

## Original article

# Frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) in peripheral blood of pediatric patients with SARS CoV-2 Infection: a pilot study

**Background:** Cytokine storm has been observed in some patients with SARS-CoV-2 due to excessive pro-inflammatory response. Foxp3<sup>+</sup> regulatory T cells (Tregs) are a distinct population of CD4<sup>+</sup> lymphocytes identified by their expression of transcription factor forkhead homeobox protein-3 (Foxp3). These cells down-regulate immune responses in inflammatory and autoimmune diseases. **Objective:** This pilot study was aimed to investigate the levels of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in children with SARS-CoV-2. **Methods:** frequency of Tregs was measured by flow cytometry in 20 patients with SARS-CoV-2, 6 months to 15 years old; 6 had COVID-19 and 14 had multisystem inflammatory syndrome in children (MIS-C). They were compared to 20 age- and sex-matched healthy children as a control group. **Results:** There was no significant difference between patients with SARS-CoV-2 infection and healthy control children in the frequency of Tregs ( $P=0.068$ ). Decreased numbers of Tregs was found in only 10% of SARS-CoV-2 patients. Patients with severe SARS-CoV-2 were comparable to those with moderate disease in terms of Tregs' levels. The frequency of Tregs correlated negatively with neutrophil counts in our series ( $p=0.036$ ). Attempts of correlation with other inflammatory markers of SARS-CoV-2 were insignificant. **Conclusion:** Decreased levels of Tregs were found in only 10% of our SARS-CoV-2 infected children. The frequency did not correlate with the disease severity or levels of routine inflammatory markers of SARS-CoV-2. Thus, Tregs expression does not seem to have a role in the up-regulated immune response seen in moderate and severe SARS-CoV-2 infection. Our conclusions are limited by the sample size.

Keywords: CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs, COVID-19, MIS-C, SARS-CoV-2.

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## INTRODUCTION

Clinical evidence indicates that the mortalities observed with severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) infection often result from alveolar injury that restricts airway capacity and may provoke multi-organ failure. Both of which are associated with hyperproduction of cytokines, which in turn, triggers excessive immune reaction and multisystem hyperinflammation. This immune hyper-reaction is known as a cytokine storm or cytokine release syndrome. Thus, early diagnosis and treatment of cytokine storm are of crucial importance. Therapies that target the cytokine excessive production and curtail cytokine storm in SARS-CoV-2 infection have become a focus of recent clinical trials.<sup>1,2</sup>

T cells are classified into 3 main groups of cells: CD8<sup>+</sup> cytotoxic T lymphocytes, CD4<sup>+</sup> T cells and

memory T cells. CD4<sup>+</sup> T cells have been also subtyped, according to their specific cytokine profiles, into 2 essential lines: CD4<sup>+</sup> helper T (Th) lymphocytes, which in turn have subdivisions (Th1, Th2, Th9, Th17, Th22 and follicular Th cell subsets), and CD4<sup>+</sup> regulatory T lymphocytes (Tregs) which express the biomarkers CD4, FOXP3, and CD25.<sup>3,4</sup>

Tregs function in 2 major ways. They serve to modulate immunosuppression with an active regulatory role in peripheral tolerance mechanisms<sup>5,6</sup> and they also participate in the resolution and repair of damaged tissue through the expression of the growth factor amphiregulin.<sup>7,8,9</sup> Adult patients who suffer from acute respiratory distress syndrome were found to have numerous Tregs in the bronchoalveolar lavage.<sup>10</sup>

Clear guidance for the identification and analysis of Tregs is lacking. It has been suggested that the most reliable method for the whole Treg compartment identification is the flow cytometry

analysis of a pattern of specific surface antigens.<sup>4,11</sup> Transcription factor Foxp3, which is known to be specifically expressed by the CD4<sup>+</sup>CD25<sup>+</sup> Tregs, coordinates Treg development and functions.<sup>12</sup> CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells are classified, based on their development, into naturally occurring thymus-derived Tregs (tTregs or natural Tregs) and peripherally-derived Tregs (pTregs, induced or adaptive Tregs). Natural Tregs development is a part of the process of T cell maturation inside the thymus, which occurs when the T cell receptors get exposed and engaged to self-antigens. These cells play a key role in the maintenance of self-tolerance. Conversely, pTreg cells are generated predominantly at peripheral lymphoid organs after antigen priming during infections and inflammation. This can explain the pivotal role of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in preventing autoinflammatory and autoimmune diseases.<sup>13,14</sup> Systemic lupus erythematosus is an example of autoimmune diseases associated with low levels of Tregs.<sup>15</sup>

