Association of immune abnormalities with symptom severity in Egyptian autistic children

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Background
Autism spectrum disorder (ASD) is considered a spectrum of behavioral anomalies described by having impaired social interaction and communication. It is usually accompanied by repetitive and stereotyped behavior. ASD likely develops from a combination of genetic and environmental factors. Among suggestions, one has been persistently proposed where an immune dysfunction was related to certain forms of ASD. Growing evidence of autoimmune phenomena in patients with ASD may represent the occurrence of changed or perhaps unsuitable immune responses in this disorder.

Objectives
This study was aimed to evaluate cell-mediated as well as humoral immunity in children with ASD. This was through evaluation of lymphocyte count (CD3, CD16, and CD19) and estimation of the serum immunoglobulin levels (IgG, IgM, and IgA).

Patients and methods
This study was carried out in the National Research Centre. A total of 50 newly diagnosed children with ASD were included (33 males and 17 females), with an age range from 3 to 8 years, in addition to 50 apparently healthy age-matched and sex-matched participants as a control group. CD3, CD16, and CD19 were estimated using flow cytometry. Serum Ig levels were measured using immunonephelometry.

Results and conclusion
The study results revealed that children with autism had significantly lower CD19 in comparison with the control group ($P<0.001$). Autistic children also had considerably higher numbers of CD16 ($P<0.001$) and significant increased absolute lymphocyte count ($P=0.034$) in comparison with healthy controls. CD3% and absolute CD16 count were significantly positive correlated with Childhood Autism Rating Scale score in children with ASD ($P=0.004$ and 0.025, respectively).
An increased total lymphocyte and natural killer cell count together with decreased B lymphocyte count and positive correlations in CD3 and absolute CD16 count with Childhood Autism Rating Scale score in participants with ASD revealed the impaired cell-mediated immunity in participants with ASD, and these tests might be useful as diagnostic markers for ASD and its degree of severity.

Keywords:
autism, immunoglobulin, lymphocyte, natural killer

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Introduction

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder characterized by impairments in social communication and restricted, repetitive patterns of behavior, interests, or activities [1]. Adding to these principal diagnostic features, children with ASD are usually presented with a host of linked behavioral concerns, such as intellectual disability and epilepsy, in addition to problems in sleeping and feeding. ASD prevalence is around 1/59, with a considerably higher ratio in males [2].

The human immune system is a group of proteins and immune cells responsible for protecting the body from foreign antigens, and immune responses vary from cell-mediated to humoral [3]. Increasing evidence proposes that immune system dysregulation might be involved in the pathophysiology of ASD [4,5]. Moreover, growing evidence of autoimmune phenomena suggests the presence of altered or perhaps inappropriate immune responses in this disorder. The immune abnormalities in autism may be cellular such as the changes in the numbers and activities of macrophages, T-cells, B-cells, and natural killer cells [6], or humoral as explained by Gupta *et al.*

[7], who reported that patients with autism have elevated immunoglobulin E (IgE) levels.

Thus, dysfunction of the immune system may be a new target for treatment [8]. Many studies concerning autism have stated altered antibody responses to brain and central nervous system proteins and skewed responses of Igs, such as increased isotype IgG4 levels despite decreased total serum IgG [9]. Several potential biomarkers are evolving for autism, and many studies have attempted to support an 'immunology-based' foundation of autism pathophysiology [4,9,10].

Despite this, neither a biochemical nor immunological foundation has been acknowledged to be efficiently translated into the clinic [11]. Such knowledge if accessible would provide much valuable information, which may contribute in both treatment plan as well as patient prognosis [12]. In the same context, researchers reported that adaptive and innate immune response alterations have been described in patients with ASD [5,13] and that these defects unequivocally accompany the performance of both arms of the cellular immune system [14,15].

However, additional research is necessary to investigate the relationship between ASD symptoms and immune profiles [16] to achieve more awareness concerning potentially useful biomarkers for ASD symptom severity and provide evidence for possible causative associations that might describe ASD subgroups. The pathophysiological importance of commonly recognized comorbidities in ASD, such as sleep disorder and gastrointestinal (GI) dysfunction, in relation to the identified immune system abnormalities, is similarly unidentified [17-19].

