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Alterations in Plasma Cytokine Levels in Korean Children with Autism Spectrum Disorder

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Purpose: Numerous studies have supported the role of the immune dysfunction in the pathogenesis of autism spectrum disorder (ASD); however, to our knowledge, no study has been conducted on plasma cytokine levels in children with ASD in South Korea. In this study, we aimed to analyze the immunological characteristics of Korean children with ASD through plasma cytokine analysis.

Materials and Methods: Blood samples were collected from 94 ASD children (mean age 7.1; 81 males and 13 females) and 48 typically developing children (TDC) (mean age 7.3; 30 males and 18 females). Plasma was isolated from 1 mL of blood by clarifying with centrifugation at 8000 rpm at 4°C for 10 min. Cytokines in plasma were measured with LEGENDplex HU Th cytokine panel (BioLegend, 741028) and LEGENDplex HU cytokine panel 2 (BioLegend, 740102).

Results: Among 25 cytokines, innate immune cytokine [interleukin (IL)-33] was significantly decreased in ASD children compared with TDC. In acute phase proteins, tumor necrosis factor α (TNF-α) was significantly increased, while IL-6, another inflammation marker, was decreased in ASD children compared with TDC. The cytokines from T cell subsets, including interferon (IFN)-γ, IL-5, IL-13, and IL-17f, were significantly decreased in ASD children compared to TDC. IL-10, a major anti-inflammatory cytokine, and IL-9, which modulates immune cell growth and proliferation, were also significantly decreased in ASD children compared to TDC.

Conclusion: We confirmed that Korean children with ASD showed altered immune function and unique cytokine expression patterns distinct from TDC.

Key Words: Autism spectrum disorder, immune dysfunction, blood cytokine, Korean

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INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder primarily characterized by deficits in social interactions and patterns of repetitive restricted behavior.[1] The prevalence of ASD is estimated to be one in 36 children (2.8%) aged 8 years in the United States.[2] In South Korea, it was estimated to be 2.64%, according to a 2011 report, and is expected to increase.[3] As the prevalence of ASD increases, social and economic burdens also greatly increase. A recent study has reported a considerable increase in the household economic burden of ASD in South Korea, with total related costs reaching $9645502 in 2015.[4]

Although the exact etiology of ASD remains unknown, numerous recent studies have supported the role of immune dysfunction in ASD pathogenesis.[5] For example, postmortem studies have revealed that the proinflammatory cytokines, including tumor necrosis factor α (TNF-α) and interleukin (IL)-6, were notably increased in the brains of patients with ASD.[6] In the peripheral system, the levels of inflammatory cytokines were also considerably increased in the plasma of patients with ASD compared to those in the control group.[7] In addition to the production of skewed cytokines, other studies have demonstrated immune abnormalities in ASD, such as changes in immune genetics, altered T-cell function, and imbalanced immune responses.[8] Moreover, Careaga, et al.[9] have reported that immune activation is associated with more severe behavioral impairments in children with ASD and have suggested that children with ASD may be characterized by their immune profiles.

These findings suggest that cytokine levels may serve as biomarkers to distinguish ASD from typical groups or ASD subtypes. Although some results such as decreased levels of IL-10, an anti-inflammatory cytokine, in ASD have been repeatedly reported by various researchers, the results of previous studies remain inconsistent, likely owing to various factors, including experimental design, age and race of the participants, and the heterogeneity of ASD.[10]

Current evidence on the role of immune dysfunction in the pathophysiology of ASD has led to an increased interest in unraveling therapeutic approaches through immune regulation. Several randomized placebo-controlled studies have shown the effectiveness of immunoregulatory and/or anti-inflammatory agent, such as prednisolone, via the inhibition of microglial proinflammatory activation and restoration of the regulatory T cells/T-helper type 17 (Treg/Th17) imbalance.[11] Although these studies had several limitations, they were still noteworthy as they have shown improvements in the core and associated symptoms of ASD through the immunomodulatory function of drugs currently in use. Using drugs whose safety has been confirmed in other diseases for treating ASD is extremely efficient; therefore, understanding the immunological characteristics of the subjects is important.

