Journal of Neuroimmunology 337 (2019) 577071

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Th17/Treg cells imbalance and their related cytokines (IL-17, IL-10 and TGF-β) in children with autism spectrum disorder

Mai Moaaz[a,*], Sara Youssry[a], Amr Elfatatry[b], Mohammed Abd El Rahman[c]

[a] Department of Immunology and Allergy, Medical Research Institute, Alexandria University, Alexandria, 21561, Egypt
[b] Department of Neuropsychiatry, Faculty of Medicine, Alexandria University, Alexandria, 21131, Egypt
[c] Department of Clinical Pathology, Alexandria Armed Forces Hospital, Alexandria, 21615, Egypt

ARTICLE INFO

Keywords:
Autism spectrum disorders (ASD)
Th17 lymphocytes
Treg lymphocytes
Cytokines

ABSTRACT

We aimed in this study to investigate a possible involvement of Th17/Treg cells imbalance in autism spectrum disorders (ASD). Using flowcytometry to determine circulating Th17 and Treg cells percentages, RT- PCR and ELISA for cytokine expression, we demonstrated that Th17/Treg balance in ASD children was significantly skewed toward a Th17 response compared to their control. Th17 cells and the ratio of Th17/Treg cells had a significantly positive correlation with disease severity whereas Treg cells had a negative correlation. The imbalance of Th17, Treg cells and their related cytokines may play a vital role in the progression of the disease.

1. Introduction

Autism is a neurodevelopmental disorder characterized by social shortfalls, stereotypic behavior patterns, and deficits in verbal and nonverbal communication that has been folded into the broader classification of autism spectrum disorders (ASD) (American Psychiatric Association, 2015). Prevalence of ASD has increased dramatically (≈150%) since the year 2000, making this disorder a public health concern. The prevalence among children aged 8 years has been estimated to be 16.8/1000 (1/59), with a striking 4:1 male to female ratio (Baio et al., 2018). In Egypt, it has been suspected that 23.8% of toddlers enrolled in Primary Health Care Units were diagnosed as having ASD with a male: female ratio of 3:1 (Mohamed et al., 2016).

Although a number of improvements have been made regarding causes of ASD, its precise etiology remains unclear (Gottfried et al., 2015). Immunological dysfunction has been considered as a recognized feature in ASD for several decades, where alterations in functions of central and peripheral immune system have been observed. These alterations include improper stimulation of immune cells, generation of autoantibodies, cytokine/chemokine imbalance, and increased permeability of the blood-brain barrier (Gładysz et al., 2018).

Several immunological diseases occur at an increased rate among primary family members of individuals with autism. Moreover, prenatal exposure to maternal immune activation (MIA) has been implicated as an environmental risk factor for ASD (Lombardo et al., 2018). Consequently, examination of familial immunity in autism provides an important insight into the disorder.

Reports have been linking ASD to altered serum immunoglobulin levels (Enstrom et al., 2010) and the existence of antibodies reactive to brain proteins (Wills et al., 2009). Improper peripheral T cell activation (Warren et al., 1995) and changes in lymphocyte subsets have been also suggested (Denney et al., 1996).

T helper 17 (Th17) is a T cell subset with pro-inflammatory action that is normally beneficial to host defense through the secretion of its effector cytokine; interleukin-17 (IL-17) (Cai et al., 2016). Th17 cells have been identified as major inducers of autoimmunity and their exaggerated functions can cause tissue inflammation (Kamali et al., 2019).

Alternatively, the preservation of immune homeostasis and the inhibition of immunopathology are mediated by subsets of T cells called regulatory T cells (Treg) (Ward-Hartstonge and Vasanthakumar, 2018). Treg (CD4 + CD25 + foxp3+) cells play an important role in immunological self-tolerance and the prevention of autoimmunity, being closely related to the generation of Th17 cells. Transforming growth factor (TGF)-β1 induces the differentiation of Treg cells, whereas its combination with interleukin-6 (IL-6) or IL-21 results in the differentiation of Th17 cells (Bettelli et al., 2006).

Treg cell differentiation and function are driven by the transcription factor forkhead box P3 (FoxP3) that has been shown to be reduced in individuals who develop inflammatory neurologic diseases. Meanwhile, reduced functionality of Treg cells is associated with upregulation of retinoid-acid receptor-related orphan receptor gamma-t (RORγt) (Yu

* Corresponding author at: Departement of Immunology and allergy, Medical Research Institute, Alexandria University, Alexandria 21561, Egypt.
E-mail address: mai.mouaz@alexu.edu.eg (M. Moaaz).

https://doi.org/10.1016/j.jneuroim.2019.577071
Received 5 August 2019; Received in revised form 19 September 2019; Accepted 20 September 2019

et al., 2015).

Accumulated evidence has demonstrated that Th17 and Treg cells have opposite roles in the immune response. It has been reported that the imbalance of Th17 and Treg cells plays a vital role in inflammatory reaction, graft versus host disease, autoimmune disorders and tumors (Noack and Miossec, 2014; Whiteside, 2014). Despite these reports, the role of this imbalance in autism has not been completely elucidated. Therefore, we aimed in this study to explore the role of Th17/Treg balance in children with autism, which may provide significant insight regarding the association between immune dysfunction and autism.

2. Materials and methods

2.1. Ethics

The study protocols as well as the collection and use of blood samples were previously approved by the Medical Ethical Committee of Alexandria University and bioethical research committee approval was taken. The study confirms with the principles outlined in the Declaration of Helsinki for use of human tissue or subjects. Signed informed consents were obtained from all parents of the participants.

2.2. Subjects

Four populations of subjects were investigated. (1) 44 children with ASD diagnosed according to the DSM-5 criteria (American Psychiatric Association, 2015) in the Neuropsychiatry clinic in Main Alexandria University Teaching Hospital, Faculty of Medicine, Alexandria, Egypt; and from the private specialized clinic. (2) 45 age and sex matched healthy children with no ASD were voluntarily recruited to the study as a control group. (3) and (4) Mothers of ASD and non-ASD children were also enrolled in this study to assess maternal immune response.

The severity of autistic symptomatology was measured by the Childhood Autism Rating Scale (CARS). It consists of 15 categories, each rated on a four-point scale. The child is considered non-autistic when his total score falls in the range of 15–29, mildly-to-moderately autistic when his total score falls in the range of 30–36.5, and severely autistic when his total score falls in the range of (> 36.5–60) (Rellini et al., 2004). The Social Responsiveness Scale (SRS) was also completed (a 65-item rating scale measuring social interaction, language and repetitive/restricted behaviors and interests in the child). The SRS provides a total score and individual scores on five subscales: awareness, cognition, communication, motivation and mannerisms.

Children included in this study had no associated neurological diseases or metabolic disorders (e.g., phenylketonuria) that may influence the results and with no clinical findings suggestive of immunological or other neuropsychiatric disorders.

2.3. Peripheral blood mononuclear cells (PBMCs isolation)

Collected venous blood samples were diluted and over layered gently over Ficoll-Hypaque (1077) (Sigma-Aldrich Chemical Company) (Fuss et al., 2009). After centrifugation 30 min. at 1800 rpm, the interface cells containing PBMCs were carefully aspirated, pelleted and resuspended in 1 ml RPMI (1640) to determine cell count and viability using Trypan blue.

2.4. Cell stimulation

PBMCs were cultured in supplemented media stimulated with phytoheamagglutinin (PHA) (10 µg/ml; Sigma), anti-CD3 and anti-CD28, for 24 h at 37 °C in 5% CO2. Following culture, plates were centrifuged before supernatants were harvested and stored at −80 °C until cytokine analysis and cells were processed for flow cytometry.

Table 1
Primers for RT-PCR.

Gene	Primer
IL-17	Forward: CAACCGATCCACCTCACCTT Reverse: GGCACTTTGCCTCCCAGAT
FOXP3	Forward: TGCCTCCTCTTCTTCCTTGAAC Reverse: TCCTGGAGGAGTGCCTGTAAGT T
TGF- β	Forward: GAACCCAATGCCAACCCTAG Reverse: TTCTTGGTTTTGAGGTCAAGG
GAPDH	Forward: GCCATCAAAGAAGCCCTGAA Reverse: GCGGGTCTGCACACATGTTA

Table 2
Autism severity according to the CARS.

