mouse CD3 biotin (clone 17A2; Biolegend, San Diego, CA, USA), anti-mouse CD4 eFluor 506 (clone RM4-5; Invitrogen, USA), anti-mouse CD25 PECy5.5 (clone PC61.5; eBioscience, USA), while anti-mouse Foxp3 PE (clone FJK-16s; Invitrogen, USA) was used for intracellular staining. Irrelevant isotype-matched monoclonal antibodies were used as controls.

A minimum of 20,000 cells per sample was obtained and characterized on the BD FACS ARIA III flow cytometer (Becton Dickinson, USA) at a flow rate ranging between 100 and 200 cells/s. The data were analyzed using FlowJo software, version 10.8.1 (Ashland, USA). The gates for lymphocytes were set according to their specific forward scatter (FS) and side scatter (SS) properties, thereby avoiding dead cells with a low FS/SS signal. FlowJo software was also used to determine the frequencies of T cells, CD4+ lymphocytes, and/or Treg cells among all peripheral blood/splenic cells. Absolute cell numbers were calculated by multiplying the indicated cell frequencies by the total number of peripheral blood/splenic cells.

Analysis of organ index

The organ index was calculated after spleen extraction as the ratio of the splenic weight to the animal body weight, following the formula:

organ index (mg/g)=organ weight (mg)/body weight (g).

Statistical analysis

The effects of CP treatment on the peripheral blood lymphocyte compartment were analyzed using repeated measures of one-way analysis of variance (RM ANOVA), followed by Tukey's post hoc test. Unpaired Student's t-test was used to analyze the differences in splenocyte subset distribution between groups of CP-treated animals and controls. Pearson's correlation analysis was used to analyze the correlation between the decrease of Tregs in the blood and spleen in CP-treated mice (GraphPad Prism version 8.00 for Windows, GraphPad Software, San Diego, CA, USA). Data are presented as means±SD, and differences were considered significant at $P \leq 0.05$.

RESULTS

Cyclophosphamide treatment and CD4+ CD25+ Foxp3+ Tregs in the blood

Since it has been demonstrated that CP treatment decreases the percentage of Tregs in human blood [22], we investigated whether the application of either a single and/or a double dose of CP impacts the prevalence of CD4+ CD25+ Foxp3+ Tregs in the blood of C57BL/6 mice.

The percentages of CD4+ and CD4+ CD25+ Foxp3+ cells and their absolute numbers in peripheral blood were monitored 48 h and 24 h after the first and the second CP adminstration, respectively (See Materials and Methods section). The percentages of CD4+ cells among WBC were unaltered throughout CP administration compared to those observed before the initiation of the treatment (control, day 0) (Fig. 1B). Similarly, the percentages of CD4+ CD25+ Foxp3+ Tregs in peripheral blood samples taken after either the first or the second CP administration remained comparable to the control values obtained at day 0 (Fig. 1B).

The absolute number of WBC was slightly decreased over the course of the treatment, although without statistical significance. However, while the absolute numbers of peripheral blood lymphocytes remained unaltered after the first CP administration, they significantly decreased ($P<0.0001$) after the second CP treatment compared to the control