To our knowledge, no study has been conducted on blood cytokine levels in children with ASD in South Korea to date. Through previous studies, it is known that the immune profile is influenced by both age and ethnicity.[12] Therefore, in this study, we aimed to analyze the immunological characteristics of Korean children with ASD using plasma cytokine analysis. Understanding the specific immunological characteristics of Korean children with ASD is expected to not only enhance our understanding of ASD pathogenesis but also contribute to the exploration of therapeutic possibilities through immunological modulation.

MATERIALS AND METHODS

Participants

This study was approved by the Institutional Review Board of the Severance Hospital, Yonsei University College of Medicine (4-2019-0926). Written informed consent was obtained from all participants and their parents before the start of the study. Blood samples were collected from 94 children with ASD (aged 4.3–13.8 years; 81 males and 13 females) and 48 typically developing children (TDC) (aged 4.3–15.2 years; 30 males and 18 females). Children with ASD met the following inclusion and exclusion criteria: 1) aged between 4 and 15 years and diagnosed by a child and adolescent psychiatrist based on the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition;[1] 2) diagnosis was supplemented by the Autism Diagnostic Observation Schedule-2 (ADOS-2), Autism Diagnostic Interview-Revised (ADI-R), and Social Responsiveness Scale (SRS);[13-15] 3) children with psychiatric disorders other than ASD were excluded based on semi-structured psychiatric interview and the Kiddie-Schedule for Affective Disorders and Schizophrenia-Present and Lifetime Version (K-SADS-PL). TDC group was recruited through bulletin boards within the hospital and external advertisements. Participants in TDC group met the following inclusion and exclusion criteria: 1) aged between 4 and 15 years and showed typical development with full-scale intelligence quotient (FSIQ) over 80; 2) ASD traits were screened using SRS; 3) children with a history of psychiatric disorder were excluded based on semi-structured psychiatric interviews and parental reports.

Sample collection and plasma isolation

Blood was collected in BD Vacutainer heparin-coated collection tubes (BD, 150-367874), and stirred on a stirrer within 2 hours after blood collection. Plasma was isolated from 1 mL of blood using centrifugation at 8000 rpm at 4°C for 10 min. The upper plasma layer was harvested, divided into 100 μL aliquot, and stored at -80°C until use.

Multiplex cytokine analysis

Cytokine in plasma was measured using LEGENDplex HU Th cytokine panel (BioLegend, 741028) for 12 cytokines [IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17a, IL-17f, IL-22, interferon (IFN)-γ, and TNF-α] and LEGENDplex Human Cytokine panel 2 (BioLegend, 740102) for 13 cytokines [thymic stromal lymphopoietin (TSLP), IL-1α, IL-1β, granulocyte macrophage colony stimulating factor (GM-CSF), IFN-α2, IL-23, IL-12p40, IL-12p70, IL-15, IL-18, IL-11, IL-27, and IL-33]. All samples were measured separately twice. Briefly, frozen plasma was thawed on ice, captured using beads, and incubated with staining antibodies at room temperature. For each sample, more than 300 beads were recorded and analyzed using the Data Analysis Software Suite for LEGENDplex, a free cloud-based program (https://legendplex.qognit.com/user/

login?next=home).

Statistical analysis

Normality and normality tests were used to define the distribution of each data point, and all data were presented as a log-normal distribution. Following the results of the normality tests, the Mann–Whitney test was applied to compare differences between the TDC and ASD groups with adjusted $p<0.05$. Statistical analyses and graph creation were performed using GraphPad Prism 10 (GraphPad Software, Boston, MA, USA).

RESULTS

Demographic characteristics

In this study, we analyzed blood samples of 142 participants, including 94 children with ASD and 48 TDC. No statistically significant differences were observed between the mean ages of the two groups. However, sex ratio, FSIQ, and SRS scores showed significant differences between ASD and TDC groups (Table 1).

