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Regular paper

Peripheral regulatory T cells and anti-inflammatory cytokines in children with juvenile idiopathic arthritis

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Background: Juvenile idiopathic arthritis (JIA) is a chronic, heterogenous inflammatory disease of unclear pathogenesis. JIA is hypothesized to be linked to a defective immune regulation. Anti-inflammatory cytokines belong to the best known regulatory factors. T-regulatory cells are a crucial cellular component of immune tolerance. One of their functions is synthesis of interleukin 10 (IL-10) and transforming growth factor beta1 (TGF-β1). The aim of this study was to determine the proportion of T-regulatory cells ($CD4^+CD25^{high}FOXP3^+$) in peripheral blood, and serum levels of TGF-β1 and IL-10 in patients with JIA. Methods: The study included 25 patients with newly diagnosed JIA: oligoarthritis (n=17) and polyarthritis (n=8). The control group was comprised of 17 healthy children. $CD4^+CD25^{high}FOXP3^+$ T cells in peripheral blood were quantified by means of three-color flow cytometry. Serum concentrations of TGF-β1 and IL-10 were estimated with ELISA. Results: The proportion of peripheral $CD4^+CD25^{high}FOXP3^+$ cells in patients with JIA was significantly higher than in the controls (p=0.04). The two groups did not differ significantly in terms of their TGF-β1 and IL-10 concentrations. Conclusions: At the time of diagnosis, children with JIA presented with an elevated proportion of T-regulatory cells ($CD4^+CD25^{high}FOXP3^+$) in peripheral blood. Anti-inflammatory cytokines, IL-10 and TGF-β1, are not upregulated in the serum of patients with JIA, and therefore should not be considered as biomarkers of this condition.

Key words: Tregs, IL-10, TGF-β1, juvenile idiopathic arthritis

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Abbreviations: IJA, juvenile idiopathic arthritis; Tregs, regulatory T cells

INTRODUCTION

Juvenile idiopathic arthritis (IJA) is the most common chronic autoimmune childhood disease. Although exact pathogenesis of this heterogenous connective tissue disorder has yet to be elucidated, it likely involves a combination of cell types in the affected joints. Similar to other autoimmune inflammatory diseases, inappropriate immune response is directed against self-antigens in genetically predisposed individuals (Ravelli & Martini, 2007). Also, some exogenous factors, e.g. viral and bacterial pathogens, have been implicated as triggers inducing the aberrant inflammatory response (Ravelli & Martini, 2007).

Defective immune regulation has been hypothesized as a potential mechanism contributing to excessive response of effector lymphocytes, especially Th1, against self-antigens. Regulatory T cells (Tregs) are a crucial component of immune regulation (Dieckmann *et al.*, 2001). Tregs express CD4 and CD25 surface antigens, as well as intracellular forkhead family transcription factor (Foxp3) which was demonstrated to be crucial for their suppressor function (Jonuleit *et al.*, 2001; Hori *et al.*, 2003). Principal biological function of Tregs is to inhibit activation and proliferation of effector cells (B, T and NK cells) by cell contact-dependent mechanism, as well as *via* anti-inflammatory cytokines, including transforming growth factor beta (TGF-β) and interleukin 10 (IL-10) (Li & Flavell, 2008; Strauss *et al.*, 2007; Yamagiwa *et al.*, 2001).

Since the discovery of Tregs, the role of these cells in the pathogenesis of inflammatory disorders, such as rheumatoid arthritis and JIA, has been studied extensively by many researchers. However, the results of these studies are inconclusive, since the number of these cells in patients with various inflammatory conditions was shown to be either unchanged or elevated/decreased. IL-10 and TGF-β are multifunctional cytokines with anti-inflammatory activity, exerting immunosuppressive effect at different levels of the immune response (Strauss *et al.*, 2007; Yamagiwa *et al.*, 2001; Huang *et al.*, 2005). They are synthesized by an array of cells, primarily by macrophages, but also by regulatory cells (Li &Flavell, 2008; Huang *et al.*, 2005). Both, IL-10 and TGF-β, are very important components of the immunoregulatory network. However, despite extensive research on IL-10 and TGF-β, still little is known about serum concentrations of these regulatory cytokines in childhood inflammatory disorders.

