

## IMMUNOMODULATORY EFFECTS OF TGF- $\beta$ IN THE PATIENTS WITH IMMUNE THROMBOCYTOPENIA

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The novel concept of immune thrombocytopenia (ITP) pathogenesis is focused on CD4<sup>+</sup> T cells, currently considered indispensable in stimulating B cells to produce anti-platelet antibodies. In this *in vitro* study, we have investigated CD4<sup>+</sup> T cell profiles of ITP patients and immunomodulatory effects of TGF- $\beta$  treatment. CD4<sup>+</sup> T cells were isolated from peripheral blood mononuclear cells of healthy controls and ITP patients. After brief incubation, the levels of T helper signature cytokines and the number of T regulatory cells (Tregs) were determined. TGF- $\beta$  immunomodulatory properties were analyzed by the alterations in cytokine production of IFN $\gamma$ , IL-4, IL-10, IL-17, and IL-2 and Treg frequency, after six-day treatment. ITP patients had decreased level of IL-4 and IL-10, increased IL-17 level and augmented both IFN $\gamma$ /IL-4 and IL-17/IL-10 ratio. Interestingly, in the six-day CD4<sup>+</sup> T cell cultures without TGF- $\beta$ , no statistically significant differences in cytokine levels between the control and ITP group were documented, except for IL-10 which was significantly lower. In ITP group treated with TGF- $\beta$ , a significant increase of IL-10 as well as a decrease in IL-17/IL-10 ratio was detected, compared to TGF- $\beta$  untreated ITP group. There was also an increase in the Treg frequency. The results of our study suggest that ITP patients have Th1 and Th17 biased cell-mediated immune response that can be corrected by enhanced TGF- $\beta$  signaling. However, it appears that long-term culturing of the CD4<sup>+</sup> T cells may not be a suitable experimental model for studying immunomodulating effects in ITP due to dynamic phenotype fluctuations of these cells in *ex vivo* conditions.

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### Introduction

Immune thrombocytopenia (ITP) is an auto-immune disease characterized by the reduced platelet number ( $< 100 \times 10^9/L$ ) due to their enhanced degradation and compromised production, which is often clinically manifested by the hemorrhagic diathesis (1). The majority of ITP patients have various anti-platelet antibodies, mainly directed against membrane bound glycoprotein complexes – GPIIb-IIIa and GPIb-IX (2), thereby the breakdown of B

cell self-tolerance was considered the central pathological event in the disease pathogenesis. Regarding the nature of auto-antigens and auto-antibodies in ITP, recently, it has become clear that B cells, in order to mount anti-platelet immune response, require the assistance of auto-reactive, platelet specific CD4<sup>+</sup> T cells (3-5). Depending on the conditions of the microenvironment, naïve CD4<sup>+</sup> T cells during initial antigen priming could adopt different polarization states, including Th1, Th2, Th17, Th22, Th9, T regulatory cells, and follicular T helper cells, each with specific cytokine profile and functional characteristics.

Th1 cells are defined as IFN $\gamma$  and IL-2 producing subset of CD4<sup>+</sup> T cells that express T-bet as a master regulator, while their differentiation programme is dependant on IL-12 signaling. Th2 cells, on the other hand, predominantly produce IL-4, IL-5, and IL-13, express GATA-3 as a master transcription factor and require IL-4 for complete differentiation. The equilibrium between Th1 and Th2 cells is perturbed in numerous immune mediated diseases and ITP is not an exception. Accordingly, ITP was classified as Th1 mediated disorder, based on the higher plasma IFN $\gamma$ /IL-4 ratio, higher blood Th1/Th2 cell ratio and the ratio between the gene expression levels of lineage specific master trans-

cription factors - T-bet and GATA-3, in peripheral blood mononuclear cells (PBMCs) (6-10). Additionally, many of these parameters were found to be negatively correlated with platelet counts, suggesting pathological relevance of Th1/Th2 immune deviation in the ITP pathogenesis (9, 10).

