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# Tumor necrosis factor-alpha expression in peripheral blood mononuclear cells correlates with early childhood social interaction in autism spectrum disorder

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ABSTRACT

Autism spectrum disorder is a neurodevelopmental disorder characterized by impaired social interaction, poor communication skills, and repetitive/restrictive behaviors. Elevated blood levels of pro-inflammatory cytokines have been reported in subjects with autism spectrum disorder. On the other hand, early childhood adverse experience also increases blood levels of these cytokines. Since social experience of children with autism spectrum disorder is generally unlike to typically developing children, we hypothesized that social interaction during childhood contribute to pro-inflammatory cytokine expression in subjects with autism spectrum disorder. We compared revised Autism Diagnostic Interview scores and expression levels of pro-inflammatory cytokines in peripheral blood mononuclear cells of subjects with autism spectrum disorder (n = 30). The score of domain A on the revised Autism Diagnostic Interview, indicating social interaction impairment in early childhood, was negatively correlated with tumor necrosis factor-α mRNA expression level in peripheral blood mononuclear cells but not interleukin-1β or -6. Consistently, tumor necrosis factor-α mRNA expression was markedly low in subjects with autism spectrum disorder compared to typically developing children who presumably experienced the regular levels of social interaction. These findings suggest that the low blood levels of tumor necrosis factor-α mRNA in subjects with autism spectrum disorder might be due to impaired social interaction in early childhood.



## 1. Introduction

A growing body of evidence indicates that immune system dysfunction is associated with the mechanisms of schizophrenia, major depressive disorder, and bipolar disorder (Altamura et al., 2014; Bhattacharya et al., 2016; Gottfried et al., 2015; Khandaker et al., 2015; Kunugi et al., 2015; Monji et al., 2009; Viscogliosi, 2015). Inappropriate innate and adaptive immunological responses lead to central nervous system (CNS) dysfunction which could influence the development of those psychiatric disorders (Depino, 2013). A number of studies have shown increased levels of pro-inflammatory cytokines in serum, plasma, and blood cells, suggesting that inflammation might be involved in the mechanisms of psychiatric disorders (Lai et al., 2016; Setiawan et al., 2015). Autism spectrum disorder (ASD) is a neurodevelopmental disorder

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characterized by impaired social interaction, poor communication skills, and repetitive/restrictive behaviors, and it has been shown that immunological anomalies are implicated in the pathobiology of ASD. Postmortem brain studies revealed that some subjects with ASD demonstrate inflammation in the CNS. Vargas et al. reported the activation of astrocytes and microglia and increased expressions of transforming growth factor beta (TGF-β) and monocyte chemotactic protein-1 (MCP-1) in the brains of subjects with ASD compared to typically developing children (TDC) (Vargas et al., 2005). Another study revealed that pro-inflammatory cytokines; interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), granulocyte macrophage colony-stimulating factor, interferon-γ, and interleukin-8, were significantly increased in the brains of ASD subjects (Chez et al., 2007; Li et al., 2009). Positron emission tomography also indicated microglial activation in the brains of subjects with ASD (Suzuki et al., 2013). Subjects with ASD also demonstrate anomalous features in peripheral blood which may be associated with autistic symptoms. The expression levels of TGF-β were decreased in peripheral blood samples from subjects with ASD compared to TDC and the lower TGF-β levels correlated with more severe symptoms of ASD (Ashwood et al., 2011). In contrast, the plasma levels of macrophage inhibitory factor (MIF) expression were higher in subjects with ASD than TDC, and the highest plasma expressions levels of MIF were associated with the most severe autistic symptoms (Grigorenko et al., 2008). However, the expression levels of major inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, in blood remain controversial (Ricci et al., 2013; Singh, 1996; Suzuki et al., 2011).

On the other hand, juvenile social experience is associated with the development of a variety of psychiatric disorders and affects the expression of pro-inflammatory cytokines in blood later in life (Nemeroff, 2016). For example, child maltreatment results in higher blood levels of IL-6 and C-reactive protein (Danese et al., 2008; Lopez-Patton et al., 2016), and deficits of social interaction after weaning lead to elevated plasma levels of IL-1β, IL-6, TNF-α, and interferon-γ in rats (Ko and Liu, 2015).