JIA is classified based on ILAR criteria revised in Edmonton (Petty *et al.*, 2004). Most of the available disease activity classification systems are based on clinical symptoms, number of involved joints, CRP and erythrocyte sedimentation rate (ESR) (Consolaro *et al.*, 2009; Nordal *et al.*, 2011).

Aim of the study. The aim of this study was to determine the proportion of T-regulatory cells ($CD4^+CD25^{high}FOXP3^+$) in peripheral blood, and serum levels of TGF-β and IL-10 in patients with juvenile idiopathic arthritis, and to verify if these parameters correlate with the established clinical activity markers for this condition.

Patients and methods. Patients and controls. The study initially included 26 children, 18 girls and 8 boys, aged 1–15 years (median 4 years), with newly diagnosed JIA, prior to treatment implementation.

One patient, who presented with systemic-onset JIA was excluded from the analysis.

The group was divided into two subgroups: those with oligoarthritis (n=17) and with polyarthritis (n=8). The diagnosis of JIA was based on the International League Against Rheumatism (ILAR) criteria.

During the first hospitalization, after obtaining written informed consent, but prior to treatment implementation, 5-ml peripheral venous blood samples were collected from all the patients, to determine the studied parameters as well as ESR, CRP, ANA and RF.

Control group (HC) was comprised of 17 healthy children, 7 girls and 10 boys, aged 2–17 years (median 7 years).

Ethics. The protocol of the study was approved by the Independent Bioethical Committee of Medical University of Gdansk. Informed consent was obtained from legal guardians of all participants included in the study.

Peripheral regulatory T cells, flow cytometry. Immediately after blood sampling, peripheral CD4+ cells were isolated with Human CD4 positive selection kit (StemCell Technologies, Canada), in line with the manufacturer's instructions. Briefly, 2 ml of whole blood were placed in a 5-ml polystyrene tube and stained with CD4-positive selection cocktail. Following a 15-min incubation, magnetic nanoparticles were added and the sample was incubated for another 10 min in a magnet rack. After removal of the unwanted (non-CD4-positive) cells, the tube was removed from the rack and the remaining cells were washed twice with PBS.

Next, 100 000 cells, isolated as described above, were labeled with monoclonal antibodies against CD4 FITC (StemCell Technologies, Canada), CD3 PC7 (Beckman Coulter, USA) and CD25 (BD Biosciences, USA). Finally, anti-FOXP3 antibody (BD Pharmingen, USA) was added after previous permeabilization of the cells with Human FOXP3 Buffer Set (BD Biosciences, USA). The same procedure, with anti-CD3 and anti-CD4 antibodies, was also applied to the negative control. $CD4^+CD25^{high}FOXP3^+$ cells were quantified using the FACS Canto II flow cytometer (BD Biosciences). The results were analyzed with the Diva software.

Serum TGF-β1 and interleukin 10, ELISA. Serum samples from 42 subjects (25 patients with JIA and 17 healthy controls) were tested with commercially available enzyme-linked immunosorbent assays (ELISA) for TGF-β1 (R&D Systems, USA) and IL-10 (R&D Systems, USA), in line with the manufacturer's instructions.

Statistical analysis. The significance of intergroup differences was verified with Mann-Whitney U-test. The relationships between pairs of variables were analyzed on the basis of Spearman's rank correlation coefficients. The results of all tests were considered significant at $p<0.05$. All analyses were performed with Statistica 10 software (Stat Soft. Inc., USA).

RESULTS

Clinical characteristics of JIA patients are presented in Table1.

Peripheral CD4+CD25^{high}FOXP3+ cells

Median proportion of $CD4^+CD25^{high}FOXP3^+$ cells among $CD4^+$ T cells was significantly higher in JIA patients than in the controls ($p=0.04$; Fig. 1): 5.1% (range of 2–9.2%) *vs.* 3.9% (range of 1.7–6.4%). Patients with oligoarthritis (OA) and polyarthritis (PA) did not differ significantly in terms of the $CD4^+CD25^{high}FOXP3^+$ cell percentages ($p=0.63$). No significant correlations were found between the percentage of peripheral Tregs and other characteristics of JIA patients: CRP, ESR, ANA, number of affected joints, duration of the symptoms and age. Also, the percentages of peripheral Tregs in boys and girls did not differ significantly ($p=0.33$).