Th17 cells represent a unique subset of CD4<sup>+</sup> effector T cells which in addition to IL-17, produce IL-21 and IL-22. Their differentiation pattern involves the activation of transcription factor RORγT induced by the combination of proinflammatory cytokines IL-6, IL-1, IL-23, and TGF-β. ITP patients have elevated plasma level of IL-17 and other Th17 associated cytokines - IL-1, IL-6, and IL-23 (11, 12), but also a higher level of RORγT expression in PBMCs and frequency of Th17 cells in the blood (12-14). In addition to the aberrant Th17 immune response, both numerical and functional abnormalities of T regulatory cells (Tregs) have been documented in ITP. Tregs are CD4<sup>+</sup> T cell subset expressing the transcription factor FoxP3, which can be induced by a combination of cytokines TGF-β and IL-2. In contrast to other CD4<sup>+</sup> T cell subsets, Tregs upon antigen recognition induce active immunosuppression by producing anti-inflammatory cytokines TGF-β and IL-10 or by direct cell to cell contact via inhibitory molecules (negative co-stimulators) CTLA-4 and PDL-1. Although decreased in number (15, 16), Tregs isolated from ITP patients also have compromised suppressive functions due to decreased production of TGF-β and IL-10, while serum/plasma levels were found to be decreased and positively correlated with the platelet counts (17-20).

One of the essential characteristics of both Th17 cells and Tregs is their plasticity, i.e. the ability to modify their phenotype and acquire the characteristics and functions of other CD4<sup>+</sup> T cell subsets. Although they perform completely opposite functions, Tregs and Th17 cells share a common differentiation pattern involving TGF-β; however, the definite differentiation fate of activated CD4<sup>+</sup> T cells is determined by additional factors including the TGF-β concentration and different microenvironmental stimuli. Specifically, TGF-β alone, at higher concentrations, drives CD4<sup>+</sup> T cell differentiation towards

Tregs, but at lower concentrations and in the presence of IL-6 and IL-23, it favors Th17 cell differentiation (21). In ITP, Th17/Treg balance was found to be increased and in the positive correlation with disease activity (22). In accordance with mutual plasticity of these cell lineages, in the tumor micro-environment, Th17 cells were shown to trans-differentiate into Tregs and TGF-β involvement in this process was suggested (23). Given that the therapy of many autoimmune diseases, including ITP, is today largely based on non-specific immunosuppression, which entails numerous side effects, this finding could be of particular importance in the treatment of Th17-mediated autoimmune diseases as it opens the possibilities for modulating immune response in terms of Th17 cell conversion to Tregs.

### Aim of the study

The objective of the present *in vitro* study was to investigate CD4<sup>+</sup> T cell profiles of ITP patients and immunomodulatory effects of TGF-β treatment.

### Patients and Methods

#### *Clinical and demographic characteristics of the study participants*

The study included 6 subjects, median age 56 (32-69) years, both males and females, subdivided into two groups - a control and ITP group. The control group included 3 healthy volunteers, 2 males and 1 female with normal platelet counts (> 150 × 10<sup>9</sup>/L). The ITP group included 3 patients, 2 males and 1 female with verified ITP diagnosis according to the criteria established by the International Working Group (24). All the subjects were promptly informed about the relevant details concerning their participation in the study and gave written informed consents. This study was approved by the Ethical Committee of the Clinical Center Niš, Serbia under the identification number 16297/5. Detailed demographic and clinical characteristics of the study participants are shown in Table 1.

**Table 1.** Demographic and clinical characteristics of the study participants\*

	<b>ITP (n = 3)</b>	<b>Control (n = 3)</b>
	<b>Mean (range)</b>	<b>Mean (range)</b>
Gender (male/female)	2/1	2/1
Age (years)	67 (61-69)	39 (32-50)
Platelet counts (×10 <sup>9</sup> /L)	67 (20-110)	294(243-340)
Duration of the disease (years)	5 (1-10)	N/A
Phase of the disease	remission	N/A
Therapy	prednisone	N/A
Splenectomy	No	N/A

