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# Cytokine profile in autistic patients

Mohammad Mahdi Eftekharian[a,1], Soudeh Ghafouri-Fard[b,1], Rezvan Noroozi[c], Mir Davood Omrani[b,d], Shahram Arsang-jang[e], Maziar Ganji[b], Vajiheh Gharzi[b], Hanieh Noroozi[f], Alireza Komaki[a], Mehrdokht Mazdeh[a], Mohammad Taheri[b,d,*]

[a] Neurophysiology Research Center, Hamadan University of Medical Sciences, Hamadan, Iran
[b] Department of Medical Genetics, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[c] Phytochemistry Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[d] Urogenital Stem Cell Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[e] Clinical Research DevelopmentCenter (CRDU), Qom University of Medical Sciences, Qom, Iran
[f] Department of Education, Ahvaz, Khuzestan, Iran

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ABSTRACT

The etiology of Autism Spectrum Disorders (ASDs) as severe neurodevelopmental ailments is not known. However, several evidences point to dysregulation of immune system as an underlying cause of ASD. In the present study we evaluated the mRNA expression levels of TNF-α, TGF-β, IFN-γ, CXCL8, IL-1β, IL-2, 1L-4, IL-6, IL-17 in whole blood samples of 30 ASD patients and 41 age and sex-matched healthy subjects with means of real-time PCR. TNF-α, IL-6 and IL-17 have been shown to be significantly up-regulated in ASD patients compared with healthy subjects ($P < 0.0001$, $P = 0.001$ and $P < 0.0001$ respectively). IL-2 has been shown to be significantly down-regulated in total ASD patients ($P < 0.0001$). No significant difference has been found in expression levels of other cytokines between patients and healthy subjects. The present study provides further evidences for dysregulation of immune response in ASD patients.

## 1. Introduction

Autism Spectrum Disorders (ASDs) as neurodevelopmental disorders are outlined by inadequacy of social communication and interaction, and the existence of limited, monotonous patterns in behavior, passion, or motions [1]. Although the exact underlying mechanism is not clear, evidences have supported a role for dysregulation of immune system [2]. Numerous studies have demonstrated changes in the cytokine levels in the blood, brain, and cerebrospinal fluid (CSF) of ASD patients compared with healthy subjects [3]. Furthermore, alteration in the expression of immune factors has been associated with the severity of ASD complications such as defects in social interactions or monotonous behaviors [4]. Abnormal T helper (Th) cell responses are implicated in the ASD development as well. Th cells are divided to distinct classes based on their functions and cytokine-secretion phenotypes. Th1 cells produce interleukin (IL-2), interferon (IFN)-γ, and tumor necrosis factor (TNF)-α. Th2 cells produce IL-4, -5, -10 and -13 and participate in the development of auto-immune disorders. Th17 cells secrete IL-17, IL-17F, IL-6, IL-22 and TNF-α and contribute in tissue inflammation. On the other hand, regulatory T (Treg) cells secrete IL-10 and transforming growth factor (TGF)-β, which modify Th functions and contribute in tolerance induction [5]. In addition to these cytokines, CXCL8 (IL-8) is a small protein which is expressed in polymorphonuclear leukocytes, as well as epithelial, endothelial, fibroblasts and neurons. This chemokine participates in defense against pathogens as well as some disease-associated processes such as tissue injury, fibrosis and angiogenesis [6].

In the present study we aimed at mRNA expression analysis of pro-inflammatory (IL-1β, IL-6 and TNF-α), Th1 (IL-2 and IFN-γ), Th2 (IL-4), Th17 (IL-17) cytokines as well as CXCL8 chemokine and TGF-β (an indicator of Treg cell activity) in whole blood cells of ASD patients as compared with healthy subjects.

## 2. Materials and methods

### 2.1. Patients and control group

This case–control study consisted of 30 Iranian ASD patients (mean age of 6 ± 1.4) and 41 age, gender, and ethnic-matched healthy

* Corresponding author.
*E-mail address:* mohammad.taheri@sbmu.ac.ir (M. Taheri).
[1] The first two authors contributed equally.

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controls (mean age of 6 ± 1.4). ASD was diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders 5th edition criteria (1). The exclusion criteria were the existence of any other neurological, metabolic or auto-immune disorders. None of the ASD patients had a comorbid disorder such as attention deficit hyperactive disorder. Controls were selected from healthy volunteers in whom medical history, physical and laboratory investigations showed no evidence of any neurological disease. In addition, controls had no history of developmental delay or psychiatric disorders as stated by their parents. The control subjects did not have familial relation with the patient group and had no clinical signs of infectious disease. Written informed consent forms were acquired from all parents. The study protocol was approved by the ethical committee of Hamadan University of Medical Science.

### 2.2. Sampling and RNA extraction

Three ml of peripheral blood was obtained from patients and healthy subjects in EDTA tubes. Total RNA was extracted from whole blood samples using Hybrid-RTM blood RNA extraction Kit (Geneall Biotechnology Co Ltd, South Korea). The quantity and quality of RNA were assessed using Nanodrop equipment (Thermo Scientific, MA, USA).