Based on the previously mentioned, Treg immunotherapy has become a legitimate quest to block unwanted immune responses in autoimmune and inflammatory diseases and to rebuild immune tolerance during severe viral pneumonia. Moreover, Tregs could promote tissue repair which does not depend on the immunosuppressive activity of Tregs.<sup>16</sup> Taken together; these biological characteristics suggest that Treg cells may have multiple antiviral protection mechanisms and Treg therapy may be one strategy for the treatment of the novel SARS-CoV-2 infection though.<sup>17</sup>

This study aimed to investigate the frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in patients with SARS-CoV-2 infection, including those with multisystem inflammatory syndrome in children (MIS-C), in comparison to healthy control children. The relationship between the number of these cells and both the disease severity and inflammatory markers was studied.

## METHODS

### Study population

This cross-sectional pilot study was conducted on 20 Egyptian children with confirmed COVID-19 as defined by the Centre for Disease Control and Prevention (CDC) Case definition for COVID-19<sup>18,19</sup> over a period of 6 months from the beginning of May 2020 to the end of October 2020. Patients fulfilling the criteria for the diagnosis of MIS-C<sup>20</sup> were included. Patients were recruited from the Emergency Department, COVID-19 Isolation Section and Pediatric ICU at Children's Hospital,

Ain Shams University, Cairo, Egypt. Confirmed cases were defined as cases meeting confirmatory laboratory evidence which is the detection of SARS-CoV-2 RNA, in a clinical specimen, by using the molecular amplification detection test. We diagnosed COVID-19 from the date of first positive SARS-CoV-2 PCR swab. Based on clinical data and basic laboratory workup results, the degree of the disease severity was identified.<sup>18</sup>

### Exclusion Criteria:

- Patients with chronic inflammatory diseases, rheumatic diseases, or other autoimmune disorders.
- Patients who had malignancies.
- Patients on corticosteroid therapy or other immune-modulatory drugs and patients who have received intravenous immunoglobulins.

The patients were compared with 20 age- and sex-matched healthy siblings of children attending the outpatients pediatric clinic of the same hospital. They had no clinical evidence of a recent infection, previous COVID-19 infection, or any of the above exclusion criteria.

An informed written consent for participation in this study was signed by the parents or legal guardians of the study subjects. This work was approved by the local Ethical Committee of the Faculty of Medicine, Ain Shams University, Cairo, Egypt.

### Study Measurements

**Clinical evaluation of the studied children:** This included the following:

- Detailed history taking including contact with a COVID patient, symptoms at disease onset, duration of COVID illness, presence of fever, respiratory symptoms, gastrointestinal symptoms, anosmia, ageusia, skin rash and symptoms of organ dysfunction. Therapeutic interventions in the hospital were recorded.
- General and systematic clinical examination were done with emphasis on skin rash, signs of respiratory distress and other organ involvement. O<sub>2</sub> saturation in room air was measured by pulse oximetry.
- Outcome assessment: All patients were assessed on discharge from the hospital. They were classified according to their fate into patients with complete cure, patients with residual illness in one or more of the major organs and those who unfortunately died.

### Routine investigations of SARS-CoV-2:

- Complete blood picture (CBC): using coulter counter (Coulter MAXMUG- HL -CCI) and

Leishman-stained peripheral blood film examination for differential white blood cell counting.

- C reactive protein (CRP): using "Latex agglutination test".
- Erythrocyte sedimentation rate (ESR): using the "Westergren Method".
- Lactate dehydrogenase enzyme (LDH), liver enzymes, serum creatinine, serum ferritin and serum fibrinogen using Synchron CX7 autoanalyzer (Beckman Instruments, Brea, California, USA). D-Dimer was measured by ELISA
- When indicated, creatine Kinase (CK), CK-MB, Troponin-I (measured by ELISA), PT, INR, and PTT were done.