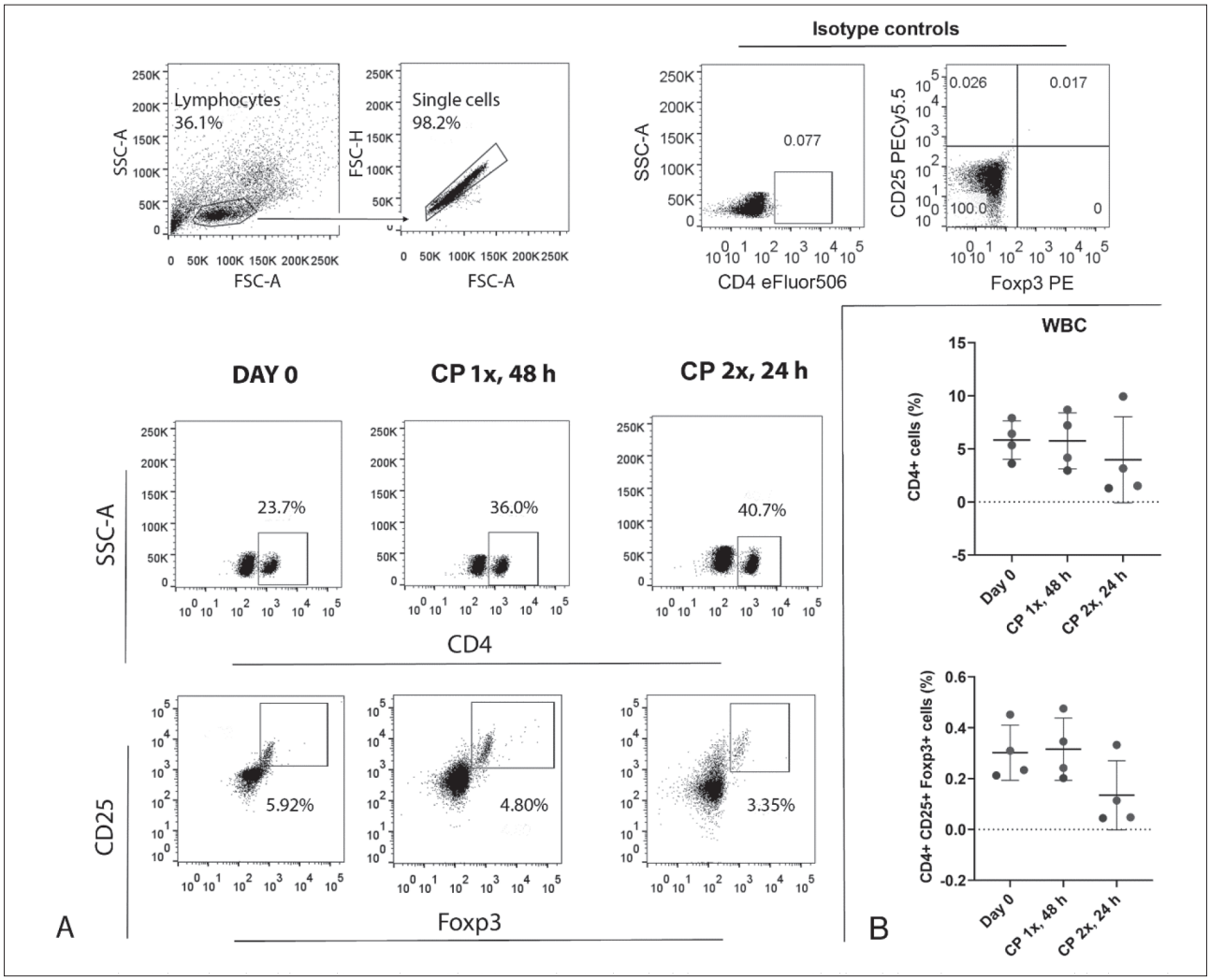

Fig. 1. The effect of cyclophosphamide (CP) application on the prevalence of CD4+ CD25+ Foxp3+ regulatory T cell (Treg) populations in peripheral blood. CP was applied intraperitoneally (100 mg/kg) twice in a 5-day interval. Blood sampling was performed on day 0 immediately before treatment initiation and repeated 48 h after the first CP administration (CP 1x, 48 h) and 24 h after the second CP application (CP 2x, 24 h). **A** – Representative flow cytometry plots from one experiment, showing the percentages of (middle panel) CD4+ cells in peripheral blood lymphocytes and (lower panel) CD25+ Foxp3+ regulatory cells in the CD4+ lymphocyte population. The upper panel indicates the gating strategy for peripheral blood lymphocytes and isotype-matched controls used to set the gating boundaries. **B** – Summarized data indicating the percentages of CD4+ and CD4+ CD25+ Foxp3+ cells in peripheral blood at different time points (untreated, treated once and twice with CP), shown as the means±SD for four animals. Repeated one-way analysis of variance was performed, followed by Tukey's post-test; differences were considered significant at P values≤0.05.

