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ORIGINAL ARTICLE

Assignment of CD4+CD25+Foxp3+ Regulatory T Cells, MOG antibodies, MAG antibodies and anti-nuclear antibodies in Autistic Children

Ghada SHOUSHA^{1*}, Azza YOUSSEF², Reham ELHOSSINY², Abeer SHEHAB³, Hala MOHAMED⁴, Amany MEHREZ⁴, Gehan MOSTAFA¹

¹ Department of Pediatrics, Pediatric Allergy, Immunology and Rheumatology Unit, Faculty of Medicine, Ain Shams University

² Pediatric Development and Behavior, Pediatrics Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt

³ Clinical Pathology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt

⁴ Ministry of Health and Population, Cairo, Egypt

KEYWORDS

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Abstract

Immune dysregulation may contribute to autism spectrum disorder (ASD) severity and progression. This cross-sectional study assessed the frequency of peripheral regulatory T cells (Tregs) and brain-related antibodies in 25 children with ASD and 25 healthy controls. Flow cytometry was used to measure CD4+CD25+ and CD4+CD25+Foxp3+ Tregs. Serum anti-nuclear antibody (ANA), anti-myelin-associated glycoprotein (MAG), and anti-myelin oligodendrocyte glycoprotein (MOG) antibodies were evaluated in both groups. Children with ASD exhibited a significant reduction in CD4+CD25+Foxp3+ Tregs compared to controls. CD4+CD25+ lymphocytes below 3.15% predicted ASD with 80% sensitivity and 73.5% specificity, while CD4+CD25+Foxp3+ Tregs below 2.55% achieved 73.3% sensitivity and 76.7% specificity. ANA and anti-MAG positivity were comparable between groups. Anti-MOG antibodies were undetectable in ASD patients. These findings suggest impaired Treg function in ASD, whereas brain-directed autoantibodies appear unaltered. Treg frequency may serve as a potential biomarker and therapeutic target in ASD.

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* Corresponding author:

Ghada Shousha

ORCID ID: 0000-0003-1621-5121

Adresse e-mail : ghada_shousha@med.asu.edu.eg

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that impacts children's behaviors and social interactions¹. There is increasing evidence suggesting that immune dysregulation plays a role in the pathogenesis and progression of ASD². Previous studies reported abnormalities, including alterations in cytokine profiles, disruption in immune cell subset distribution, changes in immunoglobulin levels, and presence of autoantibodies². These findings suggest that immune-mediated mechanisms and autoimmunity may have a role in the pathophysiology of ASD.

ASD shares several features with autoimmune disorders, including familial clustering, polygenic genetic predisposition, and gender-based differences in prevalence³. In addition, immune activation, elevated cytokines, and impaired lymphocyte function are commonly reported in ASD⁴. Brain-specific autoantibodies, including anti-myelin oligodendrocyte glycoprotein (anti-MOG) and anti-myelin-associated glycoprotein (anti-MAG), have been recorded in central nervous system autoimmunity and were suggested to have a role in ASD⁵. Antinuclear antibodies (ANAs) were found to be elevated in ASD patients, which was correlated with symptom severity, when used as a screening marker for autoimmunity⁶.

Regulatory T cells (Tregs), including CD4⁺CD25⁺FOXP3⁺ cells, are essential in maintaining immune balance by suppressing autoreactive immune responses and supporting in tissue repair⁷. Dysfunction in Tregs was previously linked to several autoimmune diseases, including systemic lupus erythematosus⁸. Reduced CD4⁺CD25⁺ Treg frequencies have been previously recorded in patients with ASD. However, there are limited studies that assessed FOXP3 expression, leaving a gap in understanding the suppressive function of these cells in ASD. This study aimed to evaluate the frequency of peripheral CD4⁺CD25⁺ and CD4⁺CD25⁺FOXP3⁺ regulatory T cells, along with serum levels of ANA, anti-MOG, and anti-MAG antibodies in children with ASD compared to healthy controls. The goal was to investigate potential immune dysregulation and its possible role in the pathogenesis of ASD, and whether they could have a diagnostic or therapeutic value as immune markers.

MATERIALS AND METHODS

Study Design and Setting

This cross-sectional study was conducted over six months, from March to September 2022, at the Developmental and Behavioral Pediatrics Clinic of the Children's Hospital of Ain Shams University. Ethical approval was obtained from the Research Ethics Committee of the Faculty of Medicine, Ain Shams University (approval number: FWA 000017585). Informed consent was obtained from the guardians of all participating children after explaining the aim and procedures of the study.