**Imaging studies:**

- Echocardiography: to detect the possible presence of endocarditis, myocarditis, pericarditis, valvular lesions, coronary vasculitis, or any other changes related to COVID-19 or MIS-C.
- CT scanning of the chest: The findings were classified according to COVID-19 Reporting and Data System (CO-RADS).<sup>21</sup>

**Assessment of the frequency of CD4+CD25+Foxp3+ Tregs:**

Ten ml of peripheral blood were withdrawn under aseptic conditions from patients and controls in heparinized vacutainers for peripheral blood mononuclear cells (PBMCs) isolation. PBMCs were isolated by Ficoll density gradient centrifugation and cells were washed with eBioscience™ Flow Cytometry Staining Buffer (Invitrogen; Thermo Fisher Scientific, Inc.), then, stained for surface markers using anti-CD4 phycoerythrin (PE), anti-CD25 allophycocyanin (APC) and anti-CD127 phycoerythrin Cy7(PE-Cy7) for 30 minutes at room temperature (BD Biosciences, San Diego, USA). Subsequently, the cells were fixed, permeabilized with 1X permeabilisation buffer (eBioscience, CA, USA), followed by intracellular staining with anti-FoxP3 antibodies (eBiosciences, CA, USA) for 30 minutes at 4°C. Foxp3 expression in CD4+CD25+ Treg cells was analyzed by flow cytometry.<sup>22,23</sup>

**Statistical Analysis:**

The results were analyzed by using the available software package (Statview, Abacus concepts, inc., Berkley, CA, USA). The normally distributed data were presented as mean and standard deviation (SD) and compared using the student's t-test. The non-parametric data were presented as median and interquartile range (IQR) that is between the 25<sup>th</sup>

and 75<sup>th</sup> percentiles and compared using the Mann–Whitney test. Chi-Square test was used to compare qualitative variables. Spearman's rho correlation coefficient "r" test was used to determine the relationship between different variables. A probability (P) value of less than 0.05 was considered to be significant for all the tests. Patients were considered to have elevated frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs if their levels were above the calculated highest cut-off values (the 95<sup>th</sup> percentiles of the healthy controls).

**RESULTS**

COVID-19 patients comprised 12 males and 8 females. Their ages ranged between 6 months and 15 years (mean ± SD = 6.28±5.35 years). The control group comprised 20 age and sex- matched apparently healthy children. They included 11 males and 9 females. Their ages ranged between 6 months and 16 years (mean ± SD =6.25 ±5.21years). Six patients had COVID-19 infection and the other 14 suffered from MIS-C. Eight patients had moderate disease, whereas 12 had severe disease and were admitted to the Pediatric ICU. Only 2/6 patients (33.3%) with COVID-19 had severe disease compared to 10/14 (71.4%) of patients with MIS–C and this was highly significant (P<0.001). Out of the 20 patients, one presented without organ dysfunctions and 19 patients had single (n=6) or multiple (n=13) organ dysfunctions in the form of cardiac, respiratory, gastrointestinal or neurological dysfunctions. Regarding the outcome of the disease, 17 patients recovered and 3 patients with MIS–C died.

According to the CO-RADS radiological classification of COVID-19 infection,<sup>21</sup> four patients had CO-RADS 1, three patients have CO-RADS 2, three patients had CO-RADS 3, five patients had CO-RADS 4, four patients had CO-RADS 5, one patient had CO-RADS 6.

Results of the basic clinical and routine laboratory data of the studied patients were presented in table 1.

Although all patients had higher frequency of Tregs than healthy control children, this elevation was not significant (P=0.068, P=0.062 and P= 0.20, respectively), table 2, figure 1. Decreased frequency of Tregs was considered in the patients if their levels were below 0.22 which was the calculated lowest cut-off value (the 5th percentile of the healthy controls). However, patients were considered to have increased frequency of Tregs if their levels were above 1.1155 which was the calculated highest cut-off value (the 95th percentile of the healthy controls). Decreased frequency of

Tregs was found in only 10% of patients whilst increased frequency of Tregs was found in 30% of patients.

