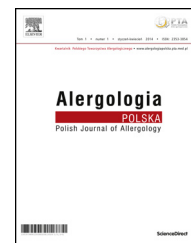


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Effects of combinatorial *in vitro* stimulation with glucocorticoids and vitamin D3 on the expression of Foxp3 in CD4+ T cells of healthy individuals

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ABSTRACT

Glucocorticoid (GC) resistance (GR) poses a serious clinical problem affecting a proportion of asthmatic patients. Thus the efforts aimed at exploring therapeutic methods allowing for overcoming GR are still warranted. To date, vitamin D3 was found to exert certain effects indicating that it can potentially supplement the effects of GC therapy in asthmatic patients. Here we wished to investigate whether treatment with GC and/or vitamin D3 can modulate the expression of one of crucial immunomodulatory molecules accounting for suppressive actions of natural regulatory T cells (Treg cells), namely forkhead box P3 (FoxP3). We demonstrated here that 24 h *in vitro* stimulation with either GC or vit. D3 (alone or in combination) did not significantly affect neither frequencies of CD4+FoxP3+ T cells or the levels of expression of FoxP3 within CD4+ T cells derived from healthy individuals. Therefore we hypothesize that anti-inflammatory actions of both GC and vit. D3 do not seem to be directly related to changes in the expression of FoxP3 in CD4+ T cells.

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Introduction

Despite numerous attempts, glucocorticoid (GC) therapy remains a mainstay therapy in management of asthma. Decreased sensitivity to GC therapy is observed in approximately 15% of asthmatic patients which warrants further efforts to investigate methods/drugs enhancing effects of GC. During last years, vitamin D3 (vit. D3) appeared as an

attractive candidate improving the effects of GC [1]. Experiments conducted over past few years suggested importance of vitamin D3 in maintaining equally balanced immune status through reducing inflammatory processes and supporting the function of regulatory T cells exhibiting IL-10+ and Foxp3+ phenotype [2]. Numerous studies focused recently on the immunomodulatory properties of vit. D3 in reference to regulation of immune responses, both in healthy states and pathological conditions – including

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asthma [3]. The most recent data suggest possible implication of vitamin D3 as supplement in treatment of severe steroid-resistant asthma supporting commonly used glucocorticosteroids via dose reduction and intensification of their activity [4-6]. Importantly, in our previous study we showed that vitamin D3 effectively reduced fraction of TNF- α -producing monocytes, and, in combination with methylprednisolone significantly depleted CD16-positive monocytes [7]. Regulatory T cells are thought to play crucial role in controlling adequate response of immune cells in the course of inflammation, and their participation in pathogenesis of asthma was confirmed as compared to healthy individuals asthmatic patients exhibited reduced number and impaired function of the T cells with immunosuppressive potential [8, 9]. Here, we investigated the effects of combined *in vitro* vitamin D3 and methylprednisolone treatment on the levels of expression of FoxP3 in CD4+ T cells.

Materials and methods

Study subjects

Study group consisted of 13 healthy volunteers aged 21-32 years (10 females and 3 males; no history of infection; non-smoking; not receiving any dietary supplements, including vitamin D) enrolled following obtaining written consent for participation in the study from each individual. Peripheral blood was collected from the participants by venipuncture into tubes containing EDTA anticoagulant (in the morning, fasting; during the period from October to March). The study was approved by local ethics committee.

Cell culture

Density gradient centrifugation technique using Ficoll (Histopaque-1077, Sigma-Aldrich) was used to isolate peripheral blood mononuclear cells (PBMC) from collected blood. The obtained cells were subsequently washed in phosphate-buffered saline (PBS, Biomed Lublin) and resuspended in RPMI-1640 media (Sigma-Aldrich), enriched with 10% fetal

bovine serum (FBS, Gibco) and 1% antibiotics (Penicillin-Streptomycin, Sigma-Aldrich). PBMC fraction was subjected to culture for 24 h (5.5% CO₂, 37 °C) in presence of 1 μ M methylprednisolone (MP) and/or 100 nM vitamin D3 (VIT.D3) (Calcitriol, Cayman Chemical).

