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Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome

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Abstract

Autism spectrum disorders (ASD) are characterized by impairment in social interactions, communication deficits, and restricted repetitive interests and behaviors. A potential role for immune dysfunction has been suggested in ASD. To test this hypothesis, we investigated evidence of differential cytokine release in plasma samples obtained from 2-5 year-old children with ASD compared with age-matched typically developing (TD) children and children with developmental disabilities other than autism (DD). Participants were recruited as part of the population based case-control CHARGE (Childhood Autism Risks from Genetics and Environment) study and included: 97 participants with a confirmed diagnosis of ASD using standard assessments (DSM IV criteria and ADOS, ADI-R), 87 confirmed TD controls, and 39 confirmed DD controls. Plasma was isolated and cytokine production was assessed by multiplex Luminex™ analysis. Observations indicate significant increases in plasma levels of a number of cytokines, including IL-1β, IL-6, IL-8 and IL-12p40 in the ASD group compared with TD controls ($p < 0.04$). Moreover, when the ASD group was separated based on the onset of symptoms, it was noted that the increased cytokine levels were predominantly in ASD children who had a regressive form of ASD. In addition, increasing cytokine levels were associated with more impaired communication and aberrant behaviors. In conclusion, using larger number of participants than previous studies, we report significantly shifted cytokine profiles in ASD. These findings suggest that ongoing inflammatory responses may be linked to disturbances in behavior and require confirmation in larger replication studies. The characterization of immunological parameters in ASD has important

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implications for diagnosis, and should be considered when designing therapeutic strategies to treat core symptoms and behavioral impairments of ASD.

Introduction

Autism Spectrum Disorders (ASD) are a heterogeneous group of neurodevelopmental disorders characterized by severe impairments in social interaction and communication, and restricted, stereotyped interests that manifest in early childhood (American Psychiatric Association, 2000). Recent epidemiologic data suggest that approximately 1% of children are diagnosed with an ASD {MMWR, 2009}. In the majority of cases, the etiology of ASD is not known and likely involves complex interactions between genetic, epigenetic and environmental factors. Recent papers have described links between genes that encode for immune-related proteins and ASD, suggesting that abnormalities in the immune system may influence aspects of brain development and synaptic functions that negatively impact clinical outcomes relevant to ASD (reviewed in Enstrom et al., 2009a). Taken together with well-established reports of cytokine mediated influences on neuronal function, differentiation, migration, proliferation, and behavioral impairments in animal models, there is an emerging view of synergistic relationships between immune dysfunction and genetic predisposition that contribute to a subset of ASD cases.

Altered immune responses in individuals with ASD have been reported for nearly 40 years including the presence of self-reactive antibodies to brain and CNS proteins (Cabanlit et al., 2007; Connolly et al., 2006; Silva et al., 2004; Todd et al., 1988; Vojdani et al., 2002; Wills et al., 2009) and evidence for increased neuroinflammation in brain and CNS specimens obtained from subjects with ASD (Vargas et al., 2005; Li et al., 2009; Garbett et al., 2008). Several studies have shown peripheral immune abnormalities in patients with ASD including abnormal or skewed T helper cell cytokine profiles (Ashwood et al., 2006; Gupta et al., 1998), decreased lymphocyte numbers (Ashwood et al., 2003), altered T cell responses to mitogens and recall responses (Molloy et al., 2006), an imbalance of serum immunoglobulin levels (Enstrom et al., 2009b), NK cell activation (Enstrom et al., 2009c), increased monocyte responses (Enstrom et al., 2010) and increased levels of complement components in ASD (Corbett et al., 2007). Taken together, these findings support alterations in the immune responses in a significant proportion of children with ASD.