\* N/A - Non Applicable

### CD4<sup>+</sup> T cell isolation and experimental protocols

Blood samples (10 mL) were collected by cubital venepuncture using EDTA as an anticoagulant. After sampling, the blood was diluted in RPMI-1640 medium (1:1) and PBMCs were isolated using centrifugation over a Ficoll-Hypaque density gradient (*Histopaque-1077*; Sigma-Aldrich, St. Louis, Missouri, USA). Upon PBMC isolation, CD4<sup>+</sup> T cells were separated as a negative fraction by immunomagnetic sorting using EasySep™ Human CD4<sup>+</sup> T Cell Isolation Kit (*Stemcell Technologies, Vancouver, Canada*) according to the manufacturer's instructions. The purity of isolated cells was verified by flow cytometry and more than 99% of isolated cells were CD4<sup>+</sup> (*data not shown*). CD4<sup>+</sup> T cells of the control and ITP group were seeded into 24-well plastic plate -  $1 \times 10^6$  cells per well in 1.5 mL of RPMI-1640 medium supplemented with 2.05 mM L-glutamine, 25 mM HEPES (*Sigma-Aldrich, St. Louis, Missouri, USA*) and 10% inactivated fetal bovine serum - FBS (*Capricorn Scientific GmbH, Ebsdorfergrund, Germany*). In order to evaluate Th profile of the tested subjects, CD4<sup>+</sup> T cells were incubated at 37°C in 5% CO<sub>2</sub> at 95% humidity for 24h, and then stimulated with phorbol myristate acetate - PMA (20 ng/mL) and ionomycin (100 ng/mL) (*Sigma-Aldrich, St. Louis, Missouri, USA*) 8h prior supernatant sampling. The remaining cells were used for Treg detection by flow cytometry. CD4<sup>+</sup> T cells were also incubated with or without human recombinant TGF- $\beta$ 1 (3 ng/mL) (*Merck KGaA, Darmstadt, Germany*) for 6 days in order to evaluate its immunomodulatory properties.

### Sample analyses

In the collected cell supernatants, IFN $\gamma$ , IL-4, IL-10, IL-17, and IL-2 levels were determined using commercial ELISA kits: Human IFN $\gamma$  Quantikine ELISA Kit, Human IL-4 Quantikine ELISA Kit, Human IL-10 Quantikine ELISA Kit, Human IL-17 Quantikine ELISA Kit, and Human IL-2 Quantikine ELISA Kit (*all obtained from R&D Systems, Minneapolis, Minnesota, USA*) according to the manufacturer's instructions. The percentage of Tregs was estimated by flow cytometry. Firstly, cells were washed in stain buffer (*BD Biosciences, Franklin Lakes, New Jersey, USA*), and then permeabilized and fixed using Transcription Factor Phospho Buffer Set (*BD Biosciences, Franklin Lakes, New Jersey, USA*). To identify Tregs, anti-CD4FITC, anti-CD25APC, and anti-FoxP3PE (*all ob-*

*tained from BD Biosciences, Franklin Lakes, New Jersey, USA*) antibodies were applied. After washing, sample analyses were performed on BD LSRFortessa using BD FACSDiva™ software.

### Statistical analyses

All statistical analyses were performed in the GraphPad software (GraphPad Software, LLC, San Diego, CA, USA). The results are presented as the average values  $\pm$  standard deviation (SD). The intergroup differences were established using the Student's t-test, and the p values less than 0.05 were considered statistically significant.

### Results

#### T helper cell profile of ITP patients

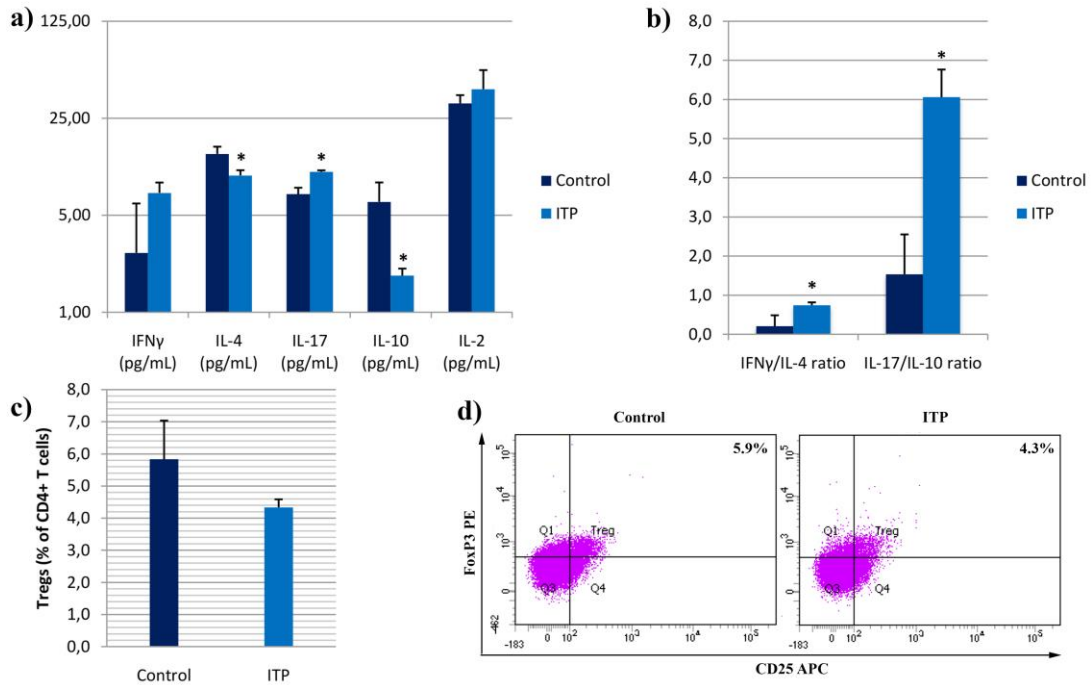
T helper (Th) cell profile of ITP patients was assessed by the level of signature cytokines in the supernatants of CD4<sup>+</sup> T cell cultures. After 24h incubation, a statistically significant decrease in the IL-4 and IL-10 was documented as well as an increase in IL-17 level. Both IFN $\gamma$ /IL-4 and IL-17/IL-10 ratios were increased; however, there was no significant difference in the frequency of Treg (Figure 1).