### 2.3. cDNA synthesis and quantitative RT-PCR

High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used for cDNA synthesis according to the manufacturer's protocol. Specific primers and probes for expression analysis of mentioned cytokines genes were designed by using Allele ID 7 for ×64 windows software (Premier Biosoft, Palo Alto, USA). The primers and probes sequences and PCR product length are shown in Table 1. *HPRT1* gene was used as normalizer.

### 2.4. Statistical analysis

To test the significance of difference in means between two groups, Bayesian estimation was used. A normal prior distribution was assumed for parameters with 200,000 iterations. Spearman rank order correlation test was used for the assessment of the correlation between cytokines relative expressions. The Analysis of Covariance (ANCOVA) was used to control the effects of possible confounding variables and to improve the statistical power. P values less than 0.05 were considered as significant.

## 3. Results

### 3.1. General data

The patient group included 30 ASD patients (Male/Female = 19/11) with age (mean ± standard deviation (SD)) of 6 ± 1.4. The control group consisted 41 healthy subjects (Male/Female = 30/11) with age (mean ± SD) of 6 ± 1.74.

### 3.2. Relative mRNA expression of CXCL8 in patients compared with healthy subjects

No significant difference has been found in CXCL8 mRNA expression between patients and healthy subjects or within age and sex subgroups (Table 2). After application of ANCOVA for adjusting the effects of sex and age, there was no statistically significant difference in CXCL8 mRNA expression between patients and healthy subjects (P = 0.227).

**Table 1**
Nucleotide sequences of primers and probes used in the study.

| Gene name | Primer and probe sequence | Primer and probe length | Product length |
|---|---|---|---|
| HPRT1 | F: AGCCTAAGATGAGAGTTC | 18 | 88 |
| | R: CACAGAACTAGAACATTGATA | 21 | |
| | FAM-CATCTGGAGTCCTATTGACATCGC-TAMRA | 24 | |
| IL-1β | F: ATAGCCTGGACTTTCCTGTTGTC | 23 | 163 |
| | R: GTGAGTAGGAGAGGTGAGAGAGG | 23 | |
| | FAM-ACACCAATGCCCAACTGCCTGCCT-TAMRA | 24 | |
| IL-2 | F: GGGATCTGAAACAACATTCATGTG | 24 | 109 |
| | R: AGTCAGTGTTGAGATGATGCTTTG | 24 | |
| | FAM-TGATGAGACAGCAACCA-TAMRA | 17 | |
| IL-4 | F: TGCTGCCTCCAAGAACACAAC | 21 | 88 |
| | R:GTCCTTCTCATGGTGGCTGTAG | 22 | |
| | FAM-CCGGAGCACAGTCGCAGCCCT-TAMRA | 21 | |
| IL-6 | F: ATGCAATAACCACCCCTGACC | 23 | 160 |
| | R: CCATGCTACATTTGCCGAAGAG | 21 | |
| | FAM-ACCACAAATGCCAGCCTGCTGACG-TAMRA | 22 | |
| CXCL8 | F:CGGAAGGAACCATCTCACTGTG | 22 | 77 |
| | R:AGAAATCAGGAAGGCTGCCAAG | 22 | |
| | FAM-TGACTTCCAAGCTGGCCGTGGCTC-TAMRA | 24 | |
| IL-17 | F: CAGCAAGAGATCCTGGTCCTG | 21 | 176 |
| | R: GGTCGGCTCTCCATAGTCTAAC | 22 | |
| | FAM-AGCCTCCACACTGCCCCAACTCCT-TAMRA | 24 | |
| IFN-γ | F: GGCAAGGCTATGTGATTACAAGG | 23 | 96 |
| | R: CATCAAGTGAAATAAACACACAACCC | 26 | |
| | FAM-AGGGGCCAACTAGGCAGCCAACCT-TAMRA | 24 | |
| TGF-β | F: GCTCCACGGAGAAGAACTGC | 20 | 101 |
| | R: GTTGGCATGGTAGCCCTTGG | 20 | |
| | FAM-CCACTTCCAGCCGAGGTCCTTGCG-TAMRA | 24 | |
| TNF-α | F: TCCACCCATGTGCTCCTCAC | 20 | 97 |
| | R: TCTGGCAGGGGCTCTTGATG | 20 | |
| | FAM-CTACCGAGTCCGTGTCTACCA-TAMRA | 21 | |

### 3.3. Relative mRNA expression of IFN-γ in patients compared with healthy subjects

No significant difference has been found in IFN-γ mRNA expression between patients and healthy subjects or within age and sex subgroups (Table 3). After application of ANCOVA for adjusting the effects of sex and age, there was no statistically significant difference in IFN-γ mRNA expression between patients and healthy subjects (P = 0.627).

### 3.4. Relative mRNA expression of IL-1β in patients compared with healthy subjects

No significant difference has been found in IL-1β mRNA expression between patients and healthy subjects or within age and sex subgroups (Table 4). After application of ANCOVA for adjusting the effects of sex and age, there was no statistically significant difference in IL-1β mRNA expression between patients and healthy subjects (P = 0.095).

### 3.5. Relative mRNA expression of IL-2 in patients compared with healthy subjects

IL-2 mRNA has been shown to be significantly down-regulated in total ASD patients (P < 0.0001) as well as all subgroups (Table 5). After application of ANCOVA for adjusting the effects of sex and age, there was statistically significant difference in IL-2 mRNA expression between patients and healthy subjects (P < 0.0001).