Given that atypical social interaction of subjects with ASD and shared alterations of pro-inflammatory cytokine expressions in blood of subjects with ASD and child maltreatment, we hypothesized that aberrant social interaction during childhood contributes to peripheral immune system status in subjects with ASD later in life. Therefore, we compared the Autism Diagnostic Interview-Revised (ADI-R) score, which is a rigorous index for evaluating social interaction at age 4, and major pro-inflammatory cytokine levels; IL-1β, IL-6, and TNF-α, in PBMCs of subjects with ASD in their post-growth period.

## 2. Materials and methods

This study was approved by the ethics committees of Hamamatsu University School of Medicine, University of Fukui, and Nara Medical University School of Medicine. All participants and their guardians were given a complete description of the study and provided written informed consent before enrollment.

### 2.1. Subjects

We recruited 30 male patients with ASD (age: 11.6 ± 2.7). All participants were of Japanese ethnicity and were born and lived in areas in central Japan including Aichi, Gifu, and Shizuoka Prefectures. Using interviews and available information including hospital records, diagnoses of ASD were made by an experienced child psychiatrist based on Diagnostic and Statistical Manual-IV-Text Revision criteria. The ADI-R was also conducted by a qualified child psychiatrist. This assessment is a semi-structured interview conducted with a parent and is used to confirm ASD diagnosis (Lord et al., 1994). Domain A of the ADI-R quantifies social interaction impairment at age 4; domain B quantifies impairment in communication; and domain C quantifies restricted, repetitive, and stereotyped patterns of behaviors and interests. Comorbid psychiatric illnesses were excluded using the Structured Clinical Interview for the DSM-IV (SCID). Participants were excluded from the study if they had any symptoms of inflammation, a diagnosis of fragile X syndrome, epileptic seizures, obsessive-compulsive disorder, affective disorders, or any additional psychiatric or neurological diagnoses. None of the participants had ever received psychoactive medications prior to this study. The medical histories of 30 male TDC (age: 11.1 ± 2.3) were comprehensively reviewed to exclude individuals with any neurological or medical disorders. The SCID was also used to identify any personal or family history of past or present mental illness. None of the initially recruited comparison subjects met any of the exclusion criteria. Whole-blood samples were collected by venipuncture from all participants. PBMCs were isolated by means of the Ficoll-Paque gradient method within 2 h after sampling.

### 2.2. Quantitative RT-PCR (qRT-PCR)

To measure mRNA levels of IL-1β, IL-6 and TNF-α in human PBMCs, total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA), and the RNA samples were further purified using the RNeasy Micro Kit (QIAGEN, Hilden, Germany). First-strand cDNA was synthesized from the RNA samples using the SuperScript III First-Strand Synthesis System (Invitrogen), and qRT-PCR analysis was performed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Relative quantification of gene expression levels was performed using the delta-delta $C_T$ method with the constitutively expressed gene *GAPDH* as an internal control. Primer sequences were as follows. *IL-1β* F: CTGTCCTGCGTGTTGAAAGA, *IL-1β* R:GAAGACAAATCGCTTTTCCA; *IL-6* F: AGTGAGGAACAAGCCAGAGC, *IL-6* R: CAGGGGTGGTTATTG CATCT; *TNF-α* F: GGCAGTCAGATCATCTTCTCG, *TNF-α* R: CAGCTT GAGGGTTTGCTACA; *GAPDH* F: ATCAGCAATGCCTCCTGCAC, *GAPDH* R: TGGCATGGACTGTGGTCATG.

### 2.3. Statistical analysis

Evaluation of the relationships between gene expression levels and clinical variables was performed using Pearson's correlation coefficient for subjects with ASD. Mann-Whitney *U* test was used to compare gene expression levels between TDC and subjects with ASD. $P < 0.05$ was considered statistically significant.

## 3. Results

We compared mRNA levels of IL-1β, IL-6, and TNF-α to ADI-R scores (domains A-C). None of the ADI-R scores correlated with IL-1β or IL-6 expression (Fig. 1A, 1B, 2A, 2B, 3A, 3B). While there were no relationships between ADI-R domain B or C scores and TNF-α expression (Fig. 2C, 3C), ADI-R domain A score negatively correlated with TNF-α expression, indicating that more severe social interaction impairment is associated with reduced TNF-α expression later in life (Fig. 1C).

We then compared TNF-α expression in PBMCs between subjects with ASD and TDC, who presumably experienced regular social interaction during early childhood. Subjects with ASD had significantly lower TNF-α expression than TDC (Fig. 4), indicating that poor social interaction during early childhood might results in low TNF-α expression in PBMCs.