Flow cytometry

Incubation of the cells was followed by immunostaining with the use of fluorochrome-conjugated monoclonal antibodies directed at extracellular CD4 (FITC, clone RPA-T4) and intracellular Foxp3 (PE, clone 259D/C7) (BD Bioscience). Acquisition of the flow cytometric data was performed on FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) and analyzed with FlowJo software (Three Star Inc.). Regulatory T cells were distinguished on the basis of forward and side scatter properties (FSC/SSC; size and internal structure respectively), and high expression of CD4 marker and Foxp3 transcription factor (Fig. 1A). Analyses included frequencies of CD4+Foxp3+ cells, total percentage CD4+ cells percentage and mean fluorescence intensity (MFI) of CD4 marker within T cells. Unstained control and isotype control were used to establish proper gating strategy for CD4+Foxp3+ T cells delineation (Fig. 1B).

Statistical analysis

Statistical analysis was performed with the use of GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA) and parametric (paired t-test) or non-parametric (Wilcoxon matched pairs test) test was used depending on the data distribution.

Results

We did not find any significant changes in frequency of CD4+Foxp3+ T cells following stimulation with 1 μ M MP and/or 100 nM VIT.D3. However, despite lack of statistical difference, we observed that combination of 1 μ M MP with 100 nM VIT.D3 led to decrease in percentage of CD4+Foxp3+

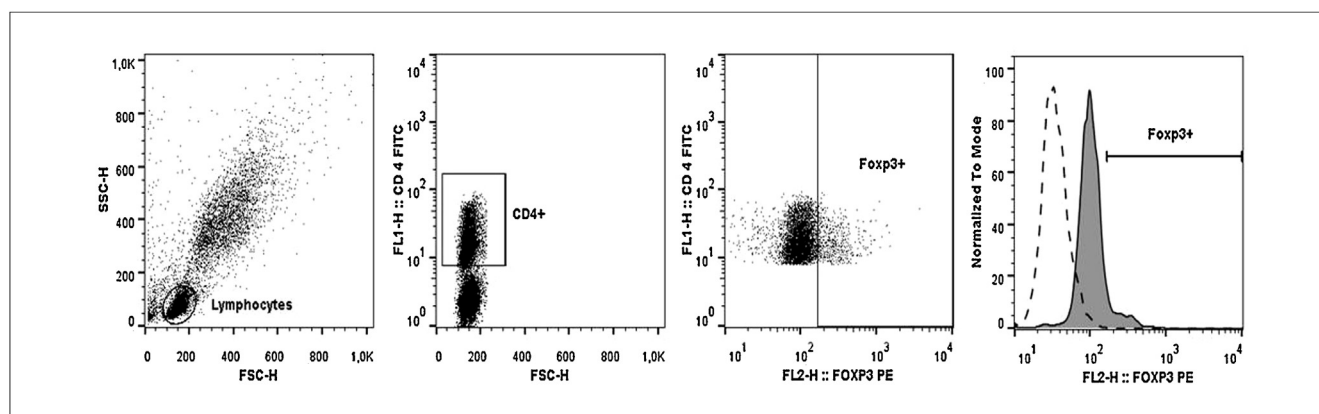


Fig. 1 – Presentation of gating strategy used to distinguish CD4+Foxp3 T cells on the basis of forward and side scatter properties (FSC/SSC) and expression of CD4 and Foxp3 (B). Proper gating was established using unstained and isotype control presented as dashed line on the histogram (A)