Table 1. Characteristics of ASD and TDC Groups

	ASD (n=94)	TDC (n=48)	*p* value
Sex			0.001*
Male	81 (86.2)	30 (62.5)	
Female	13 (13.8)	18 (37.5)	
Age (yr)	7.1±2.2	7.3±2.4	0.656
FSIQ	65.1±18.4	110.7±14.7	<0.001*
SRS (total T score)	88.4±15.9	43.1± 8.7	<0.001*
ADI-R (total)	45.8±9.2	-	
ADOS-2 (total)	14.7±5.9	-	

ASD, autism spectrum disorder; TDC, typical developing children; FSIQ, full-scale intelligence quotient; SRS, Social Responsiveness Scale; ADI-R, Autism Diagnosis Interview-Revised-Korean version; ADOS, Autism Diagnostic Observation Schedule-Korean version.
Data are presented as mean±standard deviation or n (%).
*$p<0.001$.

Altered innate immune cell cytokines and acute phase proteins in plasma of children with ASD

To identify the soluble immune features at steady state in children with ASD, we performed a cytokine assay on plasma from individuals with ASD and age matched TDC. Among the 25 cytokines, innate immune cytokines (IL-18, IL-23, and IFN-α2) remained unchanged, except for IL-33, which decreased in the plasma of individuals with ASD (Fig. 1). In acute phase proteins, only TNF-α and IL-6 levels were significantly increased or decreased in ASD compared to those in TDC, respectively (Fig. 2).

Changed basal level of cytokines secreted from T cell subsets in the plasma of children with ASD

To confirm T cell function in the steady-state immune system, we examined the cytokines secreted from T cell subsets in the plasma. The levels of signature cytokines from each T cell subset decreased in the plasma samples of children with ASD (Fig. 3). Particularly, the levels of IFN-γ (secreted by Th1 cells) (Fig. 3A), IL-5 and IL-13 (secreted by Th2 cells) (Fig. 3B), and IL-17f (secreted by Th17 cells) (Fig. 3C) were significantly decreased in the plasma of children with ASD compared to those in TDC.

Shifted levels of plasma anti-inflammatory cytokines and cytokines inducing immune growth

The immune system is maintained by balancing inflammatory and anti-inflammatory effects. IL-10, a major anti-inflammatory cytokine, was significantly lower in the plasma of children with ASD than that in TDC (Fig. 4). In addition to immune cell-secreted cytokines, the level of IL-9, which is secreted by regulatory T, Th9 cells, and mast cells, was also decreased in the plasma of children with ASD compared to that in TDC (Fig. 5).

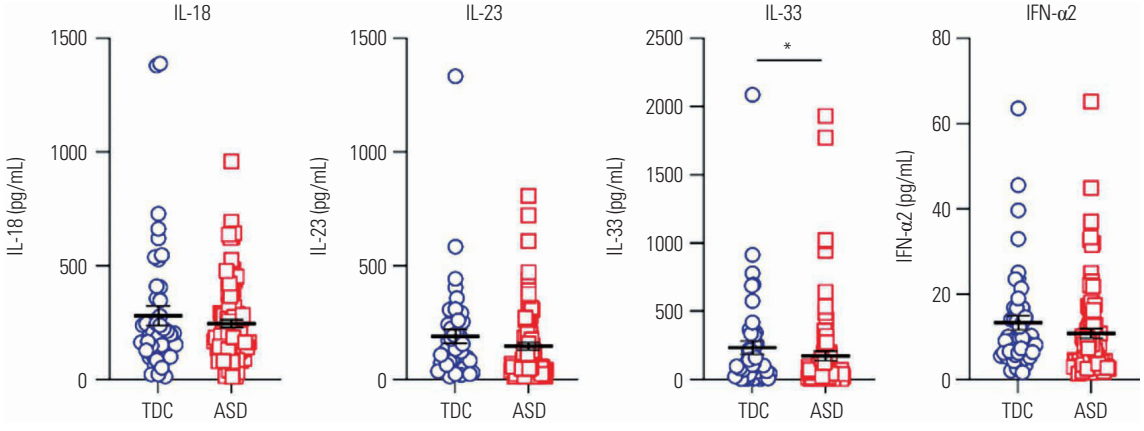

Fig. 1. Innate immune cytokine levels were altered in plasma of ASD children compared to TDC. Concentrations of cytokines secreted from innate immune cells (IL-18, IL-23, IL-33, IFN-α2) in plasma of TDC (n=48) and those with ASD (n=94) were measured. IL-33 was decreased in individuals with ASD compared to TDC. Data are means±SEMs. *P* values were calculated using a Student's t test; *$p<0.05$. ASD, autism spectrum disorder; TDC, typically developing children; IL, interleukin; IFN, interferon; SEM, standard error of the mean.