#### Immunomodulatory effects of TGF- $\beta$

Isolated CD4<sup>+</sup> T cells from the control and ITP group have been cultured for 6 days with or without TGF- $\beta$ . Interestingly, in the cultures without TGF- $\beta$ , no statistically significant differences in cytokine levels between the control and ITP group were documented, except for IL-10 which was significantly lower in the ITP group (Figure 2).

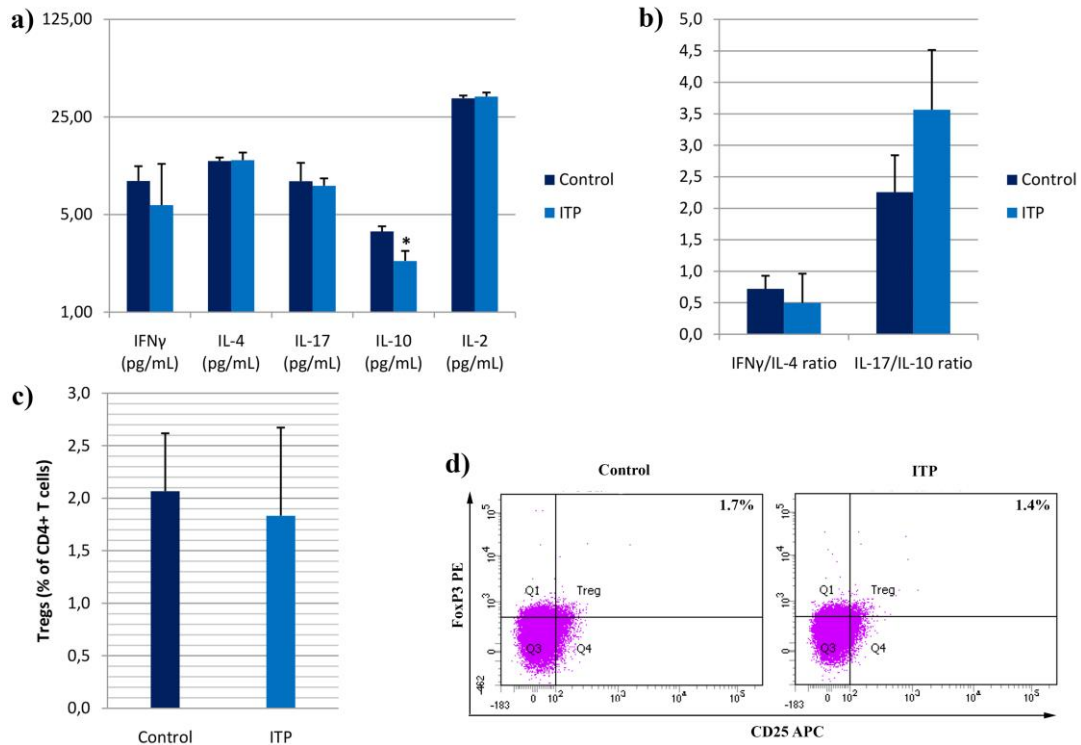
In ITP group treated with TGF- $\beta$ , a significant increase of IL-10 as well as a decrease in IL-17/IL-10 ratio was detected, compared to TGF- $\beta$  untreated ITP group. There was also a marked increase in the frequency of Tregs (Figure 3).

