RESEARCH ARTICLE

# Altered serum pro-inflammatory cytokines in children with Down's syndrome

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**ABSTRACT. There are reports showing that pro-inflammatory cytokines are dysregulated in patients with Down's syndrome (DS). However, most of these reports concern adults. We analyzed cytokine levels in serum samples from children with DS, and compared them with samples from intellectually disabled (ID), and healthy, control children.Blood samples were collected from 24 DS, 24 age-/sex-matched ID, and 24 age-/sex-matched healthy, control children. Serum levels of the cytokines IL-5, IL-10, IL-13, IFN-γ, and TNF-α were measured using a sandwich ELISA method, .The age range of the children was 1-15 years, with a mean ± SD of 5.75 ± 4.36 years.TNF-α levels were significantly higher in the DS and ID groups compared with those found in healthy, control children ($P<0.05$). The DS and ID groups had significantly higher IFN-γ levels compared with healthy, control children ($P = 0.0002$ and $P<0.01$, respectively), with significant higher levels in the DS than the ID group ($P<0.05$). Serum from the ID group showed significantly higher IL-10 levels compared with those from the DS group ($P<0.05$), but not the healthy, control group. Significant correlations were found between the differences in TNF-α and IFN-γ levels, in both ID ($rs = 0.558$; $P = 0.005$) and DS children ($rs = 0.405$; $P<0.05$).There were no significant differences found in serum levels of IL-13 between the groups, and IL-5 was not detectable in any of the serum samples.Levels of TNF-α and IFN-γ were increased, and IL-10 decreased in serum from children with DS. It may be that these differences contribute to the clinical symptoms seen in DS: consequently, these pro-inflammatory cytokines might be useful as early biomarkers of the disorders associated with DS.**

**Key words:** Down's syndrome, pro-inflammatory cytokines, TNF-α, IFN-γ

Down's syndrome (DS) is the most common chromosomal disorder. It is characterized by the presence of an extra copy of chromosome 21 (trisomy 21), which is associated with intellectual disability amongst other things. It is estimated that the incidence of Down's syndrome is 1 in every 700-1,000 birth world-wide [1]. Different clinical manifestations are associated with DS including heart defects, alterations of the immune system, increased susceptibility to infections, high risk of developing hematological malignancies, phenotypic anomalies, and an Alzheimer-like disease developing many years earlier than seen in the general population.

Changes in certain aspects of the humoral and cellular immune systems in DS have been suggested. Functional impairment of B and T lymphocytes and natural killer cells, and dysfunction of phagocytosis and chemotaxis of polymorphonuclear leukocytes have been shown in different studies [2, 3]. The total number of $CD4^+$ T cells is within the normal range, but $CD4^+$ subsets might be changed and the cytotoxic CD8+ T cell fraction might be increased [4]. B lymphocyte abnormalities also have been demonstrated associated with abnormal levels of IgG and IgM in serum [5]. Ineffective immune responses in DS lead to recurrent viral/bacterial infections and contribute to the development of various pathophysiological symptoms including cognitive impairment [6-8].

Chromosome 21 of human and chromosome 16 of mice carry genes that are involved in the function of the interferon family of cytokines and receptors[2]. Overexpression of chromosome 21-gene products causes changes in levels of inflammatory cytokines in the blood [9]. Two of these inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), have wide-ranging biological effects in the body and important regulatory roles in immune responses. In DS, abnormal pro-inflammatory cytokine production might participate in neuropathological changes such as the Alzheimer's-like mental retardation associated with DS [6, 10]. It is reported that cells of the

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neural system in DS patients are hypersensitive to the anti-cellular effects of IFNs [11] that are usually associated with the cognitive impairment seen in DS patients with increasing the age [12]. While the immune response in DS seems to be influenced by patient age [13], the majority of studies have evaluated the immune response of adult patients with DS. In this study, we investigated the possible early changes in serum proinflammatory cytokine levels in children with DS and compared them with levels found in intellectually disabled (ID) and healthy children. Early changes in proinflammatory cytokine production might be useful as a biomarker of the clinical disorders seen in DS and ID patients.

## DONORS AND METHODS

### *Participants and sample collection*

The study proposal was reviewed and approved by the local ethical committee. Potential candidates or their parents/legal guardians were informed about the study objectives and procedure, and those who were willing to participate and sign an informed consent were included.