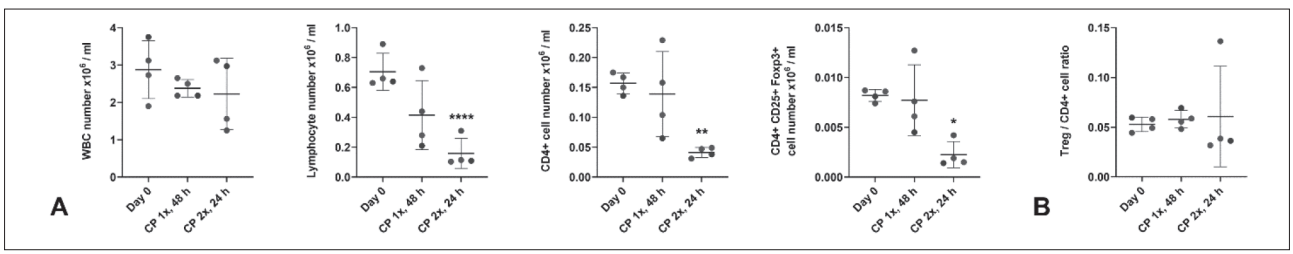

Fig. 2. The impact of cyclophosphamide (CP) treatment on the absolute numbers of white blood cell (WBC) populations at different time points regarding CP application (untreated, treated once and twice with CP). **A** – Numbers of WBC, lymphocytes, CD4+ lymphocytes, and CD4+ CD25+ Foxp3+ regulatory T cells per mL of peripheral blood. **B** – CD4+ to Treg cell ratio in peripheral blood. Summarized data are shown as mean numbers $\times 10^6$±SD for four animals. Repeated one-way analysis of variance was performed, followed by Tukey's post-test; differences were considered significant at P≤0.05. *, P<0.05; **, P<0.01; ****, P<0.0001 vs untreated mice (day 0).

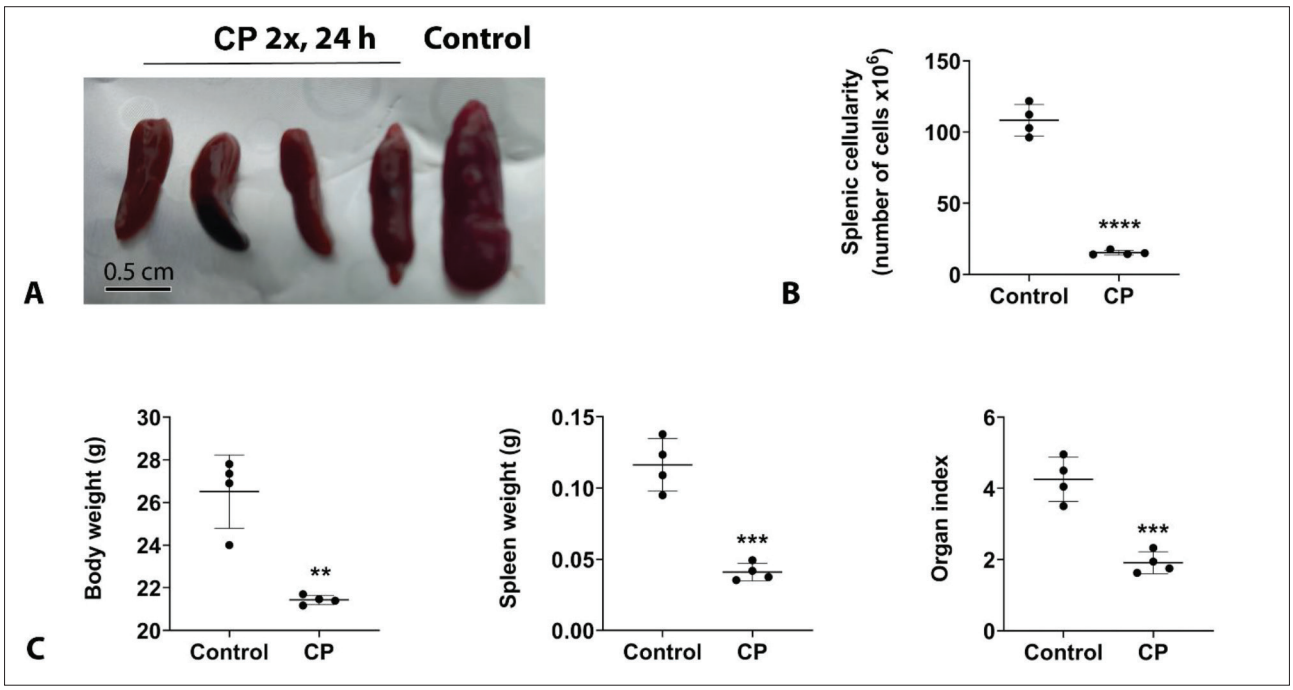

Fig. 3. Changes in the spleen of mice after treatment with cyclophosphamide (CP). CP or NaCl as for controls, was applied i.p. (100 mg/kg) twice in a 5-day interval. CP-treated mice and NaCl-injected controls were euthanized 2 days after the second treatment, and splenic immune-related parameters were measured. **A** – Spleen size. **B** – Splenic cellularity presented as a number of isolated splenocytes $\times 10^6$. **C** – Body weight, spleen weight and organ index (mg/g) for the spleen. Data are shown as the mean±SD for four animals/group. Student's t-Test was performed; differences were considered significant at P≤0.05. **, P<0.01; ***, P<0.001; ****, P<0.0001.