Consequently, description of the relationship between aberrations of the immune system, especially immune cells count, and severity of ASD symptoms is required. Therefore, the aim of this study was to assess the immune system in children with ASD in an Egyptian cohort. This includes cell-mediated immunity through evaluation of immune cells and humoral immunity through estimation of Ig serum levels in these participants with ASD.

Patients and methods

Participants

Participants and/or their legal guardians provided informed and written consent to participate in this study, and the study protocol was approved by the ethical research committee of the National Research Centre. Our study included 50 participants with ASD and 50 controls. The mean±SD age of children with ASD and control group was 5.5±2.65 and 5.0±3.3 years, respectively. All participants with ASD met the diagnostic criteria of ASDs according to DSM-5. There were 33 (66.0%) males and 17 (34.0%) females. All participants with ASD were recruited among children with ASD referred to Genetic Clinic at the Medical Research Centre of Excellency, National Research Centre, Dokki, Giza, Egypt. Detailed history was taken (personal, pregnancy, and delivery). Milestone development was recorded in addition to symptoms onset, history of vaccination and nutrition, sleep disorders, GI manifestations, as well as history of repeated infection. A pedigree was constructed up to third generation. Specific attention was drawn to likewise affect members of the family as well as to other psychiatric disorders. Childhood Autism Rating Scale (CARS) was used to rate severity, and all participants with ASD were subjected to electroencephalogram (EEG) and underwent MRI. Demographic and clinical characteristics of 50 participants with ASD are summarized in Table 1.

Blood samples

Overall, 10 ml of fresh fasting peripheral blood samples was drawn from all participants with ASD in the morning. Then, 5 ml of blood was collected in sterile EDTA-containing tubes for the procedure of flow cytometry, which was performed according to the procedure mentioned below. Another 5 ml of blood was collected into a serum separator tube and centrifuged at a rate of 3000 rpm for 10 min to separate the serum, which was collected and

Table 1. Autistic findings among case group

Variables	Mean±SD		
CARS score	38.8±6.4		
	n (%)		
Severity			
Mild	19 (38.0)		
Moderate	18 (36.0)		
Severe	13 (26.0)		
Consanguinity	12 (24.0)		
Family history of psychiatric illness	19 (38.0)		
EEG abnormalities	32 (64.0)		
MRI abnormalities	15 (30.0)	6/15	Mild
		3/15	Moderate
		6/15	Severe
GI disturbance	29 (58.0)		
Sleep disturbance	17 (34.0)		

CARS, Childhood Autism Rating Scale; EEG, electroencephalography; GI, gastrointestinal.

immediately frozen in 0.5-ml aliquots at −80°C until assayed for Igs by immunonephelometry.

Determination of immunoglobulins (IgA, IgM, and IgG)

Measurement of serum Ig was performed using immunonephelometry (Minineph, The Binding Site Ltd, Birmingham, UK) using human immunoglobulin kit (The Binding Site Ltd), according to the manufacturer's protocol. The determination of soluble antigen concentration by nephelometric methods involves a reaction with specific antiserum to form insoluble complexes. When light passes through the suspension formed, a portion of the light is scattered and detected by a photodiode. The amount of light scattered is directly proportional to the specific protein concentration in the test sample. Concentrations are automatically calculated by reference to a calibration curve stored within the instrument [20].

Flow cytometric analysis

Anticoagulated blood was stained using the whole-blood lysis method. Whole blood was stained with monoclonal antibodies directed against the surface antigens: CD3 FITC labelled MoAbs for T lymphocytes, CD16 labelled with PE for NK cells, and FITC labelled CD19 for B lymphocytes (BD Biosciences, San Jose, California, United States). Lymphocyte subset immunophenotyping was done using flow cytometry (BD Accuri C6 Cytometer, San Jose, California, United States) [21].