Twenty four (F/M = 13/11) participants with DS who were residents of an intellectual disability institute located in Tehran were included. Karyotypes confirmed trisomy 21 in the children who had been clinically diagnosed with DS. As the patient control group, 24 age-/sex- matched children with ID, other than DS, who were living in the same institute were included. As the healthy, control group, 24 age-/sex- matched, healthy children who had been referred to the Children's Medical Center were also included. C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were measured for all control groups.

Serum was separated from blood samples using centrifugation, 800 × g for 10 minutes, which had been collected for routine laboratory check-ups from each participant. Portions of the serum samples were stored at -20°C until use.

### *ELISA*

Serum samples were collected and the levels of interleukin (IL)-5, IL-10, IL-13, IFN-γ, and TNF-α (Mabtech, Stockholm, Sweden) were measured using a sandwich ELISA method. Briefly, the plates were coated with anti-IFN-γ/IL-5/IL-10/IL-13 or TNF-α mAb in PBS, pH 7.4, and incubated at 4°C overnight. After blocking the wells with buffer containing PBS plus 0.05% (v/v) Tween 20 and 0.1% (w/v) bovine serum albumin (BSA), serum samples were added to each well. Biotin-labeled mAb in incubation buffer was added to each well and as the enzyme, streptavidin-horseradish peroxidase (HRP) was used. The reaction was developed using 3,3',5,5'-tetramethyl benzidine (TMB) substrate and stopped with 0.5M $H_2SO_4$ solution. The plates were washed after each incubation step using PBS+0.05% (v/v) Tween 20. The plates were read at 450 nm using a reader (BioTek, Winooski, VT, USA). The mean optical densities (OD) of wells were compared with the standard curves prepared using recombinant IL-5, IL-10, IL-13, IFN-γ, and TNF-α. The cytokine levels represent the differences between the OD of test and background wells. The detection limit of the assays was 4 pg/mL for IL-5, 0.5 pg/mL for IL-10, 5 pg/mL for IL-13, 2 pg/ml for IFN-γ and 13 pg/mL for TNF-α.

### *Statistical analysis*

Non-parametric tests of Mann-Whitney and Kruskal-Wallis, and Dunn's post-test for paired comparisons were used for statistical analysis of the data using the SPSS version 11.5 (SPSS Inc., USA) and GraphPad Prism version 5.01 (GraphPad Software Inc., USA) software. For correlation, the nonparametric Spearman test was used, and *rs* was reported as the correlation coefficient. Nonparametric tests were chosen because the samples did not follow a Gaussian distribution. *P* value of $<0.05$ was regarded as significant.

## RESULTS

### *Demographic and clinical characteristics of participants*

The mean age ± SD was 5.75 ± 4.36 years (range: 1-15 years) for each of the three groups.

All participants in the control group had a negative CRP test and an ESR within the normal range.

The etiology of disabilities and the medical diagnoses of children with ID were as follows: developmental brain abnormalities were recorded in most cases of ID. Approximately 3/4 of the brain abnormalities were due to cerebral palsy (n = 18; 75%), while three cases (12.5%) were related to cephalic disorders including two cases (8.3%) of hydrocephaly and one (4.2%) of schizencephaly. Genetic disorders were diagnosed in two cases (8.3%), and in one case the etiology of the intellectual disability was unknown (4.2%).

### *Cytokine production assay*

Using an ELISA method, IL-5, IL-10, IL-13, IFN-γ, and TNF-α cytokines were measured in serum samples. The TNF-α levels were significantly higher in serum from the DS and ID groups compared with those from the healthy, control group ($P<0.05$) (*figure 1A*). Results of IFN-γ measurements showed that serum from the DS and ID groups had significantly higher IFN-γ levels compared with those found in the healthy control group ($P=0.0002$ and $P<0.01$, respectively). However, there was a significant difference between the DS and ID groups as regards levels of IFN-γ ($P<0.05$); significantly higher IFN-γ levels were seen in the DS group. Serum from the DS group showed significantly lower IL-10 levels compared with the ID, but not the healthy, control groups ($P<0.05$) (*figure 1B*). Levels of IL-13 in serum samples from the three groups were not significantly different. IL-5 was not detectable.The changes in TNF-α levels showed a strong correlation with IFN-γ ($rs = 0.558$; $P = 0.005$), and a strong, inverse correlation with IL-10 ($rs = -0.662$; $P<0.001$) levels in the ID group. The changes in TNF-α levels showed a moderate correlation with IFN-γ levels ($rs = 0.405$; $P<0.05$) in the DS group. Changes in IFN-γ levels showed a strong, inverse correlation with IL-10 levels ($rs = -0.574$; $P = 0.003$) in the DS group.