values. Consistently, the absolute numbers of both CD4+ lymphocytes and Tregs in peripheral blood were significantly decreased (P<0.01 and P<0.05 for CD4+ lymphocytes and Tregs, respectively) only after the second CP treatment compared to control samples. Considering that following the second dose of CP, circulating CD4+ cells and Tregs were depleted to a similar extent (about 3.3-fold and 3.8-fold, for CD4+ lymphocytes and Tregs, respectively), the ratio of Tregs to CD4+ cells in peripheral blood remained unaltered (Fig. 2) A single CP administration did not affect the counts of peripheral blood CD4+ and Treg cells, or the ratio of Tregs to CD4+ cells (Fig. 2). These findings indicated that a single dose of CP is not sufficient to induce the depletion of lymphocytes in blood and that multiple doses of CP might be more efficient.

The impact of cyclophosphamide treatment on the spleen size, organ index, and splenocyte number

Having in mind that the results presented herein revealed a downregulatory effect of two doses of CP on peripheral blood lymphocyte numbers, as well as leading to the downregulation of splenic Treg percentage in different animal model systems [15,19], we intended to correlate this finding to the impact of CP on the spleens of the treated mice. The sizes of the extracted spleens after two doses of CP were markedly decreased compared to the control group (Fig. 3A). The number of cells isolated from the spleen was significantly lower (P<0.0001) in CP-treated animals compared to the controls (Fig. 3B), as were body weights (P<0.01) and spleen weights (P<0.001) (Fig. 3C). The organ index as a ratio between the spleen and the body weight presented in mg/g, was also significantly decreased in the CP-treated group (P<0.001) (Fig 3C).

Cyclophosphamide treatment and CD4+ CD25+ Foxp3+ T regulatory cells in the spleen

Flow cytometric analysis of the percentages of lymphocytes, T cells, CD4+ T cells, and Tregs in the spleen showed that the prevalence of lymphocytes was significantly lower (P<0.0001) in CP-treated mice compared to the control animals (Fig. 4B). However, the frequency