Statistical analysis

Data were collected, coded, tabulated, and statistically analyzed. This was performed by an IBM SPSS statistics (Statistical Package for the Social Sciences) software, version 18.0 (IBM Corp., Chicago, Illinois, USA, 2009). Descriptive statistical analysis was done for quantitative data as mean±SD, whereas for qualitative data was presented as number and percentage. Inferential analyses were done for quantitative variables using independent t test and for qualitative data using χ^2 test. Correlations were done using Pearson correlation. To evaluate the performance of different tests and differentiate between groups and case severities, receiver operating characteristic (ROC) curve was used. P value less than 0.050 was considered significant, and P value less than 0.050 was considered nonsignificant. Diagnostic characteristics were calculated as follows:

(1) 1.Sensitivity=(true positive test/total positive golden)×100.
(2) 2.Specificity=(true negative test/total negative golden)×100.
(3) 3.Youden's index=sensitivity+specificity–1.

Results and discussions

This study included 50 participants with ASD, and their age ranged from 3 to 8 years old. All participants with ASD met the diagnostic criteria of autism as defined in the International Classification of Diseases, 10th edition [22]. EEG abnormality was recorded in 64.0% among the studied ASD participants. It was found in the form of epileptogenic activity either multifocal or focal epileptogenic discharge [23]. EEG changes and epilepsy were reported frequently in children with ASD [24,25], whereas GI manifestations and sleep disorders were revealed by 34.0 and 58.0%, respectively (Table 1).

The levels of IgG, IgM, and IgA were not significantly different in ASD versus control children (Table 2). The expression of CD3 and CD16 in children with ASD was not significantly different from controls. A statistically significant increase in CD16% ($P<0.001$) and absolute lymphocyte count ($P=0.034$) was observed in children with ASD versus controls. The CD19% lymphocytes significantly decreased in children with ASD compared with controls ($P<0.001$). Moreover, absolute CD19 lymphocyte significantly decreased in autistic children as compared with age-matched apparently healthy participants ($P<0.001$) (Table 2). These results were in agreement with Han *et al.* [26], who found increased total numbers of T cells linked to reduced executive function in people with ASD. Joller *et al.* [27] stated that abnormal T-cell responses or diminished elimination of activated T cells may lead to autoimmune pathology. DiStasio *et al.* [28] suggested that dysregulated cellular immunity causes damage to astrocytes at foci along the cerebrospinal fluid–brain barrier in ASD. Abnormalities in B-cell populations and antibody production have been recognized in various studies. Variation in study design and studied markers (CD20 and CD19, respectively) may explain the inconsistent results. Neither marker is comprehensive for individual B cell subsets as their expression declines as B cells mature into antibody-secreting plasma cells [29].

The current study revealed that no statistically significant difference was encountered regarding degree of severity of autism and IgG, IgM, and IgA, lymphocyte, or CD19. Yet, a high statistically significant difference was found between children

Table 2. Different laboratory parameters in autistic children versus control group

Variables	Case (*N*=50)	Control (*N*=50)	*P*
Age (years)	5.5±2.65	5.0±3.3	0.416^
Sex [*n* (%)]			
Male	33 (66.0)	28 (56.0)	0.305#
Female	17 (34.0)	22 (44.0)	
Absolute lymphocyte count ($\times10^9$/l)	4.04±1.45	3.39±1.58	0.034*^
CD3%	54.33±15.82	56.92±6.90	0.291^
Absolute CD3 ($\times10^3$/μl)	2.02±1.15	1.99±1.04	0.902^
CD16%	14.34±6.62	8.92±4.59	<0.001*^
Absolute CD16 ($\times10^3$/μl)	0.59±0.57	0.28±0.11	<0.001*^
CD19%	9.35±5.15	18.64±7.02	<0.001*^
Absolute CD19 ($\times10^3$/μl)	0.33±0.23	0.69±0.56	<0.001*^
IgA (g/l)	0.96±0.45	0.88±0.43	0.366^
IgM (g/l)	1.10±0.75	1.06±0.31	0.740^
IgG (g/l)	9.82±4.11	10.17±2.37	0.610^

Data were presented as mean±SD.
P value calculated using:
^Independent *t* test.
#χ^2 test.
*Significant *P* value less than 0.05.