However, after comparing cytokine profile of the ITP group treated with TGF- $\beta$  and untreated control group, a significant decrease in IFN $\gamma$  and IFN $\gamma$ /IL-4 ratio as well as an increase in IL-10 production was found. The frequency of Tregs was significantly higher in TGF- $\beta$  treated ITP group (Figure 4).



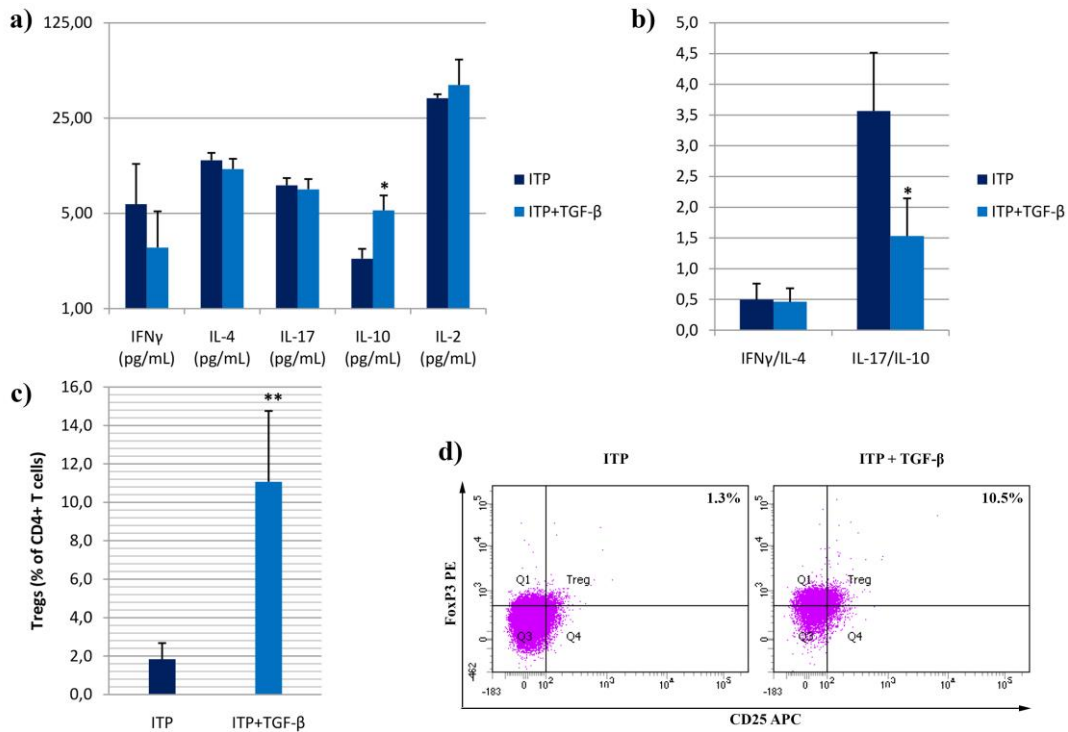
**Figure 1.** CD4<sup>+</sup> T cell profile of ITP patients after 24h incubation†

†CD4<sup>+</sup> T cells isolated from the control and ITP group were cultured for 24h and stimulated with PMA and ionomycin 8h prior supernatant sampling. In collected supernatants: a) the level of Th cell signature cytokines was determined; b) IFN $\gamma$ /IL-4 and IL-17/IL-10 ratio was calculated; c) in the remaining cells, Treg frequency was reported; d) representative flow cytometric analyses of CD4<sup>+</sup> T cells in one experiment. (\*p < 0.05 versus control)