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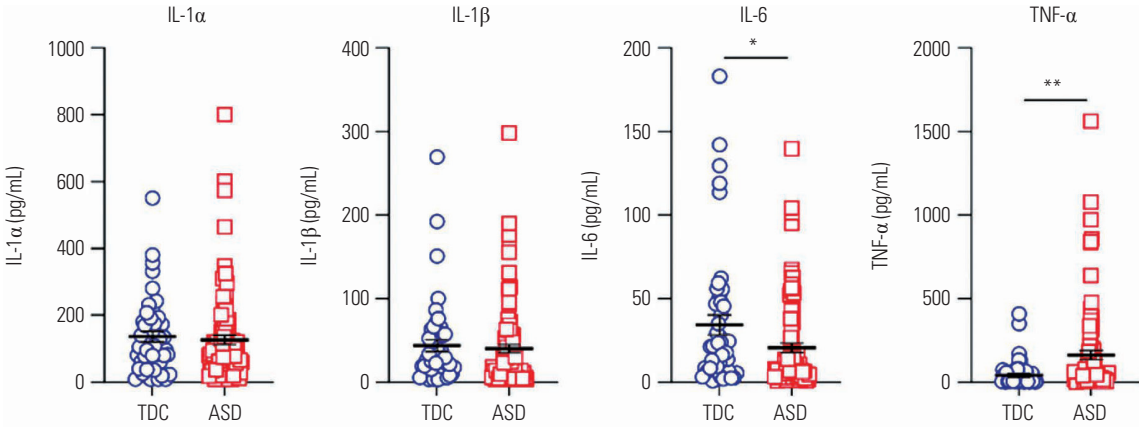

Fig. 2. Acute phase proteins showed differences in plasma of ASD children compared to TDC. Plasma level of IL-6 was lower in individuals with ASD (n=94) than in those with TDC (n=48). TNF-α was significantly increased in individuals with ASD compared to TDC. Data are means±SEMs. *P* values were calculated using a Student's t test; **p*<0.05, ***p*<0.01. ASD, autism spectrum disorder; TDC, typically developing children; TNF, tumor necrosis factor; IL, interleukin; SEM, standard error of the mean.

DISCUSSION

In this study, we aimed to characterize the patterns of plasma cytokine levels in Korean populations. We observed that TNF-α, a proinflammatory cytokine, was significantly increased at steady-state plasma in children with ASD compared with that in TDC. Notably, the levels of most other cytokines, such as IL-5, IL-6, IL-9, IL-10, IL-13, IL-17f, and IFN-γ, were significantly decreased in ASD group compared to TDC group. These results show a unique cytokine expression pattern in Korean children with ASD and suggest immunological dysfunction in children with ASD compared to TDC.

Cytokines, including ILs, chemokines, TNFs, IFNs, and growth factors, are key mediators in the regulation of immune responses. Pro- and anti-inflammatory cytokines interact with immune cells and the neuroendocrine system, including hormones and neurotransmitters. Since the expression of different cytokines can modify the immune system, it is important to maintain a balance between pro- and anti-inflammatory cytokines for proper immune function.[16]

Our finding of increased plasma TNF-α levels in patients with ASD compared to those in TDC are consistent with previous studies, supporting that heightened innate immune responses, particularly TNF-α production, may contribute to the pathophysiology of ASD. Elevated plasma TNF-α levels have been reported in Turkish children with autism with no prior history of medication,[17] and in patients with ASD compared to control groups in other studies.[18] These results suggest dysregulated immune function in ASD, characterized by a heightened proinflammatory state, which might play a role in the development or manifestation of ASD symptoms.