Childhood autism rating scale	No. (%)
Mild to moderate	14 (31.8%)
Severe	30 (68.2%)
Median (Min. – Max.)	43.5 (30.5–56)
Mean ± SD.	43.2 ± 7.7

2.5. Analysis of Th17 and Treg cells

Lymphocytes were gated using forward scatter and side scatter parameter as an indication of cell size and granularity in order to exclude non-cellular debris. We used monoclonal antibodies: anti-human CD3 phycoerythrin (PE), anti-human CD4 fluorescein isothiocyanate (FITC), anti-human CD25 (PE), anti-human foxp3 (FITC) and anti-IL-17 (PE) (BD biosciences) to determine Treg and Th17 cells within CD3 + CD4 + population. Direct fluorescent staining was done on surface molecules CD3, CD4 and CD25. Following surface staining, cells were fixed and permeabilized using fixation/permeabilization reagent (Becton Dickinson, USA) and then stained with IL-17-PE (Th17) and foxp3-FITC (Treg). Cells were tested on a BD FACS Calibur flow cytometer (FACS Calibur, Becton-dickinson, USA). They were analyzed using Cell Quest software (Becton-Dickinson).

2.6. Quantitative real-time PCR

2.6.1. RNA extraction from PBMCs

Total RNA was isolated from PBMCs from all children under study using RNeasy Mini kit and specific columns commercially available from Qiagen, USA. All extraction steps were carried out at room temperature under strictly aseptic conditions following the manufacturer's instructions. 600 µl of lysis buffer were added to pelleted leukocytes, pipetted into a Qiagen spin column in 2 ml collection tubes and centrifuged for 2 min. at 13000 rpm for proper homogenization of lysates. 600 µl of 70% ethanol were added to the homogenized lysates and were mixed and pipetted into QIAamp spin column and centrifuged. 700 µl RNA washing buffer were applied then 500 µl of pre-elution buffer (RPE) were pipetted into the QIAamp spin column and centrifuged. Pre-elution step was repeated. Each QIAamp spin column was transferred into a 1.5 ml microcentrifuge tube where 30–50 µl of RNase-free water were pipetted directly onto the QIAamp membrane, then, centrifuged for 1 min at 10,000 rpm to elute RNA, this step was repeated once.

2.6.2. Reverse transcription

The purity and quantity of RNA were assessed using a NanoDrop™ ND-1000 Spectrophotometer. High-capacity RNA-cDNA kit (Applied Biosystems, Carlsbad, CA; catalogue number 4387406) was used to make cDNA from RNA, following the manufacturer's instructions. Reverse transcription reaction was performed, using, 25 µl reaction mixture containing 2.5 µl dNTPs, 1 µl (10 pmol) primer, 2.5 µl RNA (2 mg/ml), and 0.5 unit reverse transcriptase enzyme. PCR amplification was performed in a thermal cycler programmed at 42 °C for 1 h.,

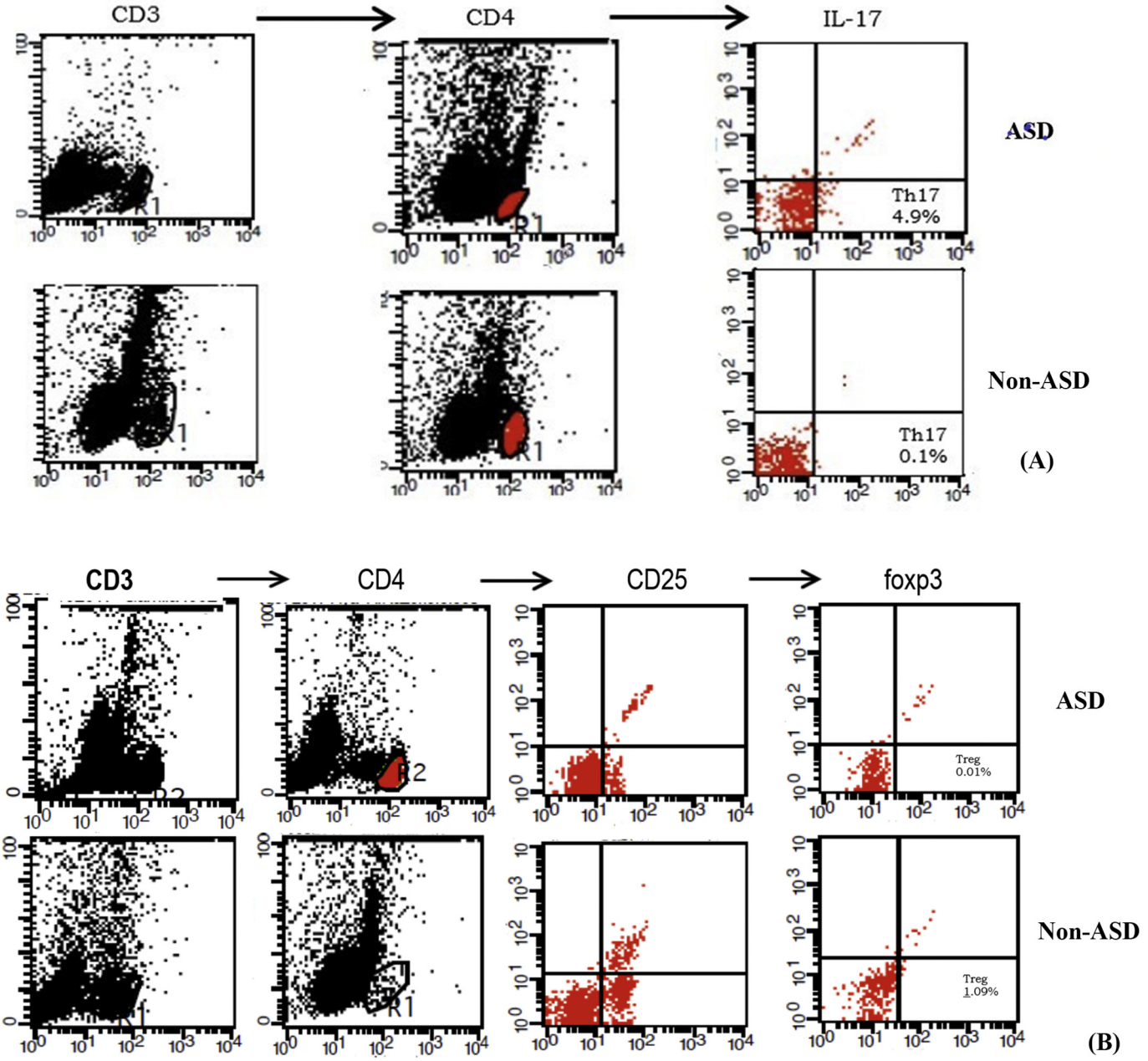


Fig. 1. The percentages of CD4 + IL-17 + (Th17) and CD4 + CD25 + foxp3 + (Treg) cells in ASD children and controls: (A) Peripheral blood mononuclear cells from children with ASD and healthy control subjects were stained with anti-CD3antibodies, anti-CD4antibodies followed by intracellular anti-IL-17 antibodies. The upper gate on the dot plots represents CD3 +, CD4 + T cells and CD4 + IL-17 + T cells in ASD children. The lower gate represents CD4 + IL-17 + T cells in non-ASD controls. (B) CD25 and intracellular foxp3 expression was gated on CD3 + CD4 + T cell population. Representative dot plots are shown from a child with ASD (upper gate) and a non-ASD control child (lower gate).

72 °C for 10 min (enzyme killing).

2.6.3. *Foxp3, IL-17 and TGF-β amplification by real time PCR*

Gene expression levels were measured by quantitative real-time PCR using Taqman gene expression assays (Applied Biosystems). The gene expression assays used were IL-17, TGF-β and Foxp3; GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used for house-keeping reactions. PCR amplification reactions were performed in a total volume of 25 µl containing; 12.5 µl Taqman Universal PCR Master Mix (1 ×), 1.5 µl of 100 nM each from forward and reverse (Table 1) Foxp3 or IL-17 or TGF-β and GAPDH, 1.5 µl of 100 nM Taqman for probes and GAPDH gene and 5 µl cDNA template, and the reaction mixtures were completed to 25 µl by RNase-free water. The preparations were then mixed by gentle pipetting up and down and centrifuged briefly to spin down the contents and to eliminate any air bubbles. The RT-PCR reaction volume (25 µl) was then transferred to wells of MicroAmp® Optical 96-well reaction plate and covered with an optical adhesive cover. The RT-PCR run was performed on an ABI Prism 7000 fast sequence with the TaqMan fluorogenic detection system.