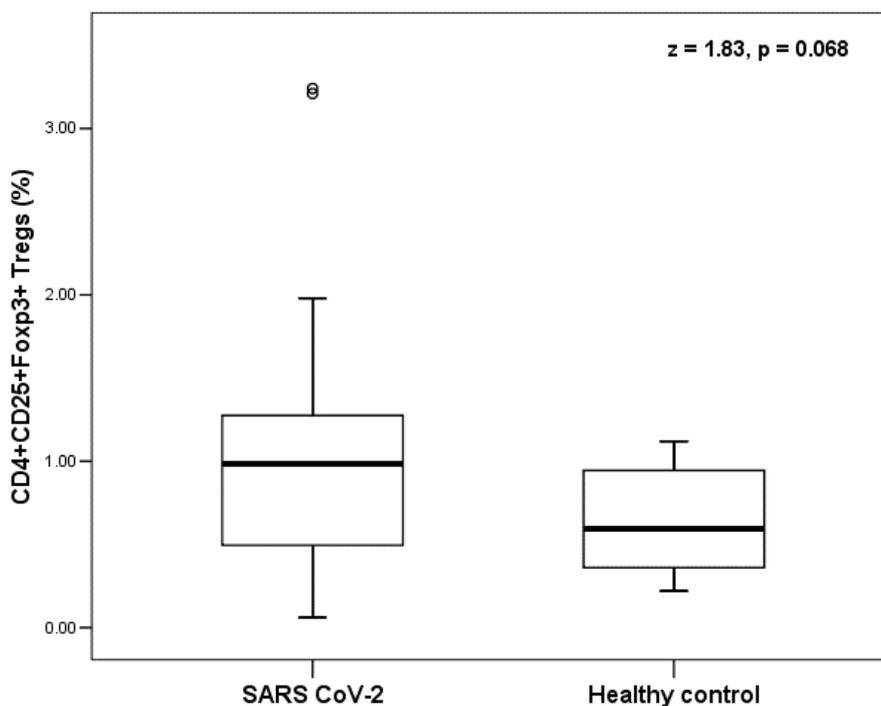
According to the pediatric normal reference range for age, elevated CRP and D-Dimer values were found in 85% and 90% respectively of these patients. Lymphopenia, neutropenia, neutrophilia, thrombocytopenia and elevated levels of ALT, ferritin and LDH were found in 30%, 10%, 60%, 30%, 20%, 45% and 70%, respectively of all COVID-19 patients.

There were non-significant differences between patients with severe and patients with moderate SARS-CoV-2 infection in the frequency of Tregs and levels of total leucocytic count, neutrophils, lymphocytes, hemoglobin, platelets, CRP, ferritin,

ALT, LDH, ESR, CK, CKMB and serum creatinine. Meanwhile, patients with severe SARS-CoV-2 infection had significantly higher values of D-dimer than patients with moderate COVID-19 (table 3).

There were non-significant differences between patients with MIS-C and patients with COVID-19 in the frequency of Tregs [median (IQR): 0.855 (1.1) and 1.035 (1.06), respectively],  $z = 0.742$  and  $P = 0.494$ .

There was a significant negative correlation between the frequency of Tregs and the absolute neutrophilic count in the studied patients (figure 2). However, this was not the case with the other inflammatory markers of SARS-CoV-2 infection (table 4).



**Figure 1.** The frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in all patients with COVID-19 versus healthy controls. The boxes enclose the interquartile ranges (IQR) which are between the 25<sup>th</sup> and the 75<sup>th</sup> percentiles. The horizontal line inside the box represents the median and the whiskers represent the non-outlier or extreme maximum and minimum values. The small open circles represent the outlier values (between 1.5 and 3 IQR).