Study population

A total of 25 children and adolescents with physician-confirmed ASD were consecutively enrolled. All patients were diagnosed based on the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision (DSM-5-TR) criteria⁹, after considering all inclusion and exclusion criteria. The inclusion criteria comprised children of both genders, with ages between 4 and 16 years, who met the diagnostic criteria for ASD. Exclusion criteria included children with a history of allergic, autoimmune, or immunodeficiency disorders, as well as those with any concomitant infections. An age- and sex-matched control group of 25 healthy children without ASD or known immune-related conditions was recruited for comparison.

Clinical and Diagnostic Assessment

All participants underwent a comprehensive assessment, including the collection of demographic data and relevant medical history such as age, gender, developmental milestones, presence of associated medical conditions, and any personal or family history of autoimmune disorders. Clinical examination was performed for all participants, with particular focus on neuropsychiatric evaluation. All diagnosis of ASD was confirmed based on THE DSM-5-TR criteria¹⁰, and ASD severity was assessed using the Childhood Autism Rating Scale (CARS)¹¹.

Laboratory Assessment

1. Sample Collection and Processing

Laboratory investigations were conducted for all participants. Peripheral blood (5 mL) was withdrawn under aseptic conditions. Half of the sample was treated with EDTA as an anticoagulant and processed within 24 hours at room temperature for flow cytometric analysis of peripheral CD4⁺CD25⁺Foxp3⁺ regulatory T cells

(Tregs). The remaining blood sample was allowed to clot, centrifuged, and serum was separated and stored at -80°C for later analysis.

2. Flow Cytometry Analysis of Tregs

Treg frequency was determined using CD4-FITC, CD25-PE, and Foxp3-PC7 monoclonal antibody kits (Beckman Coulter). Fifty μL of homogenized whole blood was mixed with 2 mL of lysis buffer, incubated for 2 minutes, and centrifuged. The supernatant was discarded, and the cell pellet was washed twice with phosphate-buffered saline (PBS). Cells were then stained with 5 μL each of CD4-FITC and CD25-PE monoclonal antibodies, incubated in the dark at room temperature for 15 minutes. After permeabilization, 10 μL of Foxp3-PC7 antibody was added, followed by another 15-minute incubation. Cells were washed and resuspended in 500 μL of PBS for flow cytometric analysis. Lymphocytes were gated using forward and side scatter, then analyzed for CD4+CD25+ expression. Foxp3 expression was subsequently assessed within the CD4+CD25+ population to determine the percentage of CD4+CD25+Foxp3+ Tregs.

3. Serological Investigations

Additional serological tests included ANA using a human ANA ELISA kit (Orgentec Diagnostika GmbH, Germany), anti-MOG antibodies via a cell-based assay (Euroimmun, Lübeck, Germany), and anti-MAG antibodies using an ELISA kit (BT LAB, Shanghai).

Statistical analysis

Statistical Package for the Social Sciences (SPSS), version 23 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Quantitative variables were expressed as mean $\pm$ standard deviation (SD) or median and interquartile range (IQR), according to data distribution. Categorical variables were presented as frequencies and percentages. Appropriate statistical tests were applied for group comparisons and correlation analyses based on data type and distribution. A p-value of <0.05 was considered statistically significant.

RESULTS

This study enrolled 25 children diagnosed with ASD, including 19 males (76%) and 6 females (24%), with a male-to-female ratio of 3.17:1. The mean age of enrolled children was 6.62 ± 2.11 years. A positive family history of ASD was recorded in 2 patients. True seizures were confirmed by electroencephalogram in 3 patients (12%). At the time of diagnosis, 64% of patients

were considered mild to moderate ASD, and 36% were considered severe (**Table 1**).

Table (1): Demographic and clinical characteristics of the studied populations

		Groups		Test of significance	
		Control (25)	Patients (25)		
		Mean ± SD N (%)	Mean ± SD N (%)	Value	p-Value
Age in years		7.36 ± 3.15	6.58 ± 2.86	t= 0.917	0.364
Gender	Male	16 (64)	19 (76)	X²= 0.857	0.355
	Female	9 (36)	6 (24)		
Family history of Allergy	No	25 (100)	24 (96)	FE	1.00
	Yes	0 (0)	1 (4)		
Family history of autoimmune diseases	No	25 (100)	25 (100)		
	Yes	0 (0)	0 (0)		
Family history of ASD	No	25 (100)	23 (92)	FE	0.49
	Yes	0 (0)	2 (8)		
Convulsions	No	22 (88)			
	Yes	3 (12)			
CARS	Mean ± SD	34.64 ± 4.15			
	Median (IQR)	34 (31-37)			
Severity of CARS	Mild to moderate	16 (64)			
	Severe	9 (36)			

ASD: autistic spectrum disorder, CARS: childhood autism rating scale, IQR: interquartile range, SD: standard deviation. *Student t-test of significance (t).