PCR amplifications were performed using the universal temperature cycles: 2 min at 50 °C (for optimal AmpErase UNG enzyme activity), then 10 min at 94 °C (to activate AmpliTaq Gold DNA Polymerase), followed by 40 cycles (15 s. at 94 °C and 1 min at 60 °C). Finally, all Foxp3, IL-17, TGF-β and housekeeping gene mRNA expression were quantified using SDS software.

Ct values were used for all manipulations and were first normalized

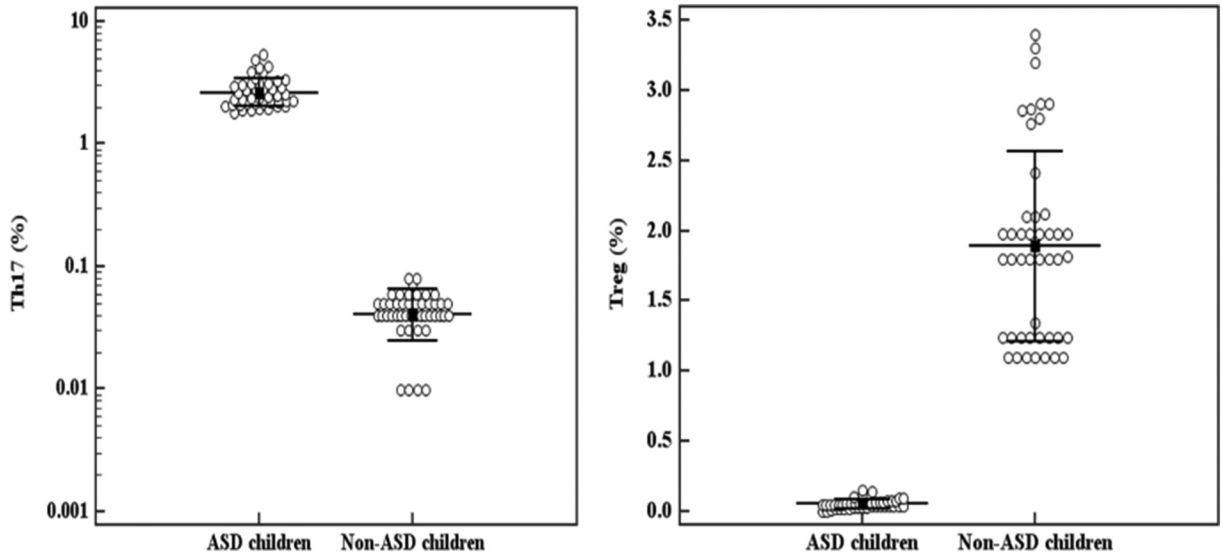


Fig. 2. Comparison between Th17 and Treg in ASD children and non-ASD children: Data represented means ± SDs and were summarized as dot plot. Each dot represents the expression percentage of either Th17 or Treg for one individual. The level of significance was set at *p ≤ 0.05.

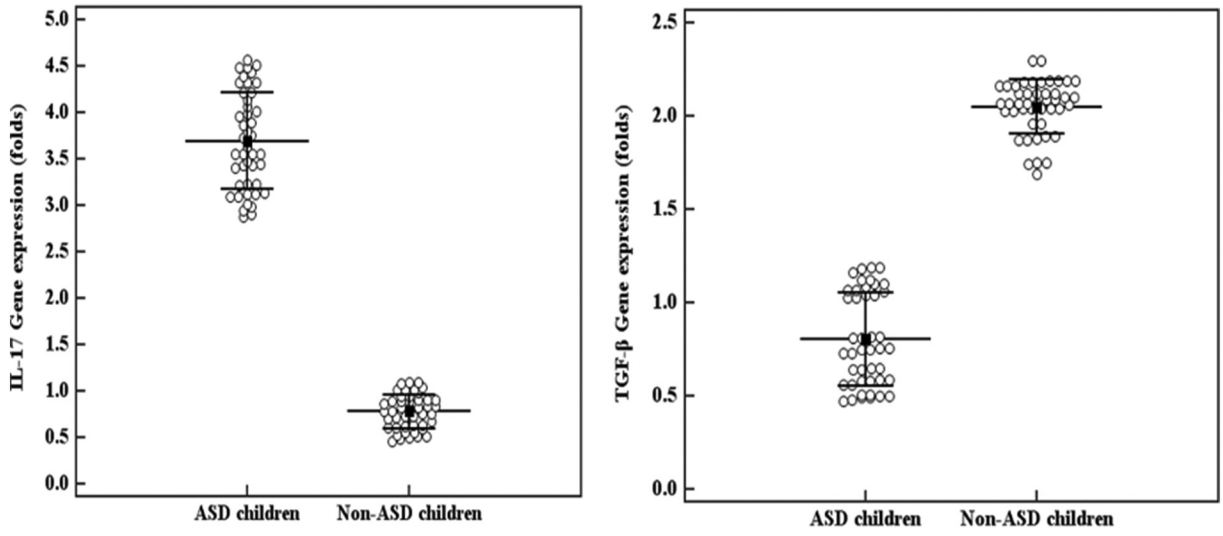


Fig. 3. Comparison between gene expression of IL-17 and TGF-β in ASD children and non-ASD children: Data represented means ± SDs and were summarized as dot plot. Each dot represents the mRNA expression of either IL-17 or TGF-β for one individual. The level of significance was set at *p ≤ 0.05.

to endogenous control levels by calculating the ΔCt for each sample. Values were then calculated relative to control to generate a ΔΔCt value. Fold change was calculated using the formula Fold Change (RQ) = $2^{-\Delta\Delta Ct}$.

2.7. C-reactive protein (CRP) assay

Venous blood samples from mothers under study were collected in plain vacutainers and left to clot for 4–5 h at room temperature, then separated by centrifugation at 1800 rpm for 10 min. Sera were collected and stored in aliquots at −80 °C until used. CRP measurements were carried out using a latex immunoassay.

2.8. Detection of IL-17, IL-10 and TGF-β cytokine levels

Both secreted and serum cytokines concentrations were detected strictly in accordance with the instructions of the commercially available ELISA kits (IL-17 ELISA kit was purchased from Abcam, while both IL-10 and TGF-β ELISA kits were purchased from Invitirogen, Thermo Fisher).

2.9. Calculations

All data were presented according to data distribution either as mean ± standard deviations (SD) (standard deviation of mean) or median, and were compared with the tabulated probability value (P value) as the 0.05 level using SPSS statistical package (SPSS Inc., Chicago, IL). P value was considered significant if it is 0.05 or less. The following statistical tests were used: Student *t*-test (for normally distributed data) and a Mann-Whitney *U* test (for non-normally distributed data). A Pearson chi-square test was used for categorical variables. Correlations between two quantitative variables were assessed using Pearson coefficient and Spearman's rho.