**Table 1.** Basic clinical and laboratory data of the studied patients.

		All patients with SARS CoV-2 (n=20)	Patients with COVID-19 (n=6)	Patients with MISC (n=14)
<b>Age (years)</b>	Range	0.5-15		
	Median (IQR)	4.5 (10.5)	3.5 (9)	6 (10.3)
	Mean±SD	6.28±5.35		
<b>Sex</b>	Female	8	1	7
	Male	12	5	7
<b>Organ Dysfunction</b>	Multiple	13	0	13
	Single	6	5	1
	Absent	1	1	0
<b>Outcome</b>	Recovered	17	6	11
	Died	3	0	3
<b>TLC</b> (x10 <sup>3</sup> /μL)	Range	4-32.7	4.4-25.2	4-32.7
	Median (IQR)	15.75 (10.4)	14.75 (13.2)	16.2 (9.7)
<b>ANC</b> (x10 <sup>3</sup> /μL)	Range	3-30	3-17	5-30
	Median (IQR)	8 (6)	8 (8)	7.9 (7)
<b>ALC</b> (x10 <sup>3</sup> /μL)	Range	1-12	1-8	1-12
	Median (IQR)	3.4 (5)	4.05 (4)	2.45 (7)
<b>Hemoglobin</b> (g/dl)	Range	8-14	9-13	8-14
	Median (IQR)	10.85 (3)	10.65 (3)	10.95 (3)
<b>Platelets</b> (x10 <sup>3</sup> /μL)	Range	69-916	148-916	69-722
	Median (IQR)	320 (296)	429 (317)	226 (248)
<b>CRP titer</b> (mg/l)	Range	2.5-370.7	5.7-127	2.5-370.7
	Median (IQR)	31 (189.7)	20.95 (46.1)	108.05 (265.6)
<b>Ferritin</b> (ng/ml)	Range	34-495	65-252	34-495
	Median (IQR)	124 (236)	94 (94.8)	169.5 (353.3)
<b>ALT</b> (IU/L)	Range	5-1720	5-69	11-1720
	Median (IQR)	21 (51)	11 (24)	30.5 (59)
<b>LDH</b> (IU/L)	Range	3-3517	3-481	147-3517
	Median (IQR)	400.5 (735)	244 (186)	529 (904)
<b>D-dimer</b> (ug/mlF)	Range	0.1-35	0.1-12.1	0.8-35
	Median (IQR)	4.28 (9.5)	1.55 (4.4)	6.570 (15.6)
<b>ESR</b> (mm/hr)	Range	14-90	25-61	14-90
	Median (IQR)	45 (33.8)	46.5 (31.5)	45 (36.3)
<b>CK-T</b> (IU/L)	Range	22-12731	27-258	22-12731
	Median (IQR)	71 (108)	68 (112)	71 (145)
<b>CK-MB</b> (U/L)	Range	13-353	14-40	13-353
	Median (IQR)	20 (18)	16 (11)	24 (20)
<b>Creatinine</b> (mg/dL)	Range	0.4-3.6	0.4-.6	0.4-3.6
	Median (IQR)	0.55 (0.3)	0.5 (0.1)	0.65 (0.4)

ALC: absolute lymphocytic count, ALT: Alanine transaminase, ANC: absolute neutrophil count, CK-MB: creatinine kinase-MB, CK-T: creatinine kinase- total, COVID 19: Corona virus disease 2019, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, IQR: Interquartile range, LDH: Lactate dehydrogenase, MIS-C: Multisystem inflammatory syndrome in children, SARS CoV-2: severe acute respiratory syndrome coronavirus-2, TLC: total leucocytic count, Tregs: T regulatory lymphocytes.

**Table 2.** Comparison of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs frequency of patients and healthy controls.

The studied children	The frequency of Tregs (%)		Z	P-value
	Range	Median (IQR)		
All patients with SARS CoV-2 (n=20)	0.06-3.24	0.985 (0.86)	1.826	0.068
Healthy controls (n=20)	0.22-1.12	0.595 (0.6)		
Patients with COVID-19 (n=6)	0.25-3.24	1.035 (1.06)	1.856	0.062
Healthy controls (n=20)	0.22-1.12	0.595 (0.6)		
Patients with MIS-C (n=14)	0.06-3.21	0.858 (1.1)	1.295	0.204
Healthy controls (n=20)	0.22-1.12	0.595 (0.6)		

COVID 19: Corona virus disease 2019, MIS-C: Multisystem inflammatory syndrome in children, SARS CoV-2: Severe acute respiratory syndrome coronavirus-2, Tregs: T regulatory lymphocytes.

**Table 3.** Inflammatory markers of COVID-19 and the frequency of CD4+CD25+Foxp3+ Tregs in patients with moderate versus severe SARS CoV-2.