Chi-Square test of significance (χ^2).

ANA and Anti-MAG levels were comparable between patients and controls (p-value = 0.429 and =1.00, respectively). Anti-MOG antibodies were detected in only one patient with ASD. CD4+CD25+Foxp3+ Tregs were significantly lower among children with ASD compared to healthy controls (p-value = 0.003) (**Table 2**).

Table (2): The studied immunological parameters among the studied populations

		Groups		Test of significance	
		Control (n=25)	Patients (n=25)	Value	p-Value
		N (%) Median (IQR)	N (%) Median (IQR)		
Anti MAG	Negative	25 (100%)	23 (92%)	FE	0.49
	Positive	0 (0%)	2 (8%)		
Anti MOG	Negative	25 (100%)	24 (96%)	FE	1.00
	Positive	0 (0%)	1 (4%)		
CD4CD25 cells (%)		4.5 (3.5 - 8.4)	2.7 (2 - 3.5)	z= - 4.077	<0.001
CD4CD25Foxp3 cells (%)		75.5 (2.8 - 89.7)	1.5 (0.5 - 3.9)	z= - 2.990	0.003
ANA		0.7 (0.6 - 0.8)	0.7 (0.6 - 0.8)	z= - 0.791	0.429

ANA: antinuclear antibodies, MAG: anti-myelin associated glycoprotein, MOG: Myelin oligodendrocyte glycoprotein. *Mann-Whitney test of significance (z).

CD4+CD25+ lymphocyte levels below 3.15% demonstrated a sensitivity of 80% and specificity of 73.5% for predicting the probability of ASD. Similarly, a FOXP3+ Treg percentage below 2.55% yielded a sensitivity of 73.3% and specificity of 76.7% (**Table 3, Figure 1**). However, no significant correlation was found between Treg levels and either ASD severity or the presence of seizures (**Table 4**).

Table (3): The predictive value of T regulatory cells for ASD patients from healthy controls

Variables	Cut off	AUC	Sensitivity	Specificity	P value	95% CI	
						Lower Bound	Upper Bound
CD4CD25 lymphocytes	< 3.15	0.816	80 %	73.5 %	<0.001	0.709	0.922
%FOXP3 Tregs	< 2.55	0.753	73.3 %	76.7 %	0.001	0.629	0.877

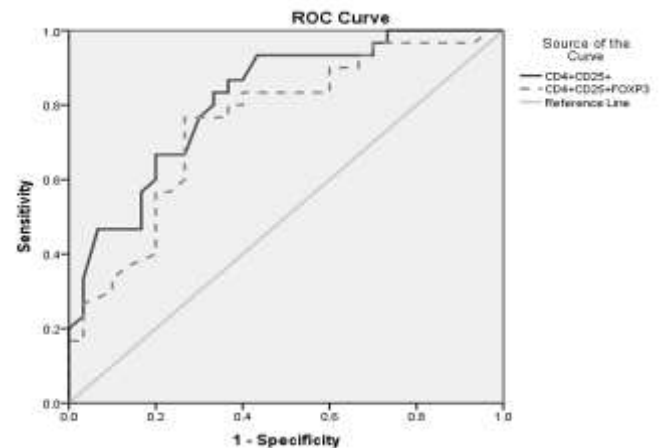

Figure (1): Receiver operating curve (ROC) of CD4+CD25+lymphocytes and CD4+CD25+FOXP3 percentage as predictors for ASD from healthy controls

Table (4): Correlations between T regulatory cells and clinical-demographic data of ASD patients

	Gender		Age		Convulsions		CARS	
	Test value ‡	P-value	r	P-value	Test value ‡	P-value	r	P-value
%CD4CD25 cells	-0.44	0.66	0.011	0.61	-0.35	0.73	0.22	0.287
%FOXP3CD4CD25 cells	-2.11	0.035	0.24	0.25	-1.32	0.19	0.19	0.355

CARS: childhood autism rating scale, IQR: interquartile range. ‡: Mann Whitney test

DISCUSSION

This study compared several immunological parameters in children with ASD to those of healthy children. The main finding was the significant reduction in the frequency of regulatory T cells (CD4+CD25+Foxp3+) among the ASD group. Children with ASD had lower levels of immunosuppressive Tregs compared to controls, suggesting an impaired immunoregulatory function in children with ASD. In contrast, classical markers of autoimmunity, such as antinuclear antibodies, did not differ between groups. Myelin-related autoantibodies, including anti-MOG and anti-MAG, were largely absent or infrequent in both ASD and control groups. These findings suggest that autoimmunity is not a common feature of ASD, aligning with previous studies that ASD is not typically characterized by the presence of pathogenic autoantibodies^{12,13}.