Serum TGF-β1

No statistically significant differences were found in median serum concentrations of TGF-β1 in JIA patients and controls ($p=0.15$; Fig. 2). Median levels of this cytokine in patients with JIA and healthy children were 23.9

Table 1. Clinical and laboratory characteristics of the juvenile idiopathic arthritis (JIA) group.

Clinical parameters		N(%)
Total number of JIA patients		26 (100)
Sex	boys	8 (31)
	girls	18 (69)
JIA onset	oligoarthritis	17 (65)
	polyarthritis	8 (31)
	systemic onset	1 (3)
Number of affected joints	1–2	8 (31)
	3–4	10 (38)
	5–6	4 (15)
	>6	4 (15)
Laboratory parameters	Median values or N (%)	of positivity
C-reactive protein (CRP)		13.27gm/l
Erythrocyte sedimentation rate (ESR)		29
Antinuclear antibodies (ANA)-positive		10 (38)
Rheumatoid factor (RF)-positive		3 (11)
Anti-citrullinated protein antibodies (ACPA)		4 (15)

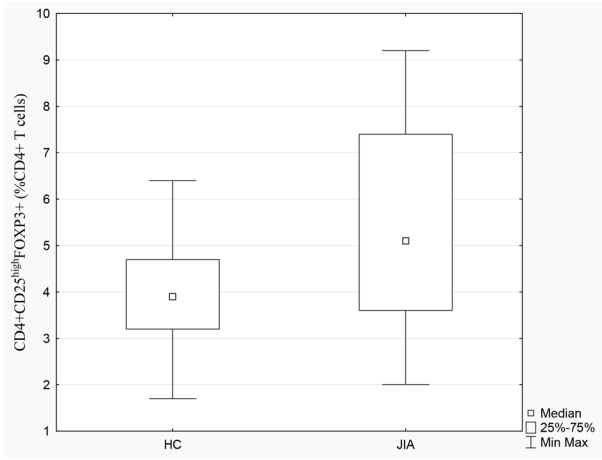

Figure 1. Frequencies of CD4+CD25^{high}FOXP3+ cells in the studied group (JIA) compared with healthy controls (HC).

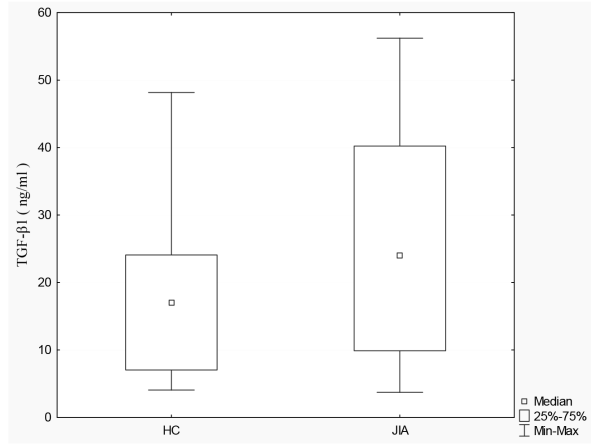

Figure 2. Serum levels of transforming growth factor β1 (TGF-β1) in the studied group (JIA) compared with healthy controls (HC).

ng/ml (range of 3.7–56.2 ng/ml) and 17.0 ng/ml (range of 4.1–48.1 ng/ml), respectively. Moreover, no statistically significant differences in serum levels of TGF-β1 were identified between JIA patients with oligoarthritis and polyarthritis (p=0.09), and between girls and boys (p=0.56). Finally, serum concentration of TGF-β1 did not correlate significantly with CRP, ESR, ANA, number of affected joints, duration of the symptoms and age of JIA patients.