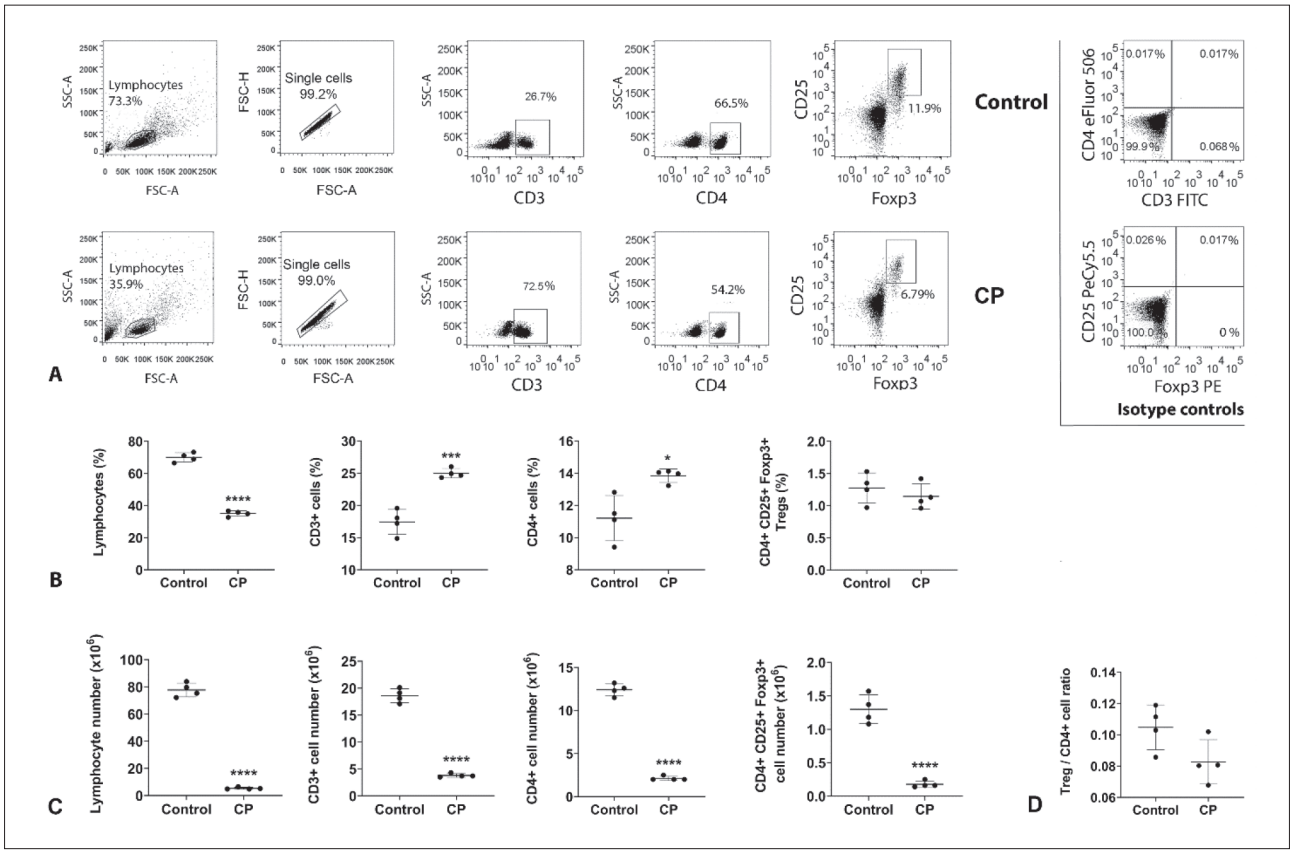

Fig. 4. Changes in the prevalence of CD4+ CD25+ Foxp3+ regulatory T cell (Treg) population in the spleen after cyclophosphamide (CP). **A** – Representative flow cytometry plots from one experiment showing the percentages of lymphocytes, as well as T cells (CD3+) among them, CD4+ cells within T lymphocytes, and CD25+ Foxp3+ Treg cells within CD4+ T lymphocytes in the spleens (lower panel) of CP-treated and control mice (upper panel). The inserted panel indicates isotype-matched controls used to set the gating boundaries; **B, C, D** – Summarized data indicating **(B)** the percentages and **(C)** the absolute numbers of splenic lymphocytes, T cells, CD4+ T cells, and Treg cells, as well as **(D)** the Treg to CD4+ cell ratio in the spleens, shown as the means±SD for four animals/group. Student's t-Test was performed; differences were considered significant at P≤0.05. *, P<0.05; ***, P<0.001; ****, P<0.0001.

of CD3+ cells in the spleen, as well as the percentage of splenic CD4+ T cells, were significantly greater (P<0.001 and P<0.05 for the frequencies of CD3+ and CD4+ T cells, respectively) in CP-treated compared to control animals (Fig. 4B). On the other hand, the percentage of CD4+ CD25+ Foxp3+ Tregs among splenic cells was unchanged in CP-treated animals compared to the control group (Fig. 4B).

As expected, considering the changes in splenic cellularity of treated versus untreated animals, the absolute numbers of lymphocytes were lower (P<0.0001) in the spleens of mice administered with CP than in those of control animals. Accordingly, the counts of T cells, CD4+ T cells, and Tregs in the spleens were significantly decreased (P<0.0001) in CP-treated mice (Fig. 4C). Similarly to peripheral blood, CP administration diminished the counts of splenic CD4+ T cells and Tregs to a comparable degree (about 5.8-fold for CD4+ T cells, and 7.3-fold for Tregs). According to Pearson's correlation analysis, there was a positive correlation between the decrease of Treg counts in the blood 24 h after the second CP application and within the spleen (the coefficient is 0.904). The ratio of Tregs to CD4+ T cells in the spleen was not significantly different between CP-treated and saline-injected control mice (Fig. 4D).