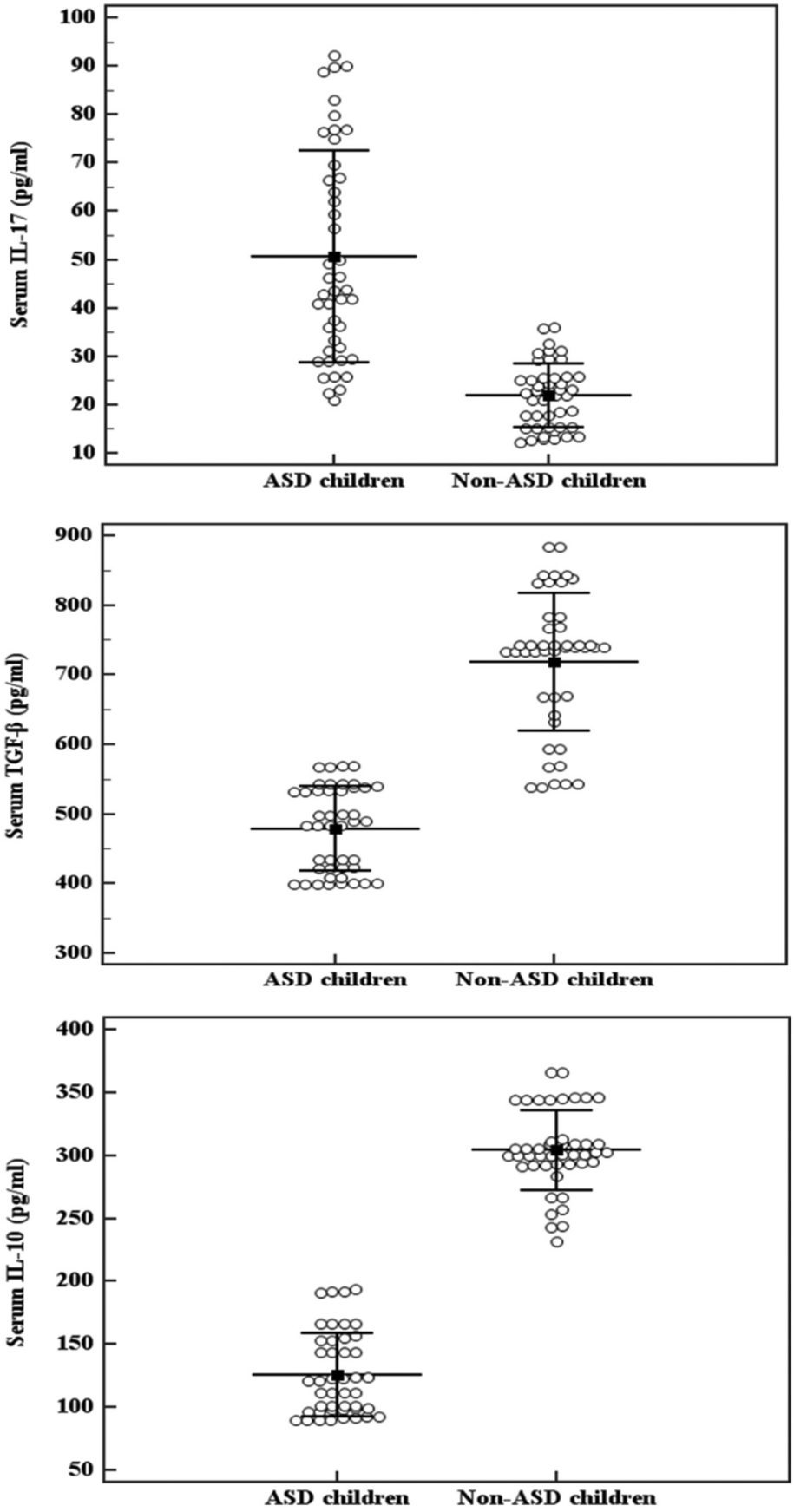


Fig. 4. Comparison between ASD children and non-ASD children according to cytokine production: A) IL-17 secreted concentration, B) TGF-β secreted concentration, C) IL-10 secreted concentration. Data represented means ± SDs and were summarized as dot plot. Each dot represents the secreted concentration of IL-17, TGF-β or Treg for one individual. The level of significance was set at *$p \leq 0.05$.

Table 3
Relation between studied immunological markers and different parameters in ASD children.

	Th17 (%)	Treg (%)	IL-17 Gene expression (folds)	TGF-β Gene expression (folds)	IL-17 conc. (pg/ml)	TGF-β conc. (pg/ml)	IL-10 conc. (pg/ml)	Foxp3 gene expression
Childhood autism rating scale (CARS)								
Mild to moderate (n = 14)	2 ± 0.1	0.1 ± 0	3.1 ± 0.1	1.1 ± 0.1	28.8 ± 4.8	542.9 ± 22.2	163.2 ± 23.5	0.8 ± 0.1
Severe (n = 30)	3.1 ± 0.8	0 ± 0	4 ± 0.4	0.7 ± 0.2	60.8 ± 18.9	449.4 ± 47.5	108.1 ± 18.9	0.6 ± 0.1
P	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
Sex								
Male (n = 35)	2.7 ± 0.9	0.1 ± 0	3.7 ± 0.5	0.8 ± 0.3	50.6 ± 22.1	478.5 ± 62.3	126.2 ± 34.7	0.7 ± 0.1
Female (n = 9)	2.7 ± 0.6	0 ± 0	3.7 ± 0.5	0.8 ± 0.2	50.6 ± 21.9	481.7 ± 54.3	123.4 ± 26.2	0.7 ± 0.1
P	0.647	0.954	0.888	0.864	0.864	0.889	0.841	0.528
Age (years)								
≤6 (n = 20)	3 ± 1.1	0.1 ± 0	3.8 ± 0.6	0.8 ± 0.3	58 ± 26.1	471.8 ± 67	125 ± 37.5	0.6 ± 0.1
> 6 (n = 24)	2.5 ± 0.4	0.1 ± 0	3.6 ± 0.5	0.8 ± 0.2	44.5 ± 15.4	485.2 ± 54.5	126.2 ± 29.3	0.7 ± 0.1
P	0.502	0.408	0.158	0.487	0.097	0.468	0.494	0.276

Data were presented as mean and standard deviation.
* Statistically significant at p ≤ 0.05.

3. Results

3.1. Subjects characteristics

Baseline characteristics of the participants showed that ASD children were predominantly males (no. =35, 79.5%) with a mean age of (7.2 ± 2.2) years, being age and sex matched with their controls (7.1 ± 2.1 years) (p = 0.875 and 0.839 for age and sex respectively). Body mass index in ASD children (mean ± SD = 19.5 ± 3) was not significantly different from non-ASD children (20 ± 3.4) (p = 0.438). In addition, total leukocyte count was significantly higher in ASD children $(9.5 \pm 2.4) \times 10^9$/L. compared to their control $(7.8 \pm 2.6) \times 10^9$/L., (p = 0.001). However, lymphocyte percentages between ASD children and controls were not significantly different (p = 0.068).

3.2. Autism severity according to CARS

ASD children were diagnosed as severe autism (n = 30, 68.2%) using CARS with a mean score of (43.2 ± 7.7) relative to 14 ASD child (31.8%) who were categorized as mild to moderate autism (Table 2).

3.3. Altered Th17/Treg ratio in ASD children

Next, to examine the involvement of Th17 and Treg in ASD, we assessed their circulating percentages. Our results showed that ASD children had significantly increased Th17 expression (1.8–5.3%) with (mean ± SD = 2.7 ± 0.8%) and reduced Treg percentage (0–0.2%; mean ± SD = 0.1 ± 0.03%) in comparison to non-ASD children (Th17: 0.01–0.1; mean ± SD = 0.1 ± 0.02%) and (Treg: 1.1–3.4; mean ± SD = 1.9 ± 0.7%) (p < 0.001) ((Fig. 1A &B) and (Fig. 2)).

In consistence with these results, Th17/Treg ratio was significantly higher in ASD children (mean ± SD = 71.5 ± 76.9) compared to their control (mean ± SD = 0.03 ± 0.01) (p < 0.001).

3.4. Altered Foxp3 expression in ASD children

We evaluated genetic expression of transcription factor Foxp3 associated with Treg expression. It showed a significant reduction in ASD children (0.4–0.9, mean ± SD = 0.7 ± 0.1 folds) compared to non-ASD children (0.7–1.3, mean ± SD = 1 ± 0.2 folds).

3.5. Gene expression of IL-17 and TGF-β

To further explore the role of Th17 and Treg in the development of autism, we assessed their cytokines on the genetic level. We used real time PCR to detect gene expression of IL-17 and TGF-β in PBMCs. We found that IL-17 mRNA expression in ASD patients was almost four fold higher than those in control. ASD = [(2.9–4.6 folds) with a mean ± SD of 3.7 ± 0.5]. Non-ASD children had reduced IL-17 with only (0.5 -1.1) folds and mean ± SD = 0.8 ± 0.2 (p < 0.001). On the other hand, only a slight amount of TGF-β was detected in autistic children with a statistically significant difference (p < 0.001) (Fig. 3).

3.6. Concentration of secreted IL-17, TGF- β and IL-10

We further assessed IL-17, TGF-β and IL-10 cytokines concentrations after being harvested from culture supernatants as shown in (Fig. 4). Our results showed that ASD children had an elevated level of IL-17 concentration (median = 43.8 (21–92.2) pg/ml) than controls (22.4 (12.2–36.1) pg/ml).