Our study showed decrease in IL-33 which plays an important role in the initiation of type-2 innate immune responses. This result means innate immune dysfunction in ASD children compared with TDC. Although an increase in innate immune cytokines in ASD has been repeatedly reported, a study by Barbosa, et al.[19] reported no significant differences in the plasma and serum levels of IL-33 between patients with ASD and controls. Similarly, Saresella, et al.[20] observed a significant reduction in IL-33 mRNA levels in patients with ASD following LPS and ATP treatment, activating an immune response in peripheral blood mononuclear cells (PBMCs). One possibility for the decreased IL-33 levels in our study is that the reduced IL-33 levels might be a consequence of the heightened proinflammatory cytokine environment frequently observed in patients with ASD. High levels of proinflammatory cytokines could potentially suppress the production or release of IL-33, leading to the observed decrease.

IL-6 is a multifunctional molecule that acts as both a pro- and anti-inflammatory cytokine. Our findings revealed that the plasma levels of IL-6 were notably reduced in children with ASD. This result is consistent with Khudiakova's report, which found lower serum IL-6 concentrations in ASD group than in TDC group.[21] However, another study reported increased levels of IL-6 in the brain and plasma of patients with ASD.[22] In addition, maternal immune activation in pregnant rodents causes ASD-like behaviors, and IL-6 is known to be involved as a critical mediator.[23] Although IL-6 is predominantly recognized as a proinflammatory cytokine, several studies have demonstrated its neuroprotective capabilities in disease models. For example, in Glial Fibrillary Acidic Protein (GFAP)-IL-6 transgenic mice, IL-6 expression is specifically targeted to astrocytes, leading to enhanced healing of the cryo-damaged brain lesions compared with their non-transgenic counterparts.[24] Considering the neuroprotective role of IL-6, the reduced IL-6 levels in the plasma of children with ASD in our study may be explained by the reduced protective effects of IL-6 in ASD children.

Functional T cells secrete cytokines that regulate the steady state of the immune system. In our analysis, we observed a significant decrease in IFN-γ, IL-5, IL-13, and IL-17f levels in the