Immune alterations are increasingly observed among patients with ASD, particularly involving impaired immune regulation and the reduced ability to control immune responses¹⁴. In the current study, there was a significant reduction in CD4+CD25+lymphocytes, the most prevalent Treg cells, in children with ASD. Moreover, the percentage of CD4+CD25+Foxp3, the most effective Treg cells, was also significantly lower in ASD. This decrease in Tregs levels is consistent with its decline in autoimmune diseases, where increased susceptibility of Tregs to Fas-mediated

apoptosis leads to their depletion following excessive exposure to self-antigens^{15,16}. Downregulation of Tregs in ASD can lead to an unopposed inflammatory cascade, highlighting Tregs as a possible therapeutic strategy in ASD patients. *Rose et al.* reported a significantly reduced percentage of CD4+CD25+Foxp3 Tregs in pediatric ASD patients (1.93%)¹⁴. Similarly, another study reported an altered immune profile, including lower expression of Foxp3+ Treg cells¹⁷. A previous study conducted on CD4+CD25+ Tregs, without Foxp3 analysis, showed a significant downregulation of Tregs among children with ASD (p-value < 0.001)¹⁸. These findings were also supported by a meta-analysis, which observed a significant decrease in Tregs cells in the peripheral blood of children with ASD compared to healthy controls (p < 0.0001). These findings suggest that Treg depletion is a consistent immunological feature of ASD. However, Treg levels did not show a significant correlation with ASD severity or with the presence of seizures in our study. This discrepancy highlights the possibility of the influence of other genetic or external factors on ASD severity.

Our study indicated that a CD4+CD25+ T-cell percentage below 3.15% can differentiate ASD cases from neurotypical children with a relatively high sensitivity and specificity. In addition, a Foxp3+ Treg percentage below 2.55% yielded a similar diagnostic accuracy. This suggests the possibility that Treg frequency could be a predictive biological marker for ASD. However, these findings need validation in larger, diverse populations. Other theories can be applied from our findings, such as the applicability of using immunomodulatory interventions in ASD, and whether restoring the immune balance could potentially control some ASD-related features¹³. Anti-MAG antibodies were detected in two children with ASD (8%). Although this percentage is relatively low, it suggests a potential autoimmune impact on the central nervous system. Brain-specific autoantibodies in ASD were evident in previous studies, where elevated levels of anti-MAG antibodies in children with ASD were reported^{19,20}. Similarly, anti-MOG antibodies were identified in one child in our ASD group. Notably, a larger study by *Mostafa and Al-Ayadhi* reported high anti-MOG percentages, reaching up to 78.5% of children with ASD. This suggests a possible link between myelin-targeting autoantibodies and the

neurodevelopmental features of autism²¹. However, this claim needs to be further investigated in larger and more diverse cohorts to determine if there is a true correlation. This finding aligns with the hypothesis that cerebral autoimmunity may contribute to ASD pathogenesis. In contrast, there was no difference between ANA levels among patients and controls in our study. This contradicts the theory that ASD could be a tone in the symphony of systemic autoimmune disorders. A previous study reported elevated ANA levels in 47% of children with ASD²². However, our findings should also be validated by a larger cohort to clarify the role of autoantibodies in the pathophysiology of ASD.

STUDY LIMITATIONS

The small sample size and the single center were major limitations that could impact the generalizability of our results. The cross-sectional study design is not the design of choice to determine causality. Additionally, the duration of illness and regimen of therapy were not questioned, which may act as confounding factors.

CONCLUSION

CD4+CD25+FOXP3+ T cells, the master promoter of Tregs, were significantly reduced in children with ASD, with expression levels below 2.5% demonstrating good sensitivity and specificity for distinguishing ASD cases. Additionally, the detection of brain-specific autoantibodies, anti-MOG and anti-MAG, in a few patients suggests a possible underlying autoimmune etiology. These findings highlight both CD4⁺CD25⁺FOXP3⁺ Tregs and brain-targeted autoantibodies as potential biomarkers and therapeutic targets.

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