Results revealed also that TGF-β concentrations were significantly lower in ASD patients (mean ± SD = 479.1 ± 60.1 pg/ml in ASD children) than controls (718.6 ± 98.9). Simultaneously, IL-10 concentrations were significantly lower (median = 116.3 (89.7–194.1) pg/ml) in ASD children than controls (300.5 (232–366)) (p < 0.001). Taken together, these findings indicate a systemic Th17 increase and Treg deficit in ASD children.

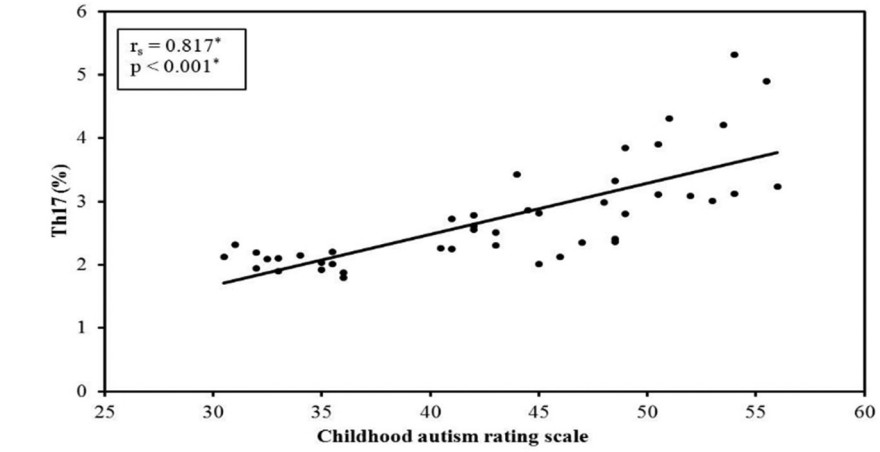
3.7. Association of immune alteration with disease severity in ASD children

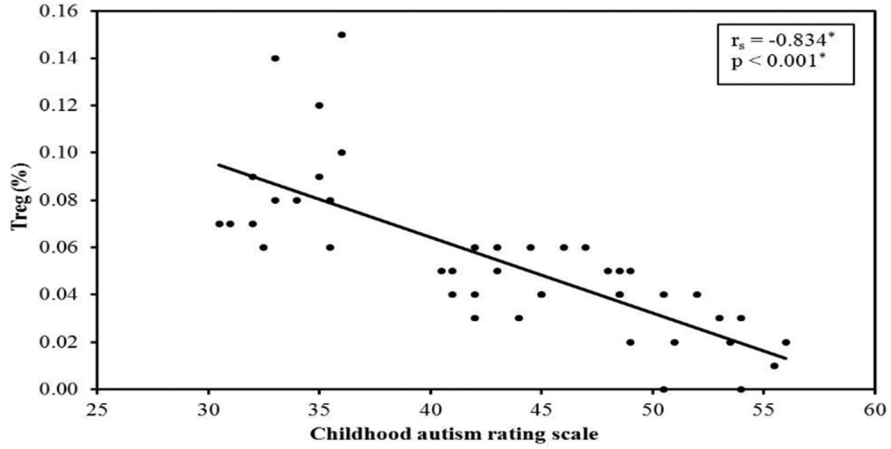
Due to the heterogeneity of ASD severity and clinical variation, we next investigated whether the observed immune alteration is a common feature of different ASD severity subtypes. To explore this relationship, we analyzed the association between studied immunological markers and CARS (Table 3). Children with severe ASD displayed significantly greater Th17 percentage, IL-17 expression and concentration, together with lower Treg percentage, Foxp3 expression and associated cytokines as TGF-β and IL-10 concentrations in comparison to those with mild to moderate ASD. Furthermore, CARS showed a positive correlation with Th17 percentage (rs = 0.817, p < 0.001) and Th17/Treg ratio (rs = 0.836, p < 0.001) and a negative correlation with Treg percentage (rs = −0.834, p < 0.001) (Fig. 5).

Our results revealed no significant association between studied immunological markers and either ASD child sex or age as shown in (Table 3).

3.8. Analysis of association between different immune markers in ASD children

Regarding the association between different immunological markers, our results showed that Th17 was negatively correlated with Treg





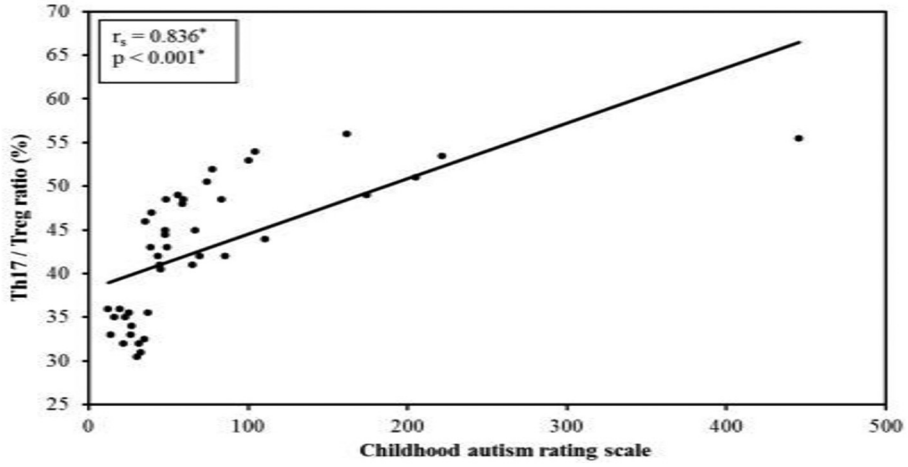


Fig. 5. Correlation between Childhood autism rating scale with Th17/Treg in ASD children (n = 44): Correlation analysis between the proportion of the two CD4+ T cell subsets and their ratio Th17/ Treg (%) with childhood autism rating scale in ASD children. a) Correlation analysis between the proportion of Th17 cells and childhood autism rating scale (rs = 0.817, $p < 0.001$); b) Correlation analysis between the proportion of Treg cells and childhood autism rating scale (rs = −0.834, $p < 0.001$). c) Correlation analysis between the ratio of Th17/Treg cells in peripheral blood of patients and childhood autism rating scale. (rs = 0.836, $p < 0.001$). Correlation was conducted by spearman coefficient. The level of significance was set at $^*p \leq 0.05$.

percentage (rs = −0.890, $p < 0.001$) fig. (6A), being further confirmed by the negative correlation between IL-17 and Treg associated cytokines as TGF-β expression and concentration (rs = −0.870, $p < 0.001$) (rs = −0.811, $p < 0.001$) and IL-10 concentration (rs = −0.811, $p < 0.001$) (Fig. 6B, C, D).

3.9. Analysis of inflammatory state and immune response in mothers of ASD children

We next investigated whether there are significant changes in C-reactive protein (CRP), IL-17 and IL-10 in mothers of ASD children in order to provide some insight on the maternal inflammatory state and immune response. Our results showed that CRP, an established inflammatory biomarker, was significantly increased in mothers of ASD children (mean ± SD = 6.3 ± 8.6) compared to mothers of non-ASD