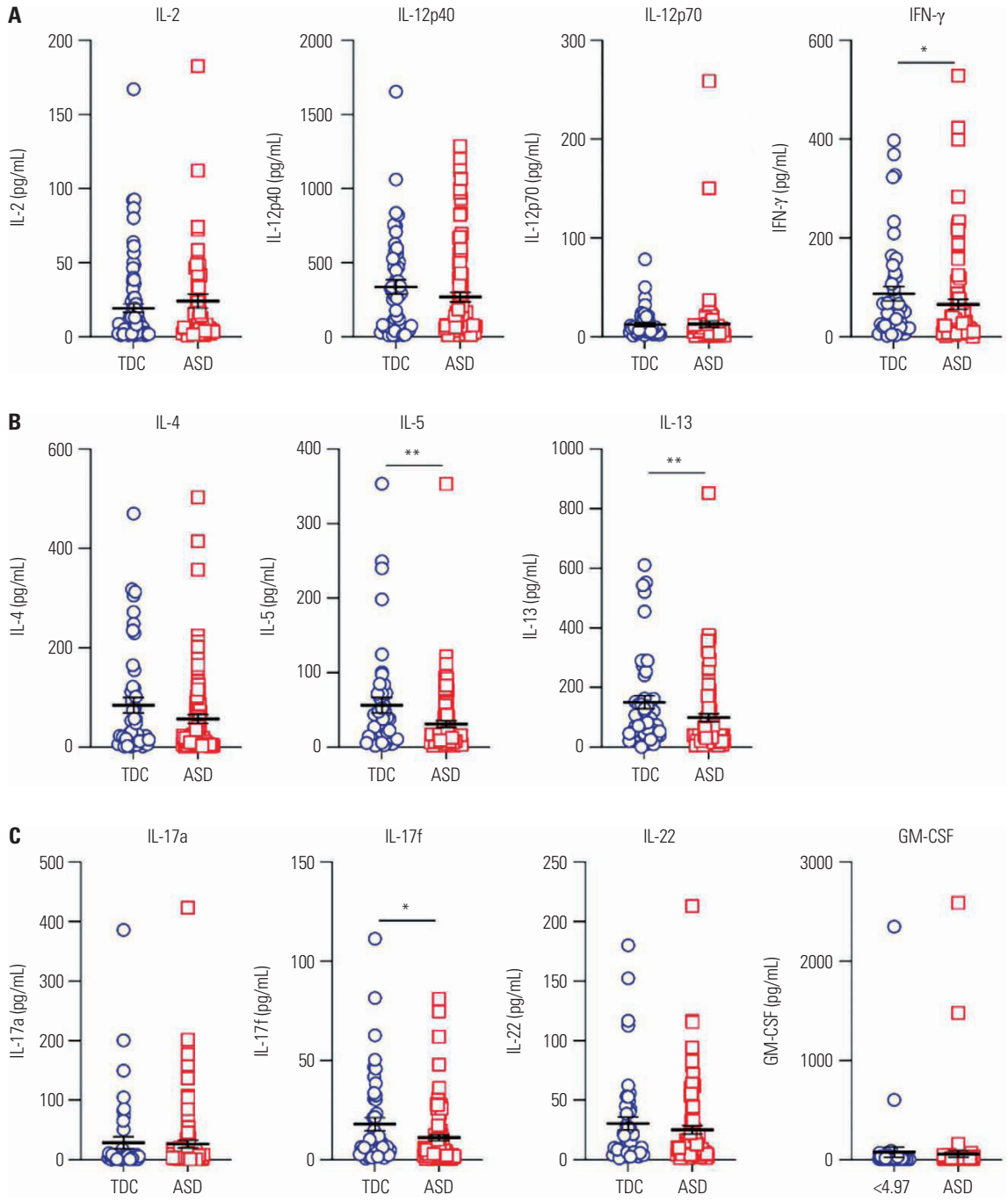

Fig. 3. T cell secreted cytokines were decreased in plasma of ASD children compared to TDC. Concentrations of IL-2 and TH1 cytokines (A), TH2 cytokines (B), and TH17 cytokines (C) in the plasma of TDC (n=48) and those with ASD (n=94) were measured using Multiplex assay. IFN-γ, IL-5, IL-13 and IL-17f were significantly decreased in ASD group compared to TDC group. Data are means±SEMs. *P* values were calculated using a Student's t test; **p*<0.05, ***p*<0.01. ASD, autism spectrum disorder; TDC, typically developing children; IL, interleukin; TH, helper T cell; IFN, interferon; SEM, standard error of the mean; GM-CSF, granulocyte macrophage-colony stimulating factor.

plasma of ASD group compared to those in TDC group, and these results were contrary to some previous studies. However, we confirmed that basal T-cell function was altered in children with ASD.

A recent meta-analysis study provided evidence for increased pro-inflammatory cytokines IFN-γ, IL-1β, IL-6, and TNF-α in patients with ASD patients compared to controls.[25] Especially, IFN-γ, secreted by Th1 cells, could induce transcriptional up-regulation of IL-6 through IRF-1 and NF-κB.[26] In contrast, IL-6 negatively regulates IFN-γ in modulating T cell activation and regulates IFN-γ-dependent immune response to infection and autoimmunity.[27] These results suggest that cytokines have a

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complex relationship in which they directly or indirectly affect each other's expressions; therefore, careful interpretation is required to explain the relationship between cytokine levels observed at a specific time point and the disease.

IL-5 and IL-13 are cytokines secreted by Th2 cells. A previous study reported that higher levels of IFN-γ, IL-4, and IL-5 in maternal serum during mid-pregnancy are linked to a 50% elevated likelihood of ASD.[28] However, another study, analyzing cytokine production against dietary proteins, reported excessive levels of TNF-α and IFN-γ produced by PBMCs, but not IL-5, in children with ASD.[29] Some studies have reported significantly higher IL-13 levels in patients with ASD than in control groups,[30] whereas others have found no difference in IL-13 levels between ASD and control groups.[7] Furthermore, a meta-analysis encompassing five studies concluded that there was no significant difference in the serum IL-13 levels between ASD and healthy control groups.[10] These diverse results highlight the need for further investigation to better understand the roles of IL-5 and IL-13 in ASD.

We also observed a decrease in plasma IL-17f levels in ASD group compared to TDC group, whereas IL-17a levels were not significantly different between the two groups in our study. Some studies have reported no significant differences in IL-17 levels between individuals with ASD and typically developing controls,[31] whereas others have found elevated IL-17a levels in high-functioning male subjects with ASD.[32] In contrast, a study from Turkey reported reduced IL-17 levels in patients with ASD.[33] Although IL-17f and IL-17a may have similar roles owing to their structural similarities, further studies are needed to elucidate the potential implications of IL-17f in the immune abnormalities observed in ASD.