Table 3. Comparison according to severity among autistic group

Variables	Mild (*N*=19)	Moderate (*N*=18)	Severe (*N*=13)	*P*
Age (years)	6.1±3.5	5.6±1.8	4.5±1.8	0.238^
Sex [*n* (%)]				
Male	16 (84.2)	10 (55.6)	7 (53.8)	0.103#
Female	3 (15.8)	8 (44.4)	6 (46.2)	
Absolute lymphocyte count ($\times10^9$/l)	3.88±1.22	3.83±1.55	4.56±1.61	0.323^
CD3%	47.51±15.85§	53.24±15.93§	65.82±8.19⌂	0.004*^
Absolute CD3 ($\times10^3$/μl)	1.72±0.78	1.89±1.18	2.63±1.40	0.076^
CD16%	12.39±5.66	16.05±7.05	14.85±7.05	0.235^
Absolute CD16 ($\times10^3$/μl)	0.40±0.34§	0.50±0.36§	0.96±0.88⌂	0.015*^
CD19%	8.88±5.21	8.71±5.63	10.94±4.34	0.442^
Absolute CD19 ($\times10^3$/μl)	0.32±0.24	0.30±0.23	0.39±0.22	0.567^
IgA (g/l)	0.98±0.47	1.10±0.48	0.75±0.30	0.104^
IgM (g/l)	1.03±0.45	1.39±1.09	0.79±0.24	0.073^
IgG (g/l)	9.09±4.37	9.95±3.73	10.73±4.32	0.541^

Data were presented as mean±SD.
P value calculated using:
^Analysis of variance test with post-hoc Tukey's test (homogenous groups had the same symbol'§ or ⌂').
#χ^2 test.
*Significant *P* value less than 0.05.

with mild, moderate, and severe ASD regarding CD3 and absolute CD16 counts, as shown in Table 3.

The American Psychiatric Association diagnosed ASD as a heterogeneous group of neurodevelopmental syndromes with persistent social communication deficits and social interaction, in addition to restricted, repetitive behavioral patterns, activities, or interests [24,26,30]. Strong influence of the immune system abnormalities to ASD has been studied. Moreover, studies among participants with ASD showed that immune system defect is frequently sustained by a strong inflammatory state [31]. In particular, increased levels of pro-inflammatory cytokines and CDs were detected by Jácome *et al.* (2016) [23].

ASD manifests early in childhood and its diagnosis is built on behavioral deficits such as decreased social activities, increased repetitive behaviors, and restricted interests [32]. The immune system has a significant role in the development of central as well as peripheral nervous systems. It regulates neuronal proliferation, formation of synapse, and plasticity, together with eliminating apoptotic neurons, and actively contributes in various neurological activities [33].

Table 4. Correlation between Childhood Autism Rating Scale score and laboratory findings in cases group

Laboratory parameters	*r*	*P*
Absolute lymphocyte count (×10^9/l)	0.181	0.209
CD3%	0.395	0.005*
Absolute CD3 (×10^3/μl)	0.264	0.064
CD16%	0.098	0.499
Absolute CD16 (×10^3/μl)	0.317	0.025*
CD19%	0.029	0.842
Absolute CD19 (×10^3/μl)	0.040	0.781
IgA (g/l)	-0.224	0.118
IgM (g/l)	-0.093	0.519
IgG (g/l)	0.188	0.192

*Significant compared with controls (by Pearson correlation).

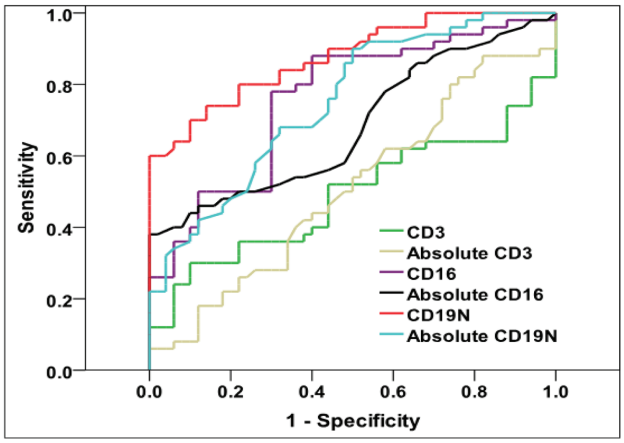
Immune system and neuroinflammation were found to play an essential role in development of ASD, in spite of particular attention about whether it causes ASD onset or regulates symptoms and pathogenesis [34].