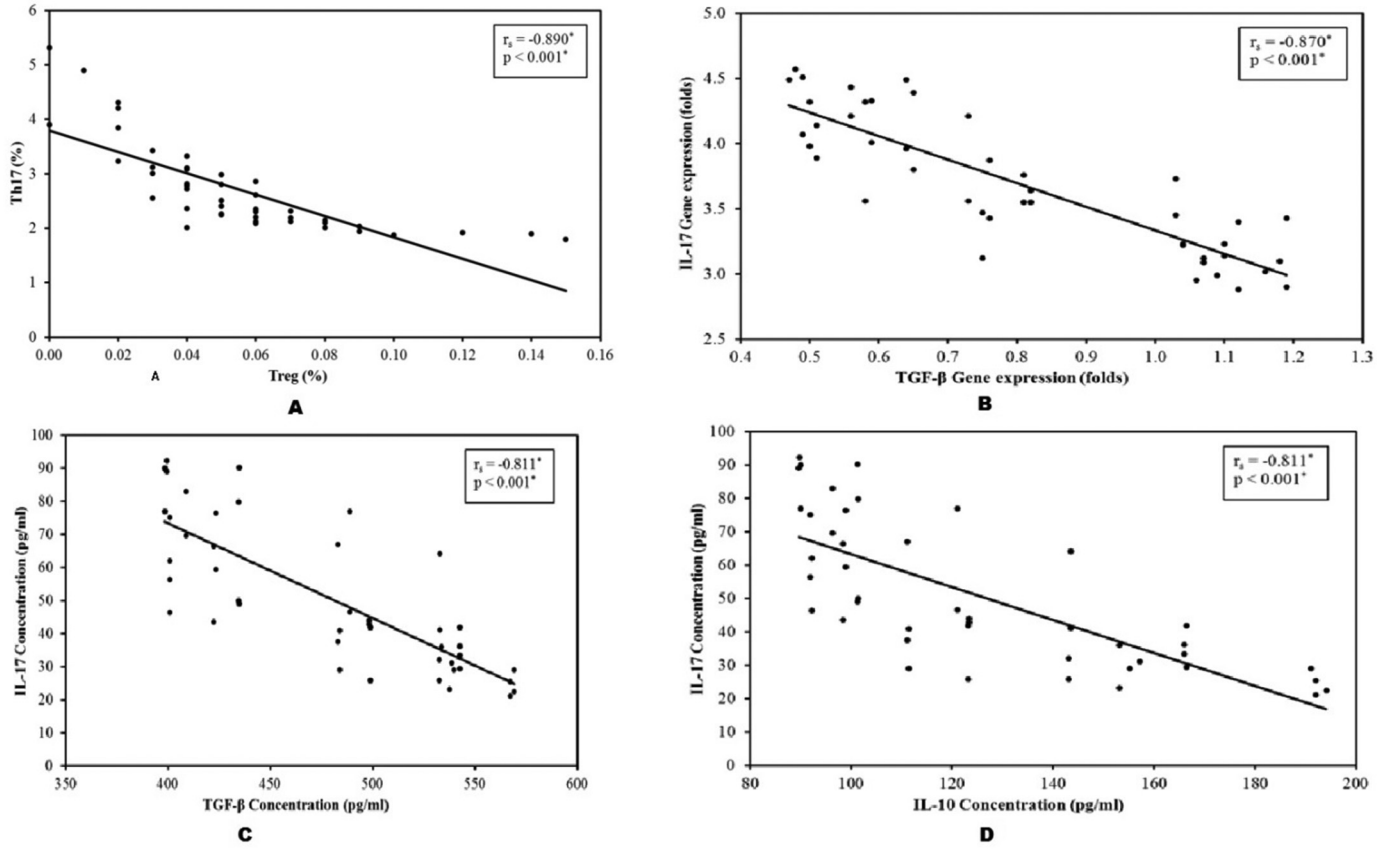


Fig. 6. Correlation between different immunological markers in ASD children (n = 44): a) Correlation analysis between the proportion of Th17 cells and Treg cells (rs = −0.890, p < 0.001); b) Correlation analysis between the gene expression of IL-17 and TGF-β (rs = −0.870, p < 0.001). c) Correlation analysis between the concentration of secreted cytokines IL-17 and TGF-β. (rs = −0.811, p < 0.001). d) Correlation analysis between the concentration of secreted cytokines IL-17 and IL-10 (rs = −0.811, p < 0.001). Correlation was conducted by spearman coefficient. The level of significance was set at *p ≤ 0.05.

children (mean ± SD = 2.3 ± 2.1) (p < 0.001), whereas no significant difference was observed regarding both IL-17 and IL-10 concentrations (Fig. 7).

We further analyzed association between maternal CRP and the studied immune markers, however, no significant association was found between CRP and either IL-17 (rs = 0.261, p = 0.088) or IL-10 concentrations (rs = 0.142, p = 0.358) (Fig. 8).

4. Discussion

The discovery of the link between immune dysfunction and behavioral traits highlighted the urge toward further analysis of the immune system alterations among individuals with ASD (Masi et al., 2017; Gottfried et al., 2015). Both chronic neurological inflammation and immune dysregulation are considered as prominent features of ASD (Xu et al., 2015). However, the detailed immunological deviations concerning for example; the balance between pro- and anti-inflammatory immune cells in this clinical disorder are still in need to be further elucidated.

The balance of Th17 and Treg cells is crucial for immune homeostasis and their imbalance plays a significant role in the inflammation reaction and autoimmune diseases (Noack and Miossec, 2014). However, limited information exists regarding the balance of Th17/Treg in ASD children.

In the present study, we investigated a possible involvement of the imbalance of Th17/Treg cells in the pathogenesis of ASD. As shown in our results, the expression of Th17 cells in the peripheral blood of ASD children was significantly higher compared to their control, being further supported by the increased both IL-17 mRNA expression and the secreted IL-17 following cell stimulation. Wong and Hoeffer (2017) demonstrated a role of maternal Th17 cells and subsequent IL-17A signaling in ASD pathogenesis. Simultaneously, a strong association of ASD-like phenotypes with Th17 cells has also been demonstrated in animal studies, where it has been found that antibody blockade of effector cytokine IL-17a prevented abnormal behaviors in the mice offspring (Choi et al., 2016). On the contrary, Hashim et al. (2013) found a nonsignificant difference in IL-17 levels in autistic children compared to their control, showing a negative correlation with the disease severity.

Notably, we found a direct correlation between Th17 and disease severity. A similar association with disease severity in subsets of ASD with co-morbid asthma was reported by Akintunde et al. (2015). These results suggest that Th17 cells and their effector cytokine may sustain inflammatory immune response in ASD pathogenesis. Additionally, it has been shown that Th17 cells can combine with receptors expressed in neurons via its secretion of IL-21, and thus induce their apoptosis (Tzartos et al., 2011).

Besides, cells of the CNS have functional receptors for IL-17, and studies have indicated that inflammation in the periphery can influence brain function and lead to pathologic changes in the CNS (Waisman et al., 2015). Furthermore, accumulated evidence indicated a proinflammatory profile in ASD, with an evidence of neuroinflammation or encephalitis (Morgan et al., 2012; Kern et al., 2016). Evidence of microglial activation or neuroinflammation has been found in at least 69% children with ASD (Tetreault et al., 2012).

Another important finding of this study is the significant reduction in Treg cells percentages in ASD children. This result was further confirmed by the downregulation of the related transcription factor (Foxp3) and cytokines (TGF-β and IL-10) in peripheral blood of ASD