Laboratory markers	Patients with moderate SARS-CoV-2 (n=8)		Patients with severe SARS-CoV-2 (n=12)		Test value t*/ z	P-value
	Range	Mean ±SD/ Median (IQR)	Range	Mean ±SD/ Median (IQR)		
<b>TLC</b> (x10 <sup>3</sup> /μL)	4-25.3	13.1±7.55	6.9-32.7	16.558±7.391	1.017*	0.323
<b>ANC</b> (x10 <sup>3</sup> /μL)	3-12	8.11±3.338	5-30	11.14±7.411	1.076*	0.296
<b>ALC</b> (x10 <sup>3</sup> /μL)	1-12	4.64±4.022	1-11	4.21±3.57	.248*	0.807
<b>Hemoglobin</b> (g/dl)	9-14	11.49±1.828	8-13	10.47±1.294	1.468*	0.159
<b>Platelets</b> (x10 <sup>3</sup> /μL)	74-530	292.25±196.72	69-916	350±243.1	0.559*	0.583
<b>CRP titer</b> (mg/l)	11-334.4	25.5 (178.4)	2.5-370.7	63 (239.7)	0.00	1.00
<b>Ferritin</b> (ng/ml)	34-183	75 (91.8)	57-495	212.5 (326)	1.312	0.208
<b>ALT</b> (IU/L)	5-69	15 (22)	11-1720	26.5 (60)	1.737	0.082
<b>LDH</b> (IU/L)	201-494	282.5 (215)	3-3517	588 (928)	1.852	0.069
<b>D-dimer</b> (ug/mIF)	0.1-30	1.45 (6.4)	1.8-35	6.57 (12.1)	<b>2.16</b>	<b>0.03</b>
<b>ESR</b> (mm/hr)	24-55	37.75±12.068	14-90	53.1±23.8	1.585*	0.115
<b>CK-T</b> (IU/L)	27-264	81 (63)	22-12731	63.5 (200)	0.386	0.734
<b>CK-MB</b> (U/L)	14-33	19 (15)	13-353	24 (31)	0.851	0.427
<b>Creatinine</b> (mg/dL)	0.4-1	0.5 (0.2)	0.4-3.6	0.65 (0.4)	1.702	0.098
<b>The frequency of Tregs</b>	0.45-3.24	0.885 (0.55)	0.06-3.21	0.985 (1.55)	0.463	0.678

P>0.05 : non significant, P<0.05: significant

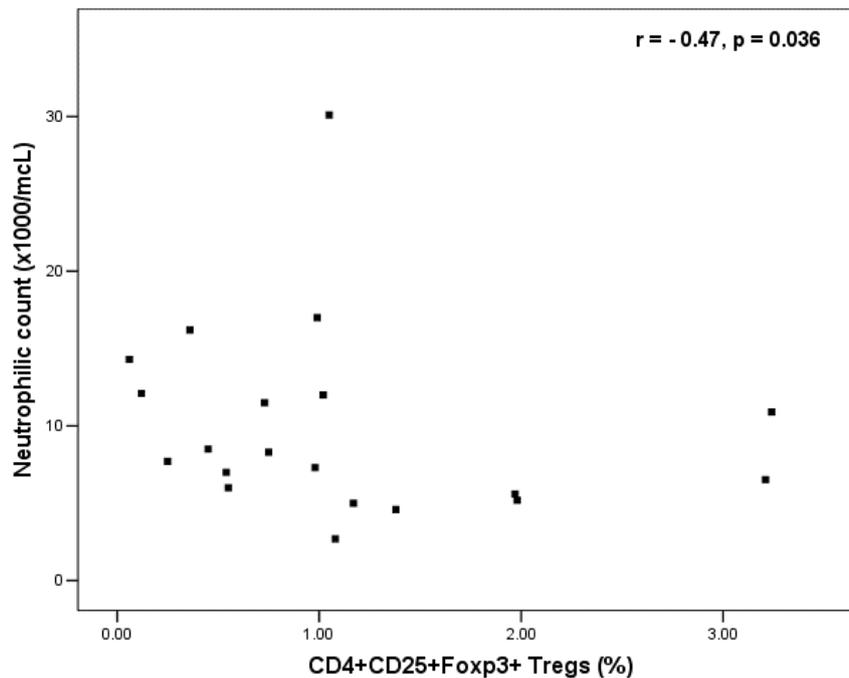
ALC: absolute lymphocytic count, ALT: Alanine transaminase, ANC: absolute neutrophil count, CK-MB: creatinine kinase-MB, CK-T: creatinine kinase- total, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, IQR: Interquartile range, LDH: Lactate dehydrogenase, SARS CoV-2: severe acute respiratory syndrome coronavirus-2, TLC: total leucocytic count, Tregs: T regulatory lymphocytes.