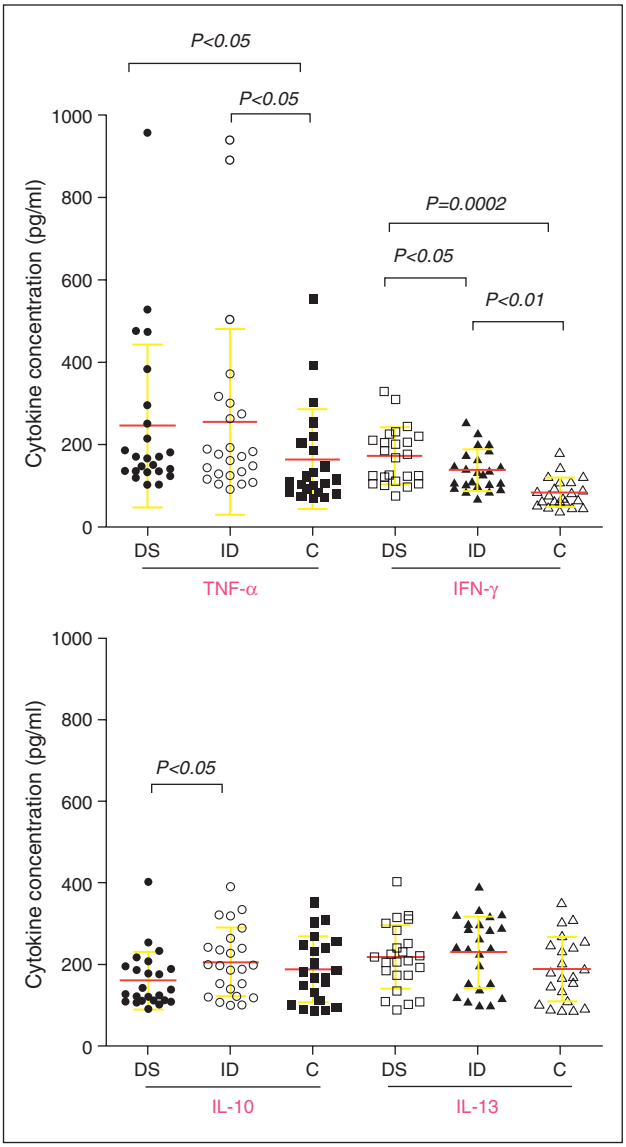


**Figure 1**
**Cytokine production levels in serum samples of children.**
Serum samples were collected and the levels of IL-5, IL-10, IL-13, IFN-γ, and TNF-α were measured using a sandwich ELISA method. The plates were coated with anti-cytokine mAb in PBS, pH 7 4, and incubated at 4°C overnight. After blocking the wells with buffer containing PBS plus 0.05% (v/v) Tween 20 and 0.1% (w/v) bovine serum albumin (BSA), serum samples were added to each well. Biotin-labeled mAb was added to each well and as the enzyme, streptavidin-HRP was used. The reaction was developed using 3,3',5,5'-tetramethyl benzidine (TMB) substrate and stopped with 0.5M $H_2SO_4$ solution. The plates were read at 450 nm and the mean optical densities (OD) of wells were compared with the standard curves prepared using recombinant cytokines. The cytokine levels represent the differences between the OD of test and background wells. Horizontal lines show the mean ± SD of TNF-α and IFN-γ (A) and IL-10 and IL-13 (B) levels from DS, age-/sex- matched ID, and age-/sex- matched healthy control children. DS = Down's syndrome; ID = intellectual disability; C = healthy controls.

## DISCUSSION

The immune system in patients with DS has been shown to be defective. There are reports showing that in DS, pro-inflammatory cytokines are increased, production of IL-2 is decreased [4, 10], and abnormal thymus anatomy is associated with overexpression of IFN-γ and TNF-α.