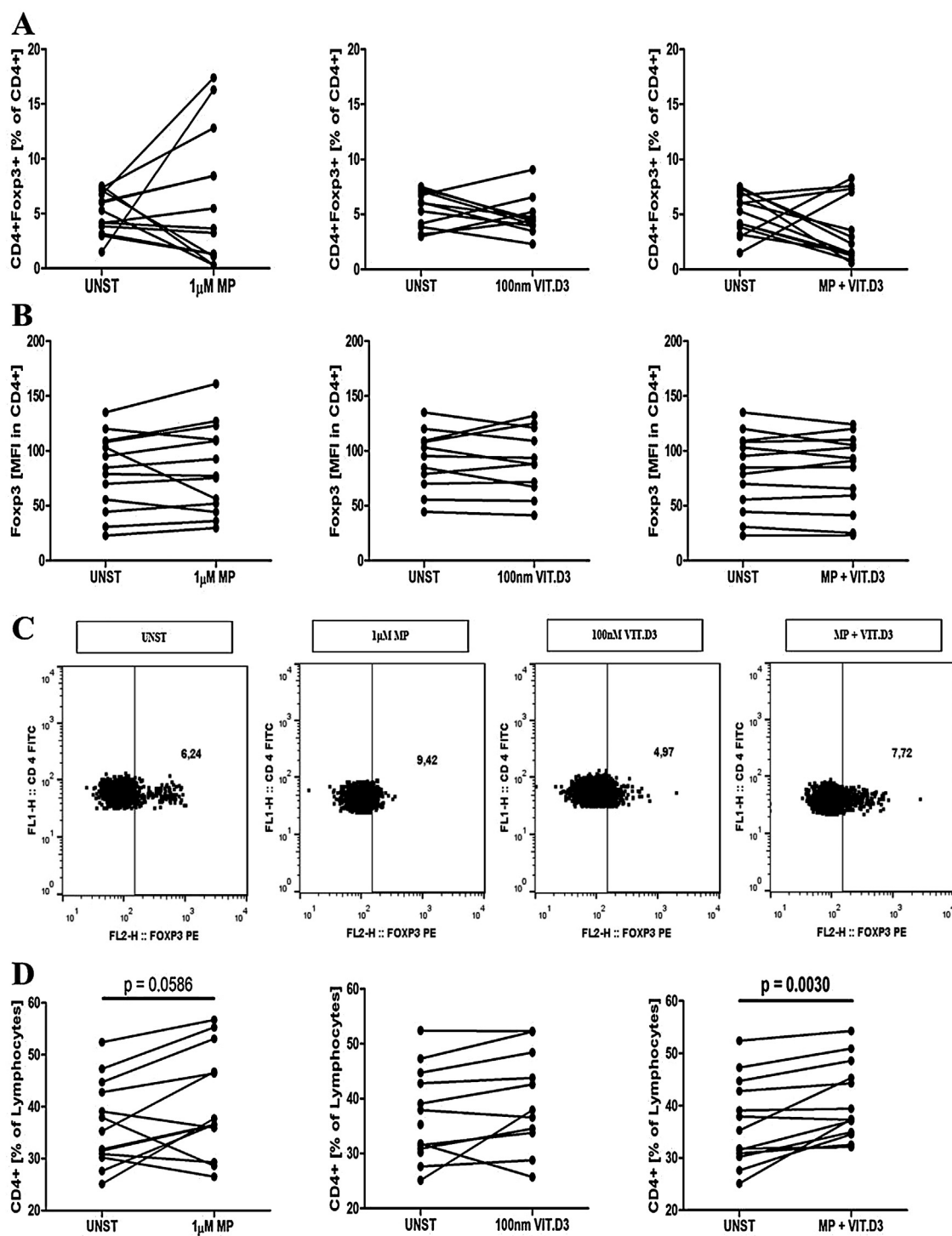


Fig. 2 – Results of 24-h culture of PBMC from healthy individuals stimulated with 1 μ M and/or 100 nM of VIT.D3 on the frequency of CD4+Foxp3+ cells (A), CD4 MFI within T cells (B) and percentage of CD4+ cells (D). Representative dot plots of the obtained data regarding CD4+Foxp3+ frequency are presented under each type of stimulation (C). UNST – unstimulated. MFI – mean fluorescence intensity

Table I – Frequency of CD4+Foxp3+ cells within CD4+ T cells after 1 μ M methylprednisolone (MP) and/or 100 nM vitamin D3 (VIT.D3), and in unstimulated (UNST) PBMC

Parameter	CD4+Foxp3+ [% of CD4+]			
	UNST	1 μ M MP	100 nM VIT.D3	MP + VIT.D3
Mean (standard deviation)	5.35 (SD = 1.85)	6.43 (SD = 5.90)	5.72 (SD = 3.66)	4.58 (SD = 5.71)
p-Value	No statistically significant differences			

Table II – Mean fluorescence intensity of Foxp3 in CD4+ T cells after 1 μ M methylprednisolone (MP) and/or 100 nM vitamin D3 (VIT.D3), and in unstimulated (UNST) PBMC

Parameter	Foxp3 [MFI in CD4+]			
	UNST	1 μ M MP	100 nM VIT.D3	MP + VIT.D3
Mean (standard deviation)	81.33 (SD = 34.97)	84.08 (SD = 40.23)	90.05 (SD = 29.73)	80.41 (SD = 34.55)
p-Value	No statistically significant differences			

Table III – Frequency of CD4+ cells within lymphocytes after 1 μ M methylprednisolone (MP) and/or 100 nM vitamin D3 (VIT.D3), and in unstimulated (UNST) PBMC