**Table 4.** Correlation between the frequency of CD4+CD25+Foxp3+ Tregs and the different studied parameters in all COVID-19 patients.

	The frequency of Tregs	
	r	P-value
<b>Age</b>	-0.082	0.73
<b>TLC</b>	-0.171	0.47
<b>ANC</b>	<b>-0.471</b>	<b>0.036</b>
<b>ALC</b>	-0.032	0.895
<b>Hemoglobin</b>	-0.266	0.257
<b>Platelets</b>	0.335	0.148
<b>CRP titer</b>	0.367	0.112
<b>Ferritin</b>	-0.003	0.99
<b>ALT</b>	-0.017	0.945
<b>LDH</b>	-0.157	0.508
<b>D-dimer</b>	-0.014	0.995
<b>ESR</b>	0.289	0.216
<b>CK-T</b>	-0.305	0.191
<b>CK-MB</b>	-0.267	0.255
<b>Creatinine</b>	-0.333	0.151

P>0.05: not significant, P<0.05: significant.

ALC: absolute lymphocytic count, ALT: Alanine transaminase, ANC: absolute neutrophil count, CK-MB: creatinine kinase-MB, CK-T: creatinine kinase- total, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, IQR: Interquartile range, LDH: Lactate dehydrogenase, TLC: total leucocytic count, Tregs: T regulatory lymphocytes.



**Figure 2.** A significant negative correlation between the frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs and the neutrophil count in all COVID-19 patients.

## DISCUSSION

Un-controlled and up-regulated immune response has been observed in some patients with SARS-CoV-2 infection. Tregs are negative modulators of the immune response to antigens in many inflammatory conditions.<sup>1,2,24</sup> We hypothesized that, alike what happens in some other autoimmune diseases,<sup>15</sup> children with moderate and severe SARS-CoV-2 infection might have reduced frequency of Tregs, which in turn, facilitates and augments the associated up-regulated immune response. and would solve the mystery of the excessive pro-inflammatory host response in these patients.

In the current study, there were non-significant differences between COVID-19 patients and healthy control children in the frequency of Tregs (P=0.068). In addition, decreased frequency of Tregs was found in only 10% of patients with COVID-19. We couldn't trace data in literature to compare our results as this is the first study that investigated the frequency of Tregs in COVID-19 patients with. Thus, decreased frequency of Tregs may not have a role in the occurrence of the up-regulated immune response in the majority of patients with moderate and severe COVID-19 who required hospitalization.

Interestingly, increased frequency of FOXP3 Tregs was detected in 30% of the studied COVID-19 patients. Tregs-associated inhibitory cytokines

such as TGF beta, IL-10 and IL-35 are known for their role in recruiting more Tregs<sup>24</sup> in a trial to downregulate the immune responses in many autoinflammatory and autoimmune diseases.<sup>5,6</sup> Nevertheless, Tregs play a key role in modulating immune reactions and motivating immunosuppression which, in turn induces the resolution and repair of damaged tissue. Part of that immunosuppressive effect occurs through Treg-expressed molecules such as CD39, CD73, CTLA-4, galectin, glucocorticoid-induced TNFR-related protein, granzyme-B, IL-10, IL-35, lymphocyte-activation gene 3, neuropilin, perforin and TGF- $\beta$ .<sup>9,25,26,27</sup> Considering the mentioned characteristics of Tregs, combined with the timing of showing up of the infected children to hospitals, it is not surprising to detect increased frequency of Tregs in some infected children with SARS-CoV-2; as an attempt to down-regulate the exaggerated inflammatory response in those patients.

The current study revealed non-significant differences between patients with severe and patients with moderate COVID-19 in the frequency of Tregs. This might refer to the limited role of Tregs frequency in determining and/or anticipating the degree of COVID-19 severity. Moreover, no significant correlations were found between the frequency of Tregs and the routine inflammatory markers in patients with SARS-CoV-2. These findings may also support our suggestion as regards

the reserved sharing of Tregs peripheral frequency in the marathon of anticipating the severity of disease in children infected with SARS-CoV-2.