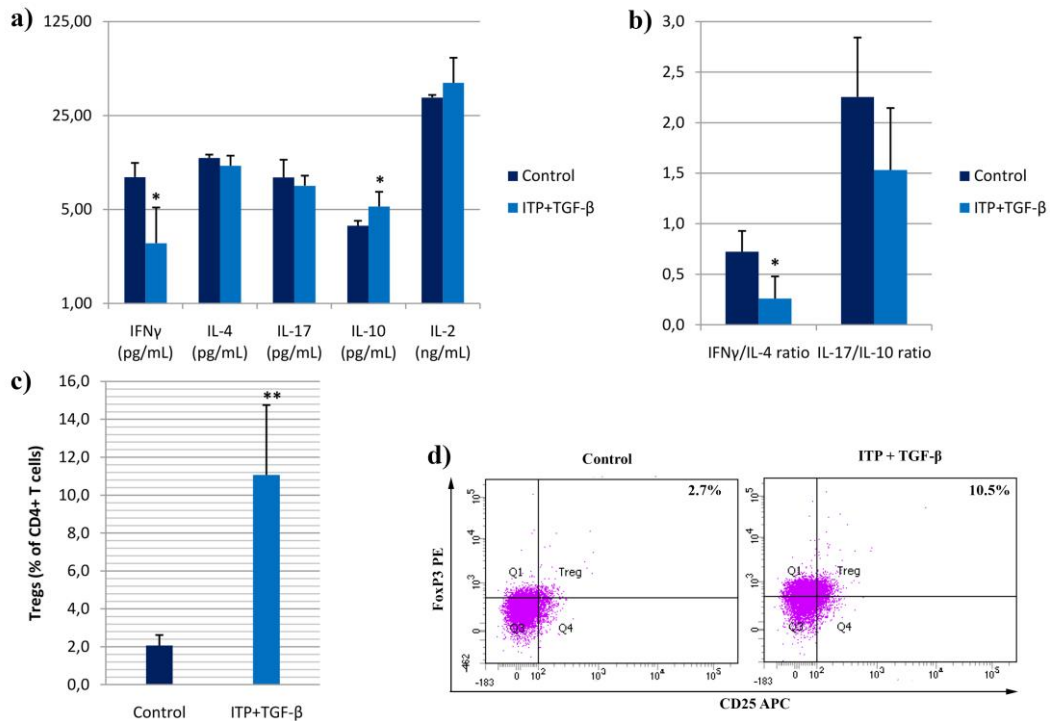
**Figure 2.** CD4<sup>+</sup> T cell profile of ITP patients after 6-day incubation without TGF- $\beta$ ‡

‡CD4<sup>+</sup> T cells isolated from the control and ITP group were cultured without TGF- $\beta$  for 6 days and stimulated with PMA and ionomycin 8h prior supernatant sampling. In collected supernatants: a) the level of Th signature cytokines was determined; b) IFN $\gamma$ /IL-4 and IL-17/IL-10 ratio was calculated; c) in the remaining cells, Treg frequency was reported; d) representative flow cytometric analyses of CD4<sup>+</sup> T cells in one experiment. (\*p < 0.05 versus control)



**Figure 3.** CD4<sup>+</sup> T cell profile of ITP patients after 6-day incubation with TGF-β<sup>◊</sup>

<sup>◊</sup>CD4<sup>+</sup> T cells isolated from the ITP group were cultured for 6 days with or without TGF-β (3 ng/mL) and stimulated with PMA and ionomycin 8h prior supernatant sampling. In collected supernatants: a) the level of Th signature cytokines was determined; b) IFNγ/IL-4 and IL-17/IL-10 ratio was calculated; c) in the remaining cells, Treg frequency was reported; d) representative flow cytometric analyses of CD4<sup>+</sup> T cells in one experiment. (\*p < 0.05; \*\*p < 0.01 versus ITP)



**Figure 4.** Immunomodulatory effects of TGF-β in ITP patients compared with healthy controls<sup>Δ</sup>

<sup>Δ</sup>CD4<sup>+</sup> T cells isolated from the ITP patients treated with TGF-β (3 ng/mL) and untreated CD4<sup>+</sup> T cells obtained from healthy controls were cultured for 6 days and stimulated with PMA and ionomycin 8h prior supernatant sampling. In collected supernatants: a) the level of Th signature cytokines was determined; b) IFNγ/IL-4 and IL-17/IL-10 ratio was calculated; c) in the remaining cells, Treg frequency was reported; d) representative flow cytometric analyses of CD4<sup>+</sup> T cells in one experiment. (\*p < 0.05; \*\*p < 0.01 versus control)