DISCUSSION

CP is widely used to induce the modulation of the immune system, and there are published data showing the effect of CP on different immune cells, which largely depends on the dose, the route, and the timing of CP

administration, as well as the animal strain used as a model system [23-26]. There is no uniform standard on the conditions for immunomodulation mediated by CP in mice, and there are insufficient published data regarding the effect of CP on peripheral blood cells in mice. To achieve the CP-mediated depleting effect on Tregs in different disease model systems based on C57BL/6 mice, we aimed to establish whether CP exerts a specific influence on Tregs by using i.p. administration of one or two intermediate referenced doses of CP (100 mg/kg). Subsequently, after each dose, the percentages of CD4+ lymphocytes and Tregs in the peripheral blood were monitored, as well as the absolute numbers of WBC, lymphocytes, CD4+ lymphocytes, and Tregs. The results obtained by other authors demonstrated the selective depletion of Tregs by CP from the peripheral blood of mice. For example, the administration of 50 mg/kg b.w. of CP selectively abrogates Tregs in the blood (and spleen and lymph nodes) when applied in 4 doses, 3 days apart, in the melanoma model in mice [27]. In addition, the continuous application of 25 mg/kg b.w. CP (every other day) in the lung cancer model restored CD4+ and CD8+ cells and reduced Tregs [28]. This was further confirmed in studies with human circulating CD4+ CD25+ Tregs, indicating that the phenomenon results from the specific response of Tregs, owing to the impaired DNA repair [22].

According to its cytotoxic effects and previous reports [10,29], the administration of two CP doses diminished the number of circulating lymphocytes. However, the composition of this compartment in terms of the frequencies of CD4+ lymphocytes and Tregs was not significantly altered, although a clear trend toward the reduction of both parameters was observed. These results are generally at odds with those obtained in previous murine studies demonstrating either decreased [10] or increased [30] frequencies of CD4+ cells, as well as decreased percentages of CD4+ CD25+ Tregs [30] in peripheral blood. As noted, the discrepancies might be related to study-to-study variability, from CP doses to the treatment regimens, to the mouse strains used [10,30]. In line with marked lymphopenia induced by two doses of CP, our results showed that the numbers of both CD4+ lymphocytes and Tregs were also markedly decreased. Generally, these findings are supported by a few murine studies [30,31] regardless of differences in experimental approaches. To elucidate whether CP treatment specifically impacts the circulating Treg subset, the ratio of Treg cells to total CD4+ lymphocytes was determined, as suggested by Motoyoshi et al. [14]. The authors postulated that a more pronounced CP-induced decrease in Treg number compared with the decrease in CD4+ T-cell counts leads to the decreased ratio of CD4+ CD25+ Treg cells to CD4+ T cells, indicating selective suppression of Tregs [14]. The finding of an unaltered Treg/CD4+ lymphocyte ratio in the peripheral blood of CP-treated mice stemming from the substantial and comparable loss of both cell subsets (about a 74% decrease) suggests that the dual intermediate dose of CP used in this study does not act selectively on Tregs. Notably, in our experimental model, a single CP dose did not affect any of the examined parameters in the peripheral blood compartment, as opposed to repeated administration. This observation indicates that for the generalized immunosuppression in C57BL/6 mice, one 100 mg/kg dose of CP is insufficient, but two doses are required.

Unlike the lack of data regarding the impact of CP on the peripheral blood lymphocyte compartment in rodents, there is more evidence of the effect of CP on immune cells from spleens and lymph nodes. The spleen is a vital immune organ related to both cellular and humoral immunity. Hence, the spleen index mirrors the state of immune regulation [16]. It was already shown that CP treatment induces both body weight and spleen weight loss in mice [17,32]; the spleen index decreases with damage of the splenic ultrastructure [16,33]. Consistent with this, our results obtained with two CP doses (100 mg/kg) showed that the body weight, the spleen weight, and the organ index decreased after the treatment. Also, the total number of cells obtained from the spleens of CP-treated mice decreased more than 7-fold compared to untreated controls, as consistently reported in studies involving a wide range of CP doses and mice of different strains [14,28,34,35]. As revealed by flow cytometry, the depletion of splenocytes was coupled to the decreased relative numbers of splenic lymphocytes. The increased frequency of total T cells in the spleen was measured following the CP administration, which could, at least partly, be related to the increased percentage of splenic CD4+ T lymphocytes. Indeed, increased relative numbers of splenic CD4+ cells have been reported in response to different modes of CP administration [28,34,35]. This finding suggested that the decrease in the splenic lymphocyte frequency mainly reflected the depletion of other lymphocyte