Serum interleukin 10

Patients and controls did not differ significantly in terms of their IL-10 levels (p=0.10; Fig. 3). Median serum concentrations of IL-10 in children with JIA and healthy controls were 6.34 pg/ml (range of 3.22–11.38) and 4.06 pg/ml (range of 1.64–10.34), respectively. No significant differences in serum levels of IL-10 were found between patients with oligoarthritis and polyarthritis (p=0.24). Moreover, the level of IL-10 did not correlate significantly with CRP, ESR, ANA, number of affected joints, duration of the symptoms and age of JIA patients. Also the difference between serum concentrations of IL-10 in boys and girls was not statistically significant (p=0.13).

Neither the serum level of IL-10 nor serum concentration of TGF-β1 correlated significantly with the proportion of T-regulatory cells in peripheral blood.

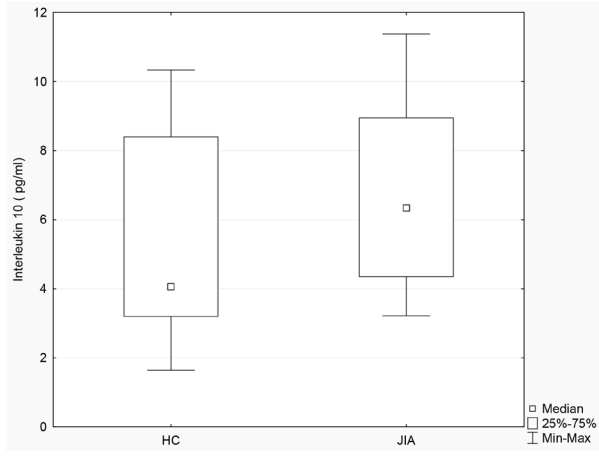

Figure 3. Serum levels of interleukin 10 (Il-10) in the studied group (JIA) compared with healthy controls (HC).

DISCUSSION

Our observation that the proportion of CD4$^+$CD25^{high}FOXP3$^+$ cells in peripheral blood of patients with JIA is higher than in healthy controls is in contrast with the results of studies dealing with the problem in question. However, previous studies addressing this issue were sparse and produced conflicting results. The results of the first study of peripheral regulatory T cells in juvenile idiopathic arthritis were published in 2004; in this study, Tregs were defined as CD4$^+$CD25^{bright} cells, without a reference to intracellular co-expression of FOXP3 (de Kleer *et al.*, 2004). The number of peripheral Tregs in children with oligoarthritis (OA) turned out to be lower than in healthy controls; the study did not include patients with polyarthritis. Importantly, the number of regulatory T cells in patients with remittent OA was higher than in children with extended OA, which implies that these cells may play a role in limitation of the disease (de Kleer *et al.*, 2004). However, it should be noted that the study included previously treated children with a relatively long history of JIA. Decreased number of Tregs, defined as CD4$^+$CD25$^+$FOXP3$^+$ cells, was also documented in another group of 12 patients with JIA, presenting either with oligoarthritis or polyarthritis; however, also participants of this study had longer history of JIA than our subjects (Stelmaszczyk-Emmel *et al.*, 2012) . Our study included treatment-naïve patients with newly diagnosed JIA. Such group better reflected immunological background of JIA and was free from potential confounding effects of treatment in the natural history of the disease. Available evidence suggests that immune regulation may deteriorate with duration of an inflammatory disease (Kugathasan *et al.*, 2007). This hypothesis is inter alia supported by an inverse correlation found between the duration of inflammatory bowel disease and the number of peripheral Tregs (Guidi *et al.*, 2013). Similar phenomenon may also occur during the course of JIA, which would explain the discrepancy between our findings and the results of previous studies. Consequently, the results of studies including patients at different stages of the disease should not be a subject of direct comparison. However, it should be noted that in

one previous study, treatment-naïve patients and healthy controls did not differ significantly in the number of circulating Tregs (Szymańska-Kałuża *et al.*, 2014).