## Discussion

Currently, the abnormalities of  $\text{CD4}^+$  T cell compartment in patients with ITP are well established (25). Accordingly, in our study, we have also observed Th1 and Th17 polarization of cellular immune response, based on higher cytokine ratio of  $\text{IFN}\gamma/\text{IL-4}$  and  $\text{IL-17}/\text{IL-10}$  in the cultures of isolated  $\text{CD4}^+$  T cells, indicating disturbed balance of both Th1/Th2 and Th17/Treg axes. Similar observations, although using different methodological approaches, such as direct cytokine detection in patient's plasma and the identification of  $\text{CD4}^+$  T cell subsets in the blood, were made by other authors (22, 26-28). Interestingly, in these studies, pulsed high-dose dexamethasone treatment was shown to correct reported Th1/Th2 and Th17/Treg immune deviations, further supporting their relevance in the disease pathogenesis (27, 28). Patients enrolled in our study were, however, in the remission induced by long-term prednisone therapy and it appeared that immunological pattern of the diseased remained unaltered. This finding might suggest that beneficial effects of prednisone treatment were achieved by non-selective immune suppression rather than the restoration of Th balance. Different non-specific acting mechanisms of prednisone have been previously proposed, including reduced phagocyte capacity to internalize antibody coated platelets (29), reduced anti-platelet antibody production due to lymphotoxic effect (30), and enhanced platelet production (31). Additionally, immunosuppressive effects of corticosteroids mainly result from the inhibition of proinflammatory transcription factors, particularly nuclear factor kappa B (NF- $\kappa$ B) and activating protein-1 (AP-1), which are active during initial T cell priming, regardless of their future differentiation fate, but also in various other cells of both innate and acquired immunity, directly suggesting their non-selective mechanism of action (32).

One of the unexpected findings of our study was that, in contrast to the short-term (24h) cultures, in six-day cultures previously observed difference in the cytokine profile of  $\text{CD4}^+$  T cells isolated from ITP patients and healthy controls did not persist, except for IL-10 level which was significantly decreased. Plausibly, the explanation of this effect could be associated with extreme plasticity of these cells, which differentiation state could be considered only in the context of microenvironment conditions. *Butch et al.* have also shown that peri-pheral blood T cell subsets do not retain an original pattern of cytokine production during extended culture periods under conventional non-polarizing conditions (33). Thus, the restoration of cytokine balance in ITP group could be a consequence of pro-longed *ex vivo* cell culturing, which do not mirror conditions present *in vivo*. Although, this experimental model has been used for investigating  $\text{CD4}^+$  T cell profile of ITP patients (34-36), our results suggest that *in vivo* studies are required in order to adequately address this issue.

Recently, it has been shown that Th17 cells can trans-differentiate into T regulatory 1 (Tr1) cells – a specific subset of IL-10 producing T cells with suppressive functions, during a physiological immune

response in the presence of TGF- $\beta$ 1 (37). Considering that we, as well as others (38), have observed a disturbed  $\text{IL-17}/\text{IL-10}$  ratio in patients with ITP, TGF- $\beta$  could be a promising immunomodulatory candidate in the restoration of immunological tolerance in ITP. Furthermore, in both adult and pediatric ITP patients, the plasma levels of TGF- $\beta$  were found to be decreased, positively correlated with the platelet counts but negatively with the levels of pro-inflammatory  $\text{IFN}\gamma$  and IL-17 (17, 18, 20). Indeed, in our study, TGF- $\beta$  treatment induced a decrease of  $\text{IL-17}/\text{IL-10}$  ratio, primarily due to enhanced production of IL-10. In order to investigate potential cellular source of enhanced IL-10 production, we have examined the frequency of Tregs which are also well established IL-10 producers, and shown a significant increase following TGF- $\beta$  treatment. When compared with the untreated control group, the TGF- $\beta$  treated ITP group had decreased  $\text{IFN}\gamma/\text{IL-4}$  ratio as well as increased IL-10 production and Treg frequency suggesting that TGF- $\beta$  induces polarization of cellular immune response toward more tolerogenic phenotype. Thus, enhancing TGF- $\beta$  signaling in ITP patients could be a promising therapeutic strategy and some initial efforts in this direction have been made. Specifically, all-trans retinoic acid (ATRA) has been shown to be capable of promoting Treg development and their suppressive capacity, simultaneously inhibiting Th17 differentiation by enhancing TGF- $\beta$  signaling (39-41). Furthermore, ATRA was able to restore macrophagal M1/M2 imbalance documented in ITP towards M2 phenotype, which development and function are also related to the TGF- $\beta$  signaling (42, 43). In the study of *Dai et al.*, ATRA therapy proved to be efficient in ITP treatment, considering significant increase of Treg percentage and IL-10 levels, which is in line with our results observed after TGF- $\beta$  treatment. Unfortunately, in this study TGF- $\beta$  levels were not evaluated (44).