subsets, e.g., B cells. To corroborate this possibility, CP administration was reported to cause a profound and long-lasting decrease in both the frequency [28,34] and the number [28,30,34,35] of B cells in the spleen. Contrary to the frequencies of total and CD4+ T cells, in our C57BL/6 murine model, two doses of CP (100 mg/kg) failed to alter the percentage of splenic Tregs, similar to the lack of effect on peripheral blood Treg frequency. This is in accordance with the findings of Shurlygina et al. [36] obtained in CBA mice and those of Salem et al. [35] in C57BL/6 animals, who showed that following a single dose of CP (200 mg/kg) the relative numbers of CD4+ CD25+ T cells in the spleen remain unchanged compared to intact controls. In contrast to these observations, there are data showing that a single low or high dose of CP in different murine strains induces a reduction in the percentage of CD4+ CD25+ Tregs in the spleen [14,28,30,34]. This discrepancy may be due to the different dosages of CP or the timing of the kill after the treatment. In support of the latter option, it has been shown that a single i.p. CP dose (200 mg/kg) in C57BL/6 mice either does not affect [35] or decrease [30] the frequency of splenic CD4+ CD25+ cells depending on whether the splenocytes were assayed for Tregs 72 or 96 h after the treatment, respectively. Many reports, however, support the application of low-dose CP for selective Treg depletion. For example, *in vitro* application of low-dose mafosfamide (CP derivative that does not need to be metabolized) preferentially reduced Treg counts and preserved other T lymphocytes [22]. The promotion of type 1 diabetes in the relatively resistant Balb/c strain of mice is possible when a low dose of 50 mg/kg bw is applied, which depletes Tregs in these mice [37]. Again, low and continuous CP administration was capable of restoring immune function via the increase of CD4+/CD8+ T cells and Treg depletion in the blood, spleen, and lung tumor [28]. With this in mind, it is possible to achieve a selective CP effect on Tregs in C57BL/6 mice by using a repetitive small dose of CP.

Despite the alterations in the proportions of splenic lymphocyte subsets, the absolute numbers of splenic lymphocytes, total T cells, CD4+ T cells, and Tregs were significantly reduced due to severe depletion of splenocyte counts. These results agree with prior reports, which demonstrated the lowering in the numbers of all major lymphocyte subsets, including CD4+ and CD8+ splenocytes [14,30,34,36], activated CD25+ [36], and CD4+ CD25+ Treg cells [14,28,30,34,35,36], as well as B lymphocytes [30] under the influence of CP treatment. Considering the focus of this study, the splenic CD4+ CD25+ Foxp3+ to CD4+ T-cell ratio was also assessed to infer whether CP treatment affects splenic Tregs more profoundly than the general CD4+ T-cell population. As both cell subsets were comparably diminished in CP-administered mice (by 80 to 90%), the splenic Treg/CD4+ T-cell ratio was not significantly affected, indicating that the CP treatment described herein lacks the specific Treg-targeting effect in the spleen [14], as shown in peripheral blood.

Overall, it seems that the two intermediate doses of CP, similar to a single high CP dose, exhibit an outright immunosuppressive action (depleting all the lymphocyte subsets to a high degree) rather than an immunomodulatory effect (acting to suppress the Treg compartment while sparing conventional T cells) characteristic of low CP dosage. Furthermore, the obtained data strongly suggest that the CP-induced effects on the peripheral blood Treg subset reflect the alterations in the splenic CD4+ CD25+ Foxp3+ Treg population. Specifically, the relative and absolute numbers of Tregs in peripheral blood mirror the situation in the spleen judging by the unaltered frequencies but diminished numbers of Treg cells in both compartments of CP-treated compared to control mice.

CONCLUSIONS

Considering the lack of a uniform standard for Treg depletion with CP in animal experimental models, this study aimed to establish if the chosen approach specifically affects Treg populations in the peripheral blood and spleen of C57BL/6 mice. Our data demonstrated that CP does not specifically deplete Treg, that CP affects both CD3+ T and B cell proportions in the spleen, and that peripheral blood can be used to count Tregs as their numbers correlate with splenic Tregs.

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Conflict of interest disclosure: The authors declare no conflict of interest.

Data availability: Data underlying the reported findings have been provided as a raw dataset which is available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Radulovic%20et%20al_Dataset[1].xlsx

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