Few studies have addressed if cytokine patterns differ in the sera or plasma of ASD (Ashwood et al., 2008; Crooenberghs et al., 2002; Enstrom et al., 2008a; Okada et al., 2007; Singh et al 1991; Singh et al., 1996; Sweeten et al., 2004; Zimmerman et al., 2005) and of these only three have attempted to evaluate whether cytokine levels are associated with core defects of ASD or impairments in associated behaviors and/or onset patterns of ASD (Ashwood et al., 2008; Enstrom et al., 2008a; Okada et al., 2007). A subset of these studies have demonstrated increased levels of cytokines that can induce inflammation in ASD, such as IFNγ or IL-12, (Sweeten et al., 2004; Singh et al 1996), or a decreased production of cytokines that negatively regulate inflammation, such as TGFβ1 (Ashwood et al., 2008; Okada et al., 2007). However, varied experimental designs, diagnostic criteria, ages of the probands and control populations (including the comparison of children with ASD with adult controls) have confounded interpretation of the results. Moreover, small sample sizes and the use of non-clinically assessed siblings of children with ASD as controls have dampened the ability to detect differences in cytokine patterns, thwarting scientific progress towards consensus regarding whether particular cytokine patterns reflect an inherent immune dysfunction in ASD.

Based on the abnormal immune dysfunction observed in ASD, we herein evaluated a comprehensive panel of inflammatory cytokines associated with general immune activation in plasma samples from a large series of well-characterized participants enrolled in a population based case-control study. To better define the immune status of children with ASD, cytokine levels were assessed in ASD children 24-60 months and compared with typically developing children and children with developmental disabilities other than ASD who were of the same age. In addition, cytokine profiles in children with ASD were investigated for any associations with clinical behavioral outcomes.

Methods

Subjects

Participants in the study were enrolled in the CHARGE (Childhood Autism Risks from Genetics and Environment) study conducted at the UC Davis M.I.N.D. Institute (Hertz-Picciotto et al., 2006). The study protocols including recruitment and behavioural assessments have been described in detail (Ashwood et al., 2008; Hertz-Picciotto et al., 2006; Enstrom et al., 2009b). In brief, after clinical evaluations, participants were placed in one of 3 groups: 1) diagnosed with autism spectrum disorders; 2) diagnosed with developmental delay but not autism; or 3) confirmed as typically developing controls. Two hundred and twenty-three (223) children were selected based on available volumes of plasma from consecutively recruited participants. Participants were not different in age or sex ratios. All children were medication free and in good health at time of blood draw. Participants included 97 children with ASD (median age 3.4 (interquartile range 2.9-4.3), 84 males), 87 TD controls (3.4(2.8-4.1), 71 males) and 39 DD controls (3.5(3.0-4.1), 28 males). This study was approved by the UC Davis institutional review board and complied with all requirements regarding human subjects. Parents gave informed consent. Autism spectrum disorder diagnosis was performed using gold-standard assessments based on Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (American Psychiatric Association, 2000) criteria by qualified trained clinicians at the M.I.N.D. Institute. To confirm and further evaluate the diagnosis, all ASD participants completed the Autism Diagnostic Interview-Revised (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS). Children confirmed as ASD were divided into subgroups of non-regressive or regressive autism based on the stage of development the child began to display autistic behavior (Hansen et al., 2008). Children with ASD were classified as having no-regression if the child exhibited traits of autism from infancy, and regressive autism if they had typical early development and later lost function in language and/or social interactions based on ADI-R scores (language loss (Q11) or any social skills loss (Q25)). There were 40 children in the ASD who had regression and 53 without regression, it was not possible to confirm onset in the remaining 4 ASD participants. Children from the TD and DD groups were screened for autism traits using the Social Communication Questionnaire (SCQ). For all children, adaptive function was assessed by parental interview using the Vineland Adaptive Behavior Scales (VABS). Additional measures of cognitive ability were determined using the Mullen Scales of Early Learning (MSEL) and abnormal behavior profiles using the Aberrant Behavior Checklist (ABC).