In this study, we measured serum TNF-α, IFN-γ, IL-10 and IL-13 levels. These are among the main T helper 1 (Th1)/Th2 and inflammatory cytokines. Levels of TNF-α were significantly higher in the DS group compared with those found in the healthy control children ($P<0.05$). Levels of IFN-γ were also significantly higher in the DS group than in the ID ($P<0.05$) and healthy, control ($P = 0.0002$) groups. The changes seen in serum TNF-α levels correlated significantly with those of IFN-γ, in both the ID and DS groups. Higher levels of TNF-α and IFN-γ cytokines in unstimulated cell–culture supernatant from adult DS patients compared with an ID group were shown in a recent experiment [10]. The overexpression of TNF-α and IFN-γ mRNA in the thymus of DS patients has been demonstrated previously using *in situ* hybridization [14]. In agreement with these findings, by intracellular cytokine staining of T cells, a significantly higher number of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells have been detected in peripheral blood samples from DS patients than in those from ID and healthy controls [12]. One study in older individuals (>30 years old) with DS showed different results for pro-inflammatory cytokine production [15]. IL-2 production by phytohemaglutinin (PHA)-stimulated monocytes was significantly decreased in older individuals with DS; levels of IL-1b, IL-2, IL-6, IL-8, and TNF-α were no different from those of age-matched controls [15].

The role of TNF in the regulation of immune cells is known, and the contribution of altered TNF production in various human disorders, including Alzheimer's disease, cancer and depression has been reported [16-18]. It has been shown that cells in DS patients are hypersensitive to the anti-cellular effects of IFNs [11]. The degenerative effects of IFN-γ on neural systems, along with β-amyloid production, has been shown in DS and the trisomy 16 mouse models [19]. These effects usually lead to cognitive impairment in DS patients with increasing the age [12]. *In vivo* administration of anti-IFN antibody to pregnant mice showed improving effects on the development of trisomy 16 fetuses, and *in vitro* use of anti-IFN-γ antibody on cell cultures of cortical neurons of trisomy 16 had inhibitory effects on the premature death of these cells [20].

In the current study, significantly lower levels of IL-10 were found in DS children compared to the ID group ($P<0.05$). Levels of IL-10 were not significantly increased, but TNF-α levels were increased in children with DS. Since it is known that IL-10 has an anti-inflammatory effect that inhibits the production of proinflammatory cytokines (such as IL-6 and TNF-α), so the reciprocal changes in the level of serum IL-10 and TNF-α are understandable in DS children. In contrast to this study, one study reported increased levels of IL-10 and decreased levels of TNF-α in children with DS [4].

Abnormal inflammatory cytokines levels were reported in DS patients with hematologic malignancy [21]. Levels of IL-1β, TNF- α, and IFN-γ were found to be different between DS neonates with or without transient abnormal myelopoiesis (TAM). The study revealed that altered levels of cytokine production might have a role in the development of the liver fibrosis and myelofibrosis seen in hematologic malignancy patients with DS [21].

In DS, the consequences of a defective immune response usually manifest later in life, even though the onset of Alzheimer-like dementia in adults with DS might present many years earlier when compared to the general population. Inflammation is involved in the neurodegeneration

[18], and it is suggested that pro-inflammatory cytokines play a role in Alzheimer's disease, and involve degenerative process of the central nervous system (CNS) in DS [6]. Study of the immune response in children with DS who were not suffering from dementia, *i.e.* before clinical manifestations of cognitive deterioration, has revealed that significantly higher levels of plasma IL-6, soluble IL-6 receptor (sIL-6R), soluble intercellular adhesion molecule-3 (sICAM-3), soluble vascular cell adhesion molecule-1 (sVCAM-1) and CRP were produced than in control children. Similarly increased levels of IL-6 and CRP were reported in aged patients with Alzheimer's disease [9]. A correlation between the degree of mental retardation and IL-6 levels was shown in DS patients [6].

In this study, we analyzed serum cytokine levels in DS children with a mean age of $5.75 \pm 4.36$ years and compared them with age-/sex- matched ID and healthy, control groups. Current data show a high level of TNF-α and IFN-γ in children with DS, and suggest a possible involvement of inflammatory cytokines in early CNS alterations. As regards the correlation between cytokines and dementia, it seems that evaluation of the immune response profile in children with DS is helpful in the early diagnosis of the clinical symptoms seen in adult patients with DS. Further study of the frequency and function of the T lymphocyte subsets in children and adult with DS is recommended.

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