Parameter	CD4+ [% of lymphocytes]			
	UNST	1 μ M MP	100 nM VIT.D3	MP + VIT.D3
Mean (standard deviation)	36.66 (SD = 8.23)	40.44 (SD = 10.22)	39.69 (SD = 8.98)	40.66 (SD = 7.28)
p-Value		$p = 0.0586^a$		$p = 0.0030^a$

^a Statistically significant as compared to unstimulated samples (UNST).

cells in 77% of individuals (64% and 54% in reference to stimulation with 100 nM VIT.D3 and 1 μ M MP alone respectively) (Fig. 2A and C, Table I). Furthermore, analysis of MFI of Foxp3 in CD4+ T cells did not show significant changes after stimulation with 1 μ M MP and/or 100 nM VIT.D3 (Fig. 2B and C, Table II).

Simultaneously, we did not observe major changes in the frequencies of CD4+ T cells except for slight increase following stimulation with 1 μ M MP alone (by 40.44%, $p = 0.0586$) and in combination with 100 nM VIT.D3 (by 40.66%, $p = 0.0030$) as compared to unstimulated PBMC (Fig. 2D, Table III).

Discussion

In this paper, in some contrast to previous reports, we did not observe any increase in CD4+Foxp3+ frequencies following either methylprednisolone or vitamin D3 *in vitro* treatment. Quite surprisingly, combinatorial treatment with GC and vitamin D3 resulted even in a decrease in percentage of Foxp3+ T cells observed in majority of individuals. These data are in some concert with our previous study, wherein 7-day oral GC treatment did not affect numbers of CD4+ T cells with putative regulatory phenotype – CD4+CD25+CD127– [10]. On the contrary, early experiments conducted in asthmatic patients showed that GC treatment significantly increased expression of Foxp3 mRNA within CD4+ T cells, and these properties of GC were also confirmed in *in vitro* cultures of freshly isolated CD4+ cells [11]. Differences between our data and those acquired by other groups might be associated with differences in experiment settings.

In the current study we analyzed samples from healthy, but not asthmatic individuals; we included all PBMC, but not sorted CD4+ T cells, and we evaluated FoxP3 at protein, but not mRNA level. In reference to vitamin D3 action the results obtained by another group indicated that active form of vitamin D predominantly affected CD4+Foxp3– T cells, therefore, we probably could not observe any significant response of regulatory CD4+Foxp3+ T cells following vitamin D3 treatment [12]. In study of Sbiera *et al.*, aside from lack of glucocorticoid-mediated induction of Foxp3+ T cells, the increase in frequency of total CD4+ cells was also observed following GC stimulation. In fact, these results support the data described in our study as we demonstrated that simultaneous use of methylprednisolone and vitamin D3 resulted in increase in the percentage of CD4+ T cells [13].

Recently we showed that methylprednisolone, similarly to the effects on FoxP3 observed in this paper, not only failed to restore the expression of IL-10 receptor (CD210) and frequency of CD4+CD210+ T cells, but on the contrary, led to decrease of these two parameters [14, 15]. We also showed that GC treatment depleted numbers of pro-inflammatory monocytes and reduced production of such pro-inflammatory mediators as TNF- α [7]. The combination of all of our data on the effects GC actions allowed us to propose a hypothesis that GC might regulate the balance between pro- and anti-inflammatory CD4+ T cell-related responses through reduction of IL-10 binding to IL-10R rather than through the changes of numbers of immunosuppressive regulatory CD4+ T cells.

In summary, the results of our study indicate that anti-inflammatory mechanisms of both GC and vitamin D3 actions does not seem to strictly depend on the changes

within fraction of Foxp3+ regulatory CD4+ T cells. However, the results of our study, due to its *in vitro* character would have to be verified by experiments performed *in vivo* in asthmatic patients. Altogether, future studies aimed at investigating possible roles of vitamin D3 and GC as regulators of immune responses in asthma-related inflammation are still warranted.

Authors' contributions/Wkład autorów

MM – study design, manuscript preparation, statistical analysis, data interpretation, literature search, funding collection. KG – study design, manuscript preparation, statistical analysis, data collection and interpretation, literature search. DL – statistical analysis, data collection.

Conflict of interest/Konflikt interesu

None declared.

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Ethics/Etyka

The work described in this article have been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform Requirements for manuscripts submitted to Biomedical journals.

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