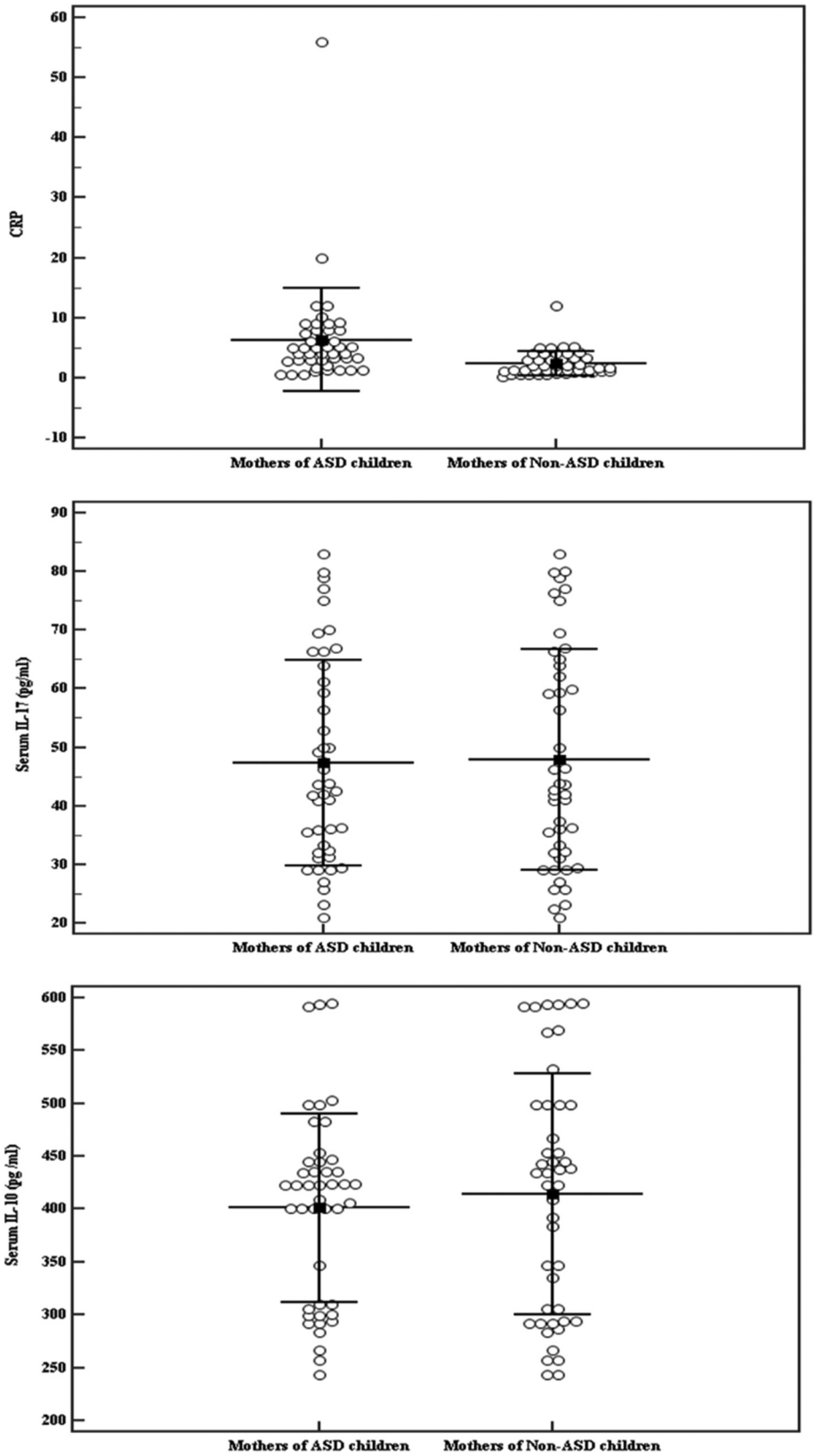


Fig. 7. Comparison between mothers of ASD children and mothers of non-ASD children according to different parameters: a) C-reactive protein level; b) IL-17 serum concentration; c) IL-10 serum concentration. Data represented means ± SDs and were summarized as dot plot. Each dot represents the concentration of CRP, IL-17 or IL-10 for one individual. The level of significance was set at $*p \leq 0.05$.

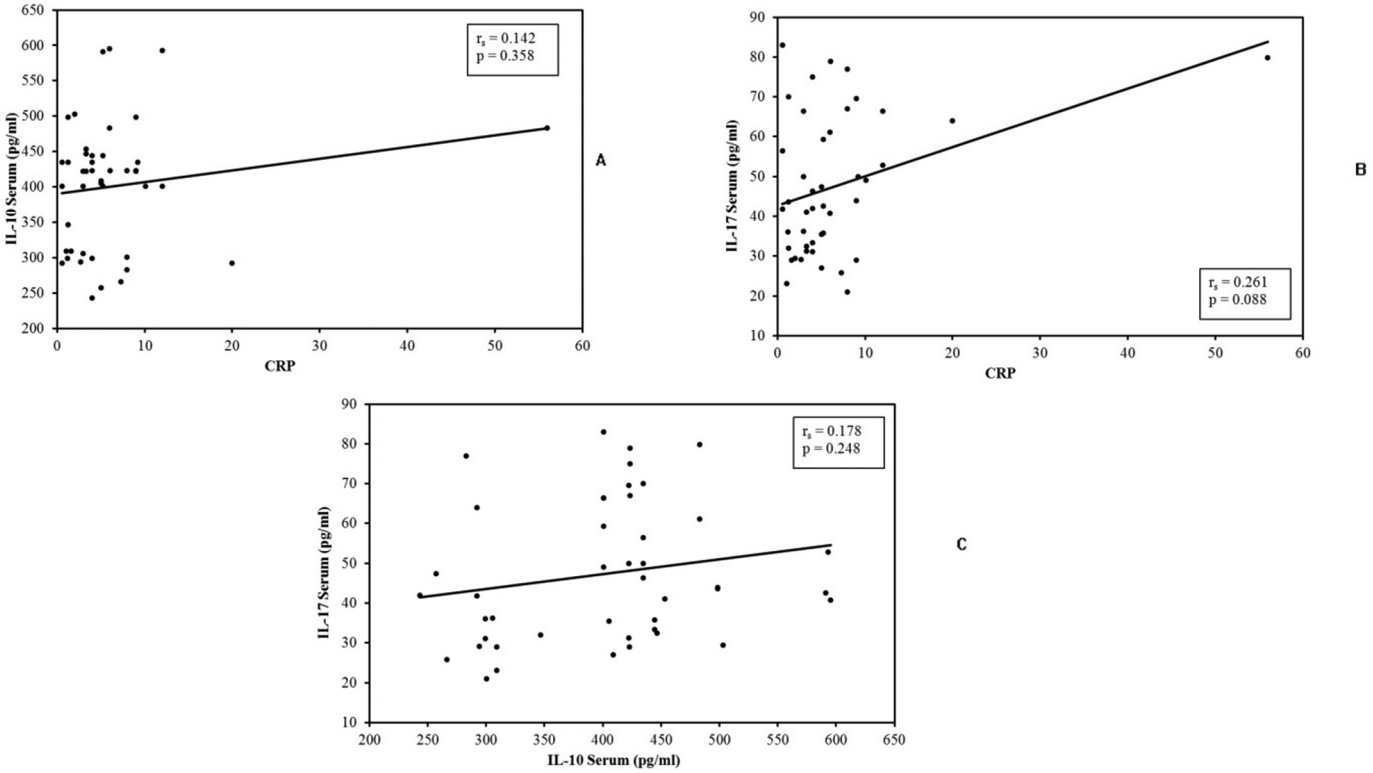


Fig. 8. Correlation between different parameters in mothers of ASD children (n = 44): a) Correlation analysis between IL-10 serum concentration and CRP (rs = 0.142, p = 0.358); b) Correlation analysis between IL-17 serum concentration and CRP (rs = 0.261, p = 0.088). c) Correlation analysis between IL-17 and IL-10 serum concentrations (rs = 0.178, p = 0.248). Correlation was conducted by spearman coefficient. The level of significance was set at *p ≤ 0.05.

children. In addition, a negative correlation was found between Treg cells and disease severity. In line with these results, Al-Ayadhi et al. (2018) reported that auditory integrative training (AIT) resulted in increased TGF-β1 levels with improvements in clinical ASD severity scores. These observations suggest that deficits in Treg cells correlate with the modulation of behaviors and core features of autism.

It has been shown that Treg cells can protect nerves by inhibiting the reaction of microglia to stimulus like nitrated α-synaptic nuclear protein (Reynolds et al., 2009). On the other hand, it has been reported that TGF-β overexpression in vivo disrupts the extracellular matrix and leads to motor incoordination, and behavioral abnormalities (Depino et al., 2011)

Regarding the association between Th17 and Treg, we found that Th17 cells were negatively correlated with Treg cells, that might be explained by the reciprocal relationship between Th17 cells and Treg, partly caused by a direct antagonistic interaction between the master transcription factors (RORγt and Foxp3) and their effector function (Zhao et al., 2010). Consistent with these results, we found that patients with ASD have an increased Th17/Treg ratio compared to their control, showing a positive correlation with the disease severity. This was in harmony with the observed upregulation of Th17 percentage and IL-17 expression and concentration.

Up to the present time, little is known about the maternal inflammatory state and immune response in ASD. Alterations in the immune system are demonstrated among family members of individuals with ASD, being evidenced by the increased prevalence of familial immune disorders in ASD (Chen et al., 2016). Our results revealed higher CRP levels in mothers of ASD children compared to their control. However, no significant difference was observed in the concentration of IL-17 and IL-10.

5. Conclusions

These results collectively suggested a role of the imbalance of Th17/Treg cells and their related cytokines in the pathogenesis and severity of ASD, providing better understanding of the role of immune alteration in ASD. They imply that recovering the balance between Th17 and Treg cells may be a potential therapeutic target in ASD. However, the functional consequences of these findings should be further investigated.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author disclosure statement

No competing financial interests exist.

Acknowledgments

We thank all subjects for participating in this study.

References

Akintunde, M.E., Rose, M., Krakowiak, P., Heuer, L., Ashwood, P., Hansen, R., Hertz-Picciotto, I., Van de Water, J., 2015. Increased production of IL-17 in children with autism spectrum disorders and co-morbid asthma. J. Neuroimmunol. 286, 33–41.