Cytokine analysis

For each subject peripheral blood was collected in acid-citrate-dextrose Vacutainers (BD Biosciences; San Jose, CA), centrifuged at 2300 rpm for 10 min, and the plasma harvested. Plasma was aliquoted and stored at -70 °C until cytokine analysis. Plasma samples were run in concordance with the instructions of the kit protocol. The quantification of the cytokines GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12(p40), IL-13, and TNF-α in the plasma was determined using human multiplexing bead immunoassays (Biosource,

Camarillo, CA) that are based on a sandwich immunoassay that utilize the Luminex® fluorescent-bead-based technology. Briefly, 50 μL of plasma sample were incubated with antibody-coupled beads. After a series of washes, a biotinylated detection antibody was added to the beads, and the reaction mixture was detected by the addition of streptavidin-phycoerythrin. The bead sets were analyzed using a flow-based Luminex™ 100 suspension array system (Bio-Plex 200; Bio-Rad Laboratories, Inc.). Unknown sample cytokine concentrations were calculated by Bio-Plex Manager software using a standard curve derived from the known reference cytokine concentrations supplied by the manufacturer. A five-parameter model was used to calculate final concentrations and values are expressed in pg/ml. The sensitivity of this assay allowed the detection of cytokine concentrations within the following ranges: GM-CSF 15-9940 pg/ml; IFN-γ 5-4740 pg/ml; IL-1β 15-6810 pg/ml; IL-2 6-7160 pg/ml; IL-4 5-4910 pg/ml; IL-5 3-5390 pg/ml; IL-6 3-5200 pg/ml; IL-8 3-6330 pg/ml; IL-10 5-4550 pg-ml; TNF-α 10-11390 pg/ml. Concentrations obtained below the sensitivity limit of detection (LOD) of the method were calculated as LOD/2 for statistical comparisons. Values obtained from the reading of samples that exceeded the upper limit of the sensitivity method were further diluted and cytokine concentrations calculated accordingly. Plasma aliquots had not undergone any previous freeze/thaws cycle.

Statistical Analysis

Descriptive statistics were computed for selected demographic variables across diagnostic groups. Covariates of interest as possible confounders included the child's age at blood draw and gender. In analyses involving questionnaire and behavioral assessment score data, diagnostic group was also evaluated as a covariate. In primary analyses, natural log-transformed cytokine levels (outcome) were compared by group (predictor) in multiple linear regression models adjusted for child's age and gender. P values were corrected for multiple comparisons using the Benjamini-Hochberg False Discovery Rate. Secondary analyses examined the association between cytokine levels (predictor) and behavioral assessment scores. These multiple linear regression models were adjusted for diagnostic group, as well as child's age, gender and multiple comparisons (as above). Findings with *P*-value <0.05 after correction were considered significant. All analyses were carried out using SAS version 9.1 (SAS Inc.; Cary, NC) and Prism 5 Software (GraphPad Software; San Diego, CA).

Results

Levels of IL-6 and IL-12p40 were significantly higher in children with ASD compared with TD and DD controls (Table 1). After adjusting for child's age and gender, levels of IL-6 were approximately two-fold higher in plasma collected from ASD children (median 20.3; interquartile range 9.5-43.5 pg/mL) when compared with TD (11.8; 3-29.2 pg/mL; $p = 0.01$), and elevated nearly seven fold compared with DD controls (3; 3-18.8 pg/mL; $p = 0.03$). Levels of IL-12p40 were significantly higher in plasma sampled from children with ASD (199.2; 162.4-277.9 pg/mL) compared with TD (171.7; 125.6-241.7 pg/mL; $p = 0.04$) and DD controls (169.7; 119.9-221.9 pg/mL; $p = 0.03$). In addition, levels of IL-8 sampled from children with ASD were significantly higher (14.1; 3.3-22.1pg/mL) compared with those from TD controls (3.9; 3-10.6 pg/mL; $p = 0.002$) and were elevated compared with DD controls but the difference did not reach statistical significance (4.9; 3-19.6 pg/mL; $p = 0.14$). Similarly, IL-1β was significantly raised in plasma samples from children with ASD (110.6; 25.7-245.8 pg/mL) compared with TD controls (62.8; 15-148.8 pg/mL; $p = 0.04$), but after correcting for multiple comparisons, differences did not reach statistical significance when compared with DD controls (46.1; 15-153.8 pg/mL; $p = 0.1$), and may be due to the relatively fewer numbers of DD subjects in our study. Finally, there was a trend showing higher, GM-CSF in children with ASD (63; 32.7-118.6 pg/mL) compared with TD

(54.2; 15-92.8 pg/mL) and DD controls (52.3; 15-123.8 pg/mL) but this was not significant after correcting for multiple comparisons. There were no differences in any cytokine measure between the TD and DD groups and cytokine levels were not related to IQ (data not shown).