## Conclusion

Our data reconfirm deviant polarization of  $\text{CD4}^+$  T cells in ITP patients and suggest that TGF- $\beta$  could restore disturbed Th1/Th2 and Th17/Treg balance, indicating that enhancing TGF- $\beta$  signaling could be a promising therapeutic strategy. However, the study also raises a concern about prolong  $\text{CD4}^+$  T cell cultures as a model of studying the phenotype characteristics of these cells, considering their spontaneous phenotype fluctuations in *ex vivo* conditions.

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## Declarations of interest

None.

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doi:10.5633/amm.2020.0201**IMUNOMODULACIJSKA SVOJSTVA TGF- $\beta$  KOD BOLESNIKA SA  
IMUNSKOM TROMBOCITOPENIJOM**Miloš Kostić<sup>1</sup>, Tanja Džopalić<sup>1</sup>, Nikola Živković<sup>2</sup>, Ana Cvetanović<sup>3</sup><sup>1</sup>Univerzitet u Nišu, Medicinski fakultet, Katedra za mikrobiologiju i imunologiju, Niš, Srbija<sup>2</sup>Univerzitet u Nišu, Medicinski fakultet, Katedra za patologiju, Niš, Srbija<sup>3</sup>Univerzitet u Nišu, Medicinski fakultet, Katedra za onkologiju, Niš, SrbijaKontakt: Miloš Kostić  
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Novi koncept patogeneze imunske trombocitopenije (ITP) fokusiran je na CD4<sup>+</sup> T ćelije, koje se trenutno smatraju ključnim u stimulisanju B ćelija na produkciju antitrombocitnih antitela. U ovoj *in vitro* studiji istraživali smo profil CD4<sup>+</sup> T ćelija obolelih od ITP i imunomodulacijska svojstva TGF- $\beta$ . CD4<sup>+</sup> T ćelije izolovane su iz mononuklearnih ćelija periferne krvi zdravih ispitanika i ispitanika obolelih od ITP. Nakon kratkotrajne inkubacije, određeni su nivoi osnovnih citokina T pomoćničkih ćelija, kao i broj T regulatornih ćelija (Treg). Imunomodulacijska svojstva TGF- $\beta$  analizirana su praćenjem izmena u produkciji citokina IFN $\gamma$ , IL-4, IL-10, IL-17 i IL-2, kao i učestalosti Treg, nakon šestodnevog tretmana. Oboleli od ITP imali su smanjen nivo IL-4 i IL-10, povećan nivo IL-17, kao i povišen odnos IFN $\gamma$ /IL-4 i IL-17/IL-10. Zanimljivo je to da u šestodnevnim CD4<sup>+</sup> T ćelijskim kulturama, bez TGF- $\beta$  tretmana, nisu evidentirane statistički značajne razlike u nivou citokina između ispitanika kontrolne i ITP grupe, osim nivoa IL-10, koji je bio značajno niži. U ITP grupi ispitanika, nakon TGF- $\beta$  tretmana, registrovano je značajno povećanje IL-10, kao i smanjenje odnosa IL-17/IL-10 u poređenju sa ispitanicima ITP grupe, koji nisu tretirani na ovaj način. Takođe, primećena je povećana učestalost Treg. Rezultati naše studije sugerišu na to da oboleli od ITP pokazuju aberantnu Th1 i Th17 polarizaciju celularnog imunskog odgovora, što se može korigovati stimulisanim TGF- $\beta$  signalizacijom. Međutim, ispostavlja se da dugotrajna kultivacija CD4<sup>+</sup> T ćelija nije pogodan eksperimentalni model za proučavanje imunomodulatornih svojstava u ITP, usled dinamičkih fluktuacija fenotipa ovih ćelija u *ex vivo* uslovima.

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**Ključne reči:** imunska trombocitopenija; TGF- $\beta$ ; imunomodulacija; IL-10, IL-17