Al-Ayadhi, L., Alhowikan, A.M., Halepoto, D.M., 2018. Impact of auditory integrative training on transforming growth factor-β1 and its effect on behavioral and social emotions in children with autism spectrum disorder. Med. Princ. Pract. 27, 23–29.

American Psychiatric Association (Ed.), 2015. Diagnostic and Statistical Manual of Mental Disorders, 5th ed. American Psychiatric Association, Arlington, VA.

Baio, J., Wiggins, L., Christensen, D.L., Maenner, M.J., Daniels, J., Warren, Z., Kurzius-Spencer, M., Zahorodny, W., Robinson Rosenberg, C., White, T., Durkin, M.S., Imm,

P., Nikolaou, L., Yeargin-Allsopp, M., Lee, L.C., Harrington, R., Lopez, M., Fitzgerald, R.T., Hewitt, A., Pettygrove, S., Constantino, J.N., Vehorn, A., Shenouda, J., Hall-Lande, J., Van Naarden Braun, K., Dowling, N.F., 2018. Prevalence of autism spectrum disorder among children aged 8 years-autism and developmental disabilities monitoring network, 11 sites, United States, 2014. MMWR Surveill. Summ. 67, 1–23.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., Kuchroo, V.K., 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441, 235–238.

Cai, C.W., Blase, J.R., Zhang, X., Eickhoff, C.S., Hoft, D.F., 2016. Th17 cells are more protective than Th1 cells against the intracellular parasite Trypanosoma cruzi. PLoS Pathog. 12, e1005902.

Chen, S.W., Zhong, X.S., Jiang, L.N., Zheng, X.Y., Xiong, Y.Q., Ma, S.J., Qiu, M., Huo, S.T., Ge, J., Chen, Q., 2016. Maternal autoimmune diseases and the risk of autism spectrum disorders in offspring: a systematic review and meta-analysis. Behav. Brain Res. 296, 61–69.

Choi, G.B., Yim, Y.S., Wong, H., Kim, S., Kim, H., Kim, S.V., Hoeffer, C.A., Littman, D.R., Huh, J.R., 2016. The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. Science 351, 933–939.

Denney, D.R., Frei, B.W., Gaffney, G.R., 1996. Lymphocyte subsets and interleukin-2 receptors in autistic children. J. Autism Dev. Disord. 26, 87–97.

Depino, A.M., Lucchina, L., Pitossi, F., 2011. Early and adult hippocampal TGF-beta1 overexpression have opposite effects on behavior. Brain Behav. Immun. 25, 1582–1591.

Enstrom, A., Onore, C., Van de Water, J., Ashwood, P., 2010. Differential monocyte responses to TLR ligands in children with autism spectrum disorders. Brain Behav. Immun. 24, 64–71.

Fuss, I.J., Kanof, M.E., Smith, P.D., Zola, H., 2009. Isolation of whole mononuclear cells from peripheral blood and cord blood. Curr. Protoc. Immunol. https://doi.org/10.1002/0471142735.im0701s85. Chapter 7:Unit7.1.

Gładysz, D., Krzywdzińska, A., Hozyasz, K.K., 2018. Immune abnormalities in autism spectrum disorder–could they hold promise for causative treatment? Mol. Neurobiol. 55, 6387–6435.

Gottfried, C., Bambini-Junior, V., Francis, F., Riesgo, R., Savino, W., 2015. The impact of neuroimmune alterations in autism spectrum disorder. Front Psychiatry 6, 121.

Hashim, H., Abdelrahman, H., Mohammed, D., Karam, R., 2013. Association between plasma levels of transforming growth factor-β1, IL-23 and IL-17 and the severity of autism in Egyptian children. Res. Autism Spectr. Disord. 7, 199–204.

Kamali, A.N., Noorbakhsh, S.M., Hamedifar, H., Jadidi-Niaragh, F., Yazdani, R., Bautista, J.M., Azizi, G., 2019. A role for Th1-like Th17 cells in the pathogenesis of inflammatory and autoimmune disorders. Mol. Immunol. 105, 107–115.

Kern, J.K., Geier, D.A., Sykes, L.K., Geier, M.R., 2016. Relevance of neuroinflammation and encephalitis in autism. Front. Cell. Neurosci. 9, 519.

Lombardo, M.V., Moon, H.M., Su, J., Palmer, T.D., Courchesne, E., Pramparo, T., 2018. Maternal immune activation dysregulation of the fetal brain transcriptome and relevance to the pathophysiology of autism spectrum disorder. Mol. Psychiatry 23, 1001–1013.

Masi, A., Glozier, N., Dale, R., Guastella, A.J., 2017. The immune system, cytokines, and biomarkers in autism spectrum disorder. Neurosci. Bull. 33, 194–204.

Mohamed, F.E., Zaky, E.A., Youssef, A., Elhossiny, R., Zahra, S., Khalaf, R., Youssef, W., Wafiq, A., Ibrahim, R., Abd-Elhakim, R., Obada, A., Eldin, W.S., 2016. Screening of Egyptian toddlers for autism spectrum disorder using an Arabic validated version of M-CHAT; report of a community-based study (stage I). Eur. Psychiatry 34, 43–48.

Morgan, J.T., Chana, G., Abramson, I., Semendeferi, K., Courchesne, E., Everall, I.P., 2012. Abnormal microglial-neuronal spatial organization in the dorsolateral prefrontal cortex in autism. Brain Res. 1456, 72–81.

Noack, M., Miossec, P., 2014. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. Autoimmun. Rev. 13, 668–677.

Rellini, E., Tortolani, D., Trillo, S., Carbone, S., Montecchi, F., 2004. Childhood autism rating scale (CARS) and autism behavior checklist (ABC) correspondence and conflicts with DSM-IV criteria in diagnosis of autism. J. Autism Dev. Disord. 34, 703–708.

Reynolds, A.D., Stone, D.K., Mosley, R.L., Gendelman, H.E., 2009. Nitrated {alpha}-synuclein-induced alterations in microglial immunity are regulated by CD4+ T cell subsets. J. Immunol. 182, 4137–4149.

Tetreault, N.A., Hakeem, A.Y., Jiang, S., Williams, B.A., Allman, E., Wold, B.J., Allman, J.M., 2012. Microglia in the cerebral cortex in autism. J. Autism Dev. Disord. 42 (12), 2569–2584.

Tzartos, J.S., Craner, M.J., Friese, M.A., Jakobsen, K.B., Newcombe, J., Esiri, M.M., Fugger, L., 2011. IL-21 and IL-21 receptor expression in lymphocytes and neurons in multiple sclerosis brain. Am. J. Pathol. 178, 794–802.

Waisman, A., Hauptmann, J., Regen, T., 2015. The role of IL-17 in CNS diseases. Acta Neuropathol. 129, 625–637.

Ward-Hartstonge, K.A., Vasanthakumar, A., 2018. Regulatory T-cell heterogeneity. Clin. Transl. Immunol. 7, e01012.

Warren, R.P., Yonk, J., Burger, R.W., Odell, D., Warren, W.L., 1995. DR-positive T cells in autism: association with decreased plasma levels of the complement C4B protein. Neuropsychobiology 31, 53–57.

Whiteside, T.L., 2014. Regulatory T cell subsets in human cancer: are they regulating for or against tumor progression? Cancer Immunol. Immunother. 63, 67–72.

Wills, S., Cabanlit, M., Bennett, J., Ashwood, P., Amaral, D.G., Van de Water, J., 2009. Detection of autoantibodies to neural cells of the cerebellum in the plasma of subjects with autism spectrum disorders. Brain Behav. Immun. 23, 64–74.

Wong, H., Hoeffer, C., 2017. Maternal IL-17A in autism. Exp. Neurol. 299, 228–240.

Xu, N., Li, X., Zhong, Y., 2015. Inflammatory cytokines: potential biomarkers of immunologic dysfunction in autism spectrum disorders. Mediat. Inflamm. 2015 (531518).

Yu, F., Sharma, S., Edwards, J., Feigenbaum, L., Zhu, J., 2015. Dynamic expression of transcription factors T-bet and GATA-3 by regulatory T cells maintains immunotolerance. Nat. Immunol. 16, 197–206.

Zhao, L., Qiu, D.K., Ma, X., 2010. Th17 cells: the emerging reciprocal partner of regulatory T cells in the liver. J. Dig. Dis. 11, 126–133.