Neutrophils have long been considered as a crucial player in the immune defense against invading pathogens. Accumulating evidence strongly supported the direct and indirect regulatory effects of neutrophils on adaptive immunity.<sup>28</sup> It was impressive to find in the current study, that there was a significant negative correlation between the frequency of Tregs and the absolute neutrophilic count in children infected with SARS-CoV-2. CD4+CD25+Foxp3+ Tregs have an inhibitory impact on chronic inflammatory as well as adaptive immune responses. This suppressive effect is achieved through inhibiting neutrophil accumulation and survival due to restricted expression of the neutrophil chemo-attractants CXCL1 and CXCL2.<sup>29</sup> This may explain the negative correlation between the frequency of Tregs and the neutrophilic count in our studied COVID-19 patients.

The peripheral frequency of Tregs was comparable in children who had COVID-19 and those who had MIS-C. The degree of the disease severity was not the same in both conditions. However, as patients with MIS-C had a significantly higher degree of disease severity than patients with COVID-19 ( $P < 0.001$ ). This adds strength to our previous notion that peripheral Treg frequency is not of value as COVID-19 severity determinants.

In this study, according to the pediatric normal reference range for age, elevated D-Dimer, CRP and LDH values were found in 90%, 85% and 70%, (respectively) of COVID-19 patients, highlighting their role as markers of severe COVID-19 in hospitalized patients. Abnormalities of other laboratory markers were less frequently observed. It is more likely that the course of the disease will be unfavorable if some or all of these parameters are altered.<sup>30</sup>

In the present work, not only was D-dimer elevated in most of the patients but also patients with severe COVID-19 had significantly higher values of D-dimer than those with moderate COVID-19. COVID-19 is a systemic, hypercoagulable disease. Anticoagulation is still an issue to be studied in pediatric ages, however, the parallelism between the level of D-dimer and the disease severity is to be considered as an indicative factor to start potent and effective treatment as early as possible.<sup>31</sup> The level of D-dimer was widely used and interpreted as an indicator of thrombosis.

Studies have reported an increase of the D-dimer level in the early stages of COVID-19; and they linked a 3 to 4-fold rise in D-dimer levels to poor prognosis. Measuring the level of D-dimer and coagulation parameters at the early stages of the disease can be valuable in controlling and managing of COVID-19.<sup>32</sup>

Similarly, elevated CRP values were noticeable in most of our series regardless of the severity of the disease. A unique interaction between SARS-CoV-2 and the immune system has led to diverse clinical manifestations of the COVID-19 disease. While adaptive immune responses are essential for SARS-CoV-2 clearance, the innate immune cells, such as macrophages, may contribute, in some cases, to the disease progression. Macrophages have shown a significant production of IL-6; thus, they may contribute to the excessive inflammation in COVID-19 disease. Macrophage activation may explain the high serum levels of CRP, which are normally lacking in viral infections.<sup>33</sup>

The breakdown of the Treg-immune control has been linked to a reduced frequency of the Tregs, the impairment of the Treg's suppressive function, as well as the enhanced reactivity and resistance to the self-reactive effector T cell regulatory. IL-6 which is known to be up regulated in COVID-19 has a role in inhibiting or subverting Tregs function.<sup>24</sup> Thus, Studies, on large scales, to investigate the relationship between Tregs function and SARS-CoV-2 are required.

A major limitation of this study is the small number of the studied children, so we could not investigate the relationship between the frequency of Tregs and the occurrence of organ dysfunction, or their impact on the outcome of SARS-CoV-2 infection.

In conclusion, decreased frequency of Tregs was found in only 10% of patients with COVID-19. In addition, the frequency of these cells neither correlated with the disease severity nor the routine inflammatory markers of SARS-CoV-2. Thus, decreased frequency of Tregs may not have a role in the occurrence of the up-regulated immune response in most patients with moderate and severe COVID-19 who needed hospitalization. Studies, on large scales, to investigate the relationship between Tregs function and SARS-CoV-2 infection, including the disease severity, the occurrence of organ dysfunction and the disease outcome, are required.

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