Although the number of peripheral Tregs was relatively more often determined in patients with rheumatoid arthritis (RA), the most common arthritis in adults, the results of these studies are also quite conflicting. While according to some authors, the proportion of circulating Tregs in patients with RA was lower than in healthy controls (Lawson *et al.*, 2006; Jiao *et al.*, 2007; Sempere-Ortells *et al.*, 2009), others reported an increase (van Amelsfort *et al.*, 2004; Han *et al.*, 2008), or did not find significant changes in this cellular population (Liu *et al.*, 2005; Lin *et al.*, 2007; Ji *et al.*, 2013). Although increase in the proportion of Tregs does not constitute a direct argument for the theory of defective immune regulation, it also is not a rationale against this hypothesis.

First, although present in a large amount, Tregs may be functionally defective, as already documented in both, JIA and RA patients (Stelmaszczyk-Emmel *et al.*, 2012; Ehrenstein *et al.*, 2004). Moreover, the expansion of these cells is not supposed to be adequate for inflammatory status. It has been demonstrated, that the number of peripheral Tregs in patients with RA was lower than in subjects with reactive arthritis, a condition with good prognosis, expected to exhibit undisturbed immunoregulatory function (Lawson *et al.*, 2006).

Consistently with other investigators, we did not find statistically significant differences in the proportion of Tregs in patients with oligoarthritis and polyarthritis; however, this might be also a consequence of a relatively small size of our sample. Our study included only 8 patients with polyarthritis and 17 children with oligoarthritis, which reflects actual incidence of these two subtypes of JIA. The only patient presenting with systemic onset of the disease was not included in the analysis.

Aside from the proportion of Tregs, we also analyzed serum concentrations of two principal anti-inflammatory cytokines, constituting another vital component of immunosuppressive network, IL-10 and TGF-β1. Children with JIA did not differ from healthy controls in terms of their serum levels of these cytokines. Serum concentration of IL-10 in patients with JIA was a subject of only one published study, which also did not document significant differences between the patients and controls (Prahalad *et al.*, 2008). To the best of our knowledge, serum TGF-β1 has not been studied in JIA patients thus far. In rheumatoid arthritis one publication reported elevated serum levels of IL-10, but this observation was not further confirmed by other researchers (Cush *et al.*, 1995). In the only study analyzing serum level of TGF-β1 in patients with RA, this parameter did not differ significantly from that found in healthy individuals (Muñoz-Valle *et al.*, 2012). Our hereby presented findings imply that JIA is not associated with a deficit in anti-inflammatory cytokines. The lack of increase in serum concentrations of IL-10 and TGF-β1 may imply that anti-inflammatory response in JIA is not adequate to the inflammatory status. However, it should be emphasized that serum levels of cytokines do not necessarily reflect their local concentrations at the site of ongoing inflammation. Indeed, many previous studies demonstrated presence of TGF-β1 and IL-10 in synovium, and immunoregulatory function of these cytokines was documented in some experiments.

Nevertheless, the hereby presented findings imply that serum concentrations of IL-10 and TGF-β1 should not be considered as biomarkers of JIA.

We have not investigated the immunoregulatory parameters in the site of inflammation, which might be regarded as a limitation of the study. It has to be noted, that synovial sampling, which is not recommended as a diagnostic procedure in juvenile idiopathic arthritis would be ethically controversial.

In this study, serum levels of IL-10 and TGF-β1 did not correlate with the proportion of regulatory T cells in peripheral blood from JIA patients and healthy controls. Although synthesis of anti-inflammatory cytokines belongs to the activities of Tregs, IL-10 and TGF-β1 are produced by many other cell types, which can easily explain the lack of correlations between these parameters.

The hereby presented results, demonstrating increased proportion of peripheral Tregs and unchanged serum levels of Il-10 and TGF-β1, do not confirm the hypothesis of deficient immune regulation in juvenile idiopathic arthritis, although these results are also not sufficient to negate it.

Further research should focus on the function of regulatory T cells in this condition.

CONCLUSIONS

At the time of the diagnosis, children with JIA presented with elevated proportion of regulatory T cells ($CD4^+CD25^{high}FOXP3^+$) in peripheral blood. Anti-inflammatory cytokines, IL-10 and TGF-β1, are not upregulated in the serum of patients with JIA, and therefore should not be considered as biomarkers of this condition.

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