IL-10 level was significantly lowered in plasma of children with ASD compared with that in TDC. This finding was consistent with that of a recent meta-analysis conducted by Saghazadeh, et al.[10] In this meta-analysis, eight previous studies were included which provided evidence for a moderate decrease in plasma levels of IL-10 in patients with ASD. A Danish study also showed that children who later developed ASD were more likely to have reduced neonatal IL-10 levels.[34] However, several studies have found no differences in IL-10 expression in the plasma and brain between ASD and control groups.[32]

Analysis of the expression of plasma cytokines related to immune cell growth and proliferation confirmed that the IL-9 levels were lower in ASD group than in TDC group. Similar to our results, Pecorelli, et al.[35] reported that serum IL-9 levels were significantly higher in patients with Rett syndrome, a genetic disorder that shares some symptoms with ASD, but found no such difference between ASD and TDC groups. Conversely, Ahmad, et al.[36] found increased IL-9 positive immunostaining in all cells, specifically CD4+ cells, in children with ASD compared to that in TDC. The decrease in IL-9 is not exclusive to

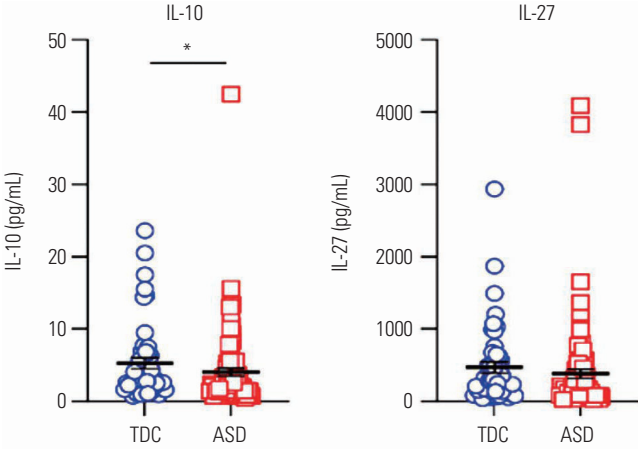

Fig. 4. Anti-inflammatory cytokines were changed in plasma of ASD children compared to TDC. The level of IL-10 in ASD children (n=94) was significantly lower than that in TDC (n=48). Data are means±SEMs. *P* values were calculated using a Student's t test; *$p<0.05$. ASD, autism spectrum disorder; TDC, typically developing children; IL, interleukin; SEM, standard error of the mean.

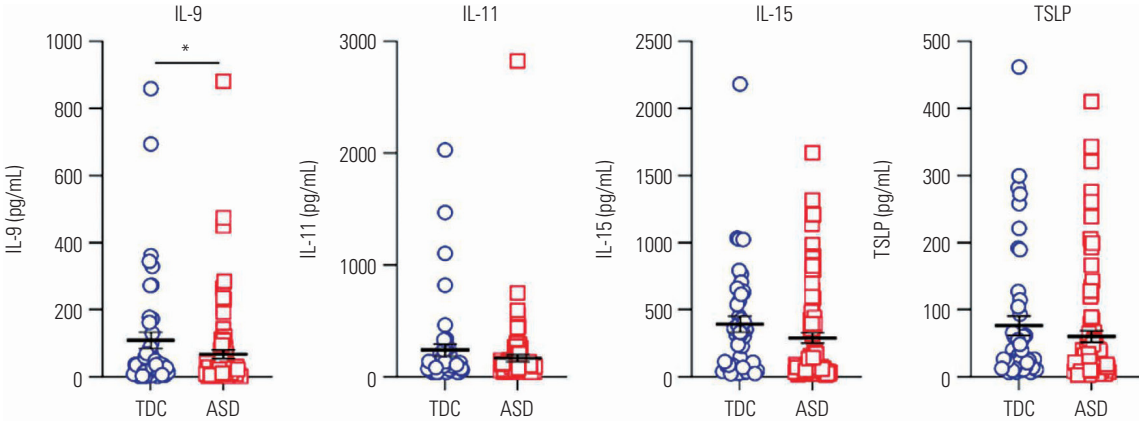

Fig. 5. Plasma levels of immune cell growth and proliferation factors showed differences between ASD and TDC groups. IL-9 was significantly lower in individuals with ASD (n=94) than in TDC (n=48). Data are means±SEMs. *P* values were calculated using a Student's t test; *$p<0.05$. IL, interleukin; ASD, autism spectrum disorder; TDC, typically developing children; SEM, standard error of the mean; TSLP, thymic stromal lymphopoietin.