Several studies have noted altered immune responses in ASD [35]. Several people with ASD commonly complain of infections as well as allergies, asthma, and seizures. Persistent intestinal yeast infections and unexplained skin rashes have been also recognized [36,37]. Likewise, individuals diagnosed with ASD frequently show signs of immune tolerance loss in the form of allergies and altered response to vaccines and/or infections in addition to undiagnosed autoimmune disorders [38-40]. The association of the immune system in the neurobiological pattern of ASD is now an area of intensive research.

When Pearson correlation coefficients were calculated (Table 4), it was apparent that both CD3% and absolute CD16 count were significantly positively correlated with CARS score in children with ASD [41] (P=0.005 and 0.025, respectively). This is in agreement with Ashwood *et al.* [42], who found a significant increase of total number of NK cells in children with ASD with both high and low IQ.

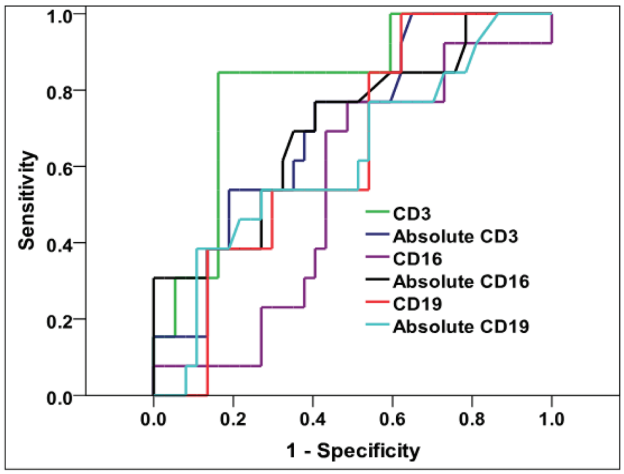
Using ROC analysis, we observed that CD19 had the highest diagnostic performance and characteristics in differentiating case group from control group with an area under the curve (AUC) value of 0.876 (95% confidence interval 0.810–0.942), with a sensitivity of 74.0%, a specificity of 86% (P<0.001), and cut off point less than or equal to 11.35 (Fig. 1). In addition, CD16 has an AUC value of 0.76 (95% confidence interval 0.665–0.854), a sensitivity of 88.0%, and a specificity of 60% (P<0.001), and a cutoff point more than or equal to 8.59 (Fig. 1).

Figure 1



ROC curve for CD cells to differentiate case group from control group. ROC, receiver operating characteristic.

Figure 2



ROC curve for CD cells to differentiate severe grade from mild/moderate grades. ROC, receiver operating characteristic.

ROC curve regarding severity differentiation presented that CD3 had high diagnostic performance and characteristics in differentiating severe grade from mild/moderate grades with an AUC value of 0.813 (95% confidence interval 0.681–0.944), a sensitivity of 84.6%, a specificity of 83.8% (P<0.001), and cut off point more than or equal to 61.52 (Fig. 2). Preceding studies have stated changes in Ig levels among individuals with ASD. Nevertheless, these reports are debatable, and no strong consensus has been adopted. IgG and IgM are either increased [43] or decreased [44] in individuals with ASD compared with healthy controls.

Conclusion

Individuals with ASD frequently have significant immune dysfunction and neuroinflammation. T

lymphocytes and natural killer cells are significantly positive correlated with CARS score in children with ASD, and these defects affect the neurological and behavioral changes in ASD, which are a key component of the diagnosis and treatment of ASD. Therefore, we recommend patients with ASD to add immune profile measurements to their laboratory investigations.

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Conflicts of interest

There are no conflicts of interest.

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