the serum; a reduction in IL-9 concentration has also been reported in the fetal brain of offspring from a maternal autoantibody-related ASD mouse model compared to that in the control.[37] This result suggests that the effects of IL-9 alterations in ASD may also involve intrauterine or early postnatal life and may not be limited to peripheral manifestations.

Our study is meaningful in that it revealed the unique immunological characteristics of Korean children with ASD; however, it has several limitations. First, the immune system can be affected by various environmental factors such as race, sex, diet, and medication. However, we could not control all variables due to the insufficient number of participants. In particular, a previous study comparing the rates of antimicrobial prescription among pediatric populations in six countries revealed extensive antimicrobial use in all age groups in South Korea.[38] Since the continuous use of antibiotics can affect serum and brain cytokine levels,[39] the unique immunological characteristics observed in this study may be attributed to high antibiotic use in South Korea. In addition, approximately one-quarter of the participants with ASD were on medications, including aripiprazole and risperidone, which can affect the immune response and cytokine production. The possibility of immunological changes caused by drugs administered to relieve ASD symptoms should be considered in the future.

Second, approximately 30% of the TDC group in this study comprised siblings of children with ASD. Typical development was confirmed through screening before participating in the study; however, since they share genetic vulnerabilities, there is a possibility that they will show a different cytokine pattern from the TDC group, with no genetic risk. Indeed, Napolioni, et al.[40] showed a lack of significant differences in plasma cytokine profiles between children with ASD and their non-autistic siblings.

Third, children with ASD with a variety of symptoms and severity participated in this study; and as a result, the heterogeneity of ASD may have influenced the results. We confirmed that the range of cytokine expression was extremely wide, even within the ASD group. Therefore, we analyzed the relationship between the severity of ASD symptoms and the cytokine expression based on the ADOS-2 and SRS score; but unfortunately, we could not find a correlation between the expression of cytokines and ASD severity. In addition, the immune profile in ASD can change depending on the presence or absence of other comorbidities such as gastrointestinal symptoms or food sensitivity.[8] Therefore, a follow-up study should be performed to confirm the results in a more homogenous ASD group by considering not only ASD severity but also comorbidities.

Although there are several limitations, this study is valuable in that it confirmed peripheral immunological characteristics through plasma cytokine analysis of ASD children in South Korea. Our results demonstrated that Korean children with ASD also show dysregulation of immune function and a unique cytokine expression pattern. In addition, various cytokine levels were observed even within the same ASD group, confirming the importance of understanding individual characteristics for therapeutic intervention, as well as revealing the etiological mechanism of common ASD through immune dysfunction.

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AUTHOR CONTRIBUTIONS

Conceptualization: Sungji Ha, Juli Choi, Ho-Keun Kwon, and Keun-Ah Cheon. Data curation: Songjoo Shim, Sungji Ha, and Juli Choi. Formal analysis: Sungji Ha and Juli Choi. Funding acquisition: Juli Choi and Keun-Ah Cheon. Investigation: Songjoo Shim, Sungji Ha, and Juli Choi. Methodology: Juli Choi and Ho-Keun Kwon. Project administration: Sungji Ha and Juli Choi. Resources: Juli Choi, Ho-Keun Kwon, and Keun-Ah Cheon. Software: Juli Choi and Ho-Keun Kwon. Supervision: Ho-Keun Kwon and Keun-Ah Cheon. Validation: Songjoo Shim, Sungji Ha, and Juli Choi. Visualization: Songjoo Shim, Sungji Ha, and Juli Choi. Writing—original draft: Songjoo Shim, Sungji Ha, and Juli Choi. Writing—review & editing: all authors. Approval of final manuscript: all authors.

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