The children with ASD were further subdivided into those who exhibited regression and those with early onset ASD (Table 2). There were no significant differences in psychological measures based on the ABC, ADI-R, ADOS, MSEL and VABS scores between ASD children who had regressed and those that had not (data not shown). In general, cytokine levels were higher in children with regression compared with those that had early onset ASD (Table 2). Higher levels of IL-1β were measured in plasma of ASD children with regression (median 144.3; range 64.6-353.3 pg/mL) compared with ASD children without regression (61; 15-241.8 pg/mL: $p = 0.04$). Observed levels of GM-CSF in children with ASD regression were also elevated (101.2; 45.8-133.4 pg/mL) compared with ASD children without regression (51.3; 15.4-81.2 pg/mL, $p = 0.01$). When compared with TD controls, the levels of IL-8 were significantly increased in the plasma of both regressive ASD children ($p = 0.001$) and non-regressive ASD children ($p = 0.05$). However, as compared with TD controls, levels of IL-1β ($p = 0.002$), IL-6 ($p = 0.008$), GM-CSF ($p = 0.05$) and IL-8 ($p = 0.001$) were only significantly raised in plasma samples obtained from ASD children with regression (Table 2) and not in non-regressive ASD children.

We then examined whether there were associations between cytokine levels and the severity of clinical behavioral outcomes. Based on ADI-R assessment scores in children with ASD, increased IL-4 was associated with greater impairments in non-verbal communication ($t = 2.61$, $p = 0.03$). There was also trends for associations between more impaired social interactions measured by ADI-R and plasma IL-β levels. ($t = 2.15$, $p = 0.09$) and IL-10 ($t = 1.82$, $p = 0.07$) but these did not reach statistical significance after correction for multiple comparisons.There was a trend for an association between elevated IL-13 levels and the degree of impaired social interactions assessed by ADOS module 1 but this did not reach statistical significance after correction for multiple comparisons($t = 2.15$, $p = 0.06$). In addition, significant associations were observed between cytokine levels and aberrant behaviors measured in the ABC such that increased lethargy ($t = 2.58$, $p = 0.03$), stereotypy ($t = 2.56$, $p = 0.02$), hyperactivity ($t = 2.89$, $p = 0.02$) as assessed by ABC were associated with increased IL-8 levels. Increased lethargy ($t = 2.41$, $p = 0.04$) and stereotypy ($t = 2.55$, $p = 0.05$) were associated with increased IL-12p40 levels. In addition, increased stereotypy ($t = 2.21$, $p = 0.05$) was associated with increased IL-6 levels and also with increased IL-1β levels ($t = 3.07$, $p = 0.01$). Finally, IL-8 was associated with MSEL scores of visual reception ($t = -2.65$, $p = 0.04$), receptive language ($t = -2.35$, $p = 0.04$) and expressive language ($t = -2.27$, $p = 0.04$) as well as VABS scores of daily living ($t = -2.32$, $p = 0.04$) such that as IL-8 decreased cognitive and adaptive ability improved.

Discussion

Although the etiology and pathogenesis of ASD remain unclear, it is suggested that there may be an association with immune dysfunction (Enstrom et al 2009a). Marked neuroinflammation and microglia cell activation is one of the most prominent ASD characteristics (Vargas et al., 2005), along with increased circulating autoantibodies to brain or CNS tissue (Wills et al., 2009). Further, altered T cell activation (Ashwood et al., 2004; Ashwood et al., 2006; Molloy et al., 2006; Onore et al., 2009; Sarasella et al., 2009), increased monocyte cell activation (Enstrom et al., 2010; Jyonouchi et al., 2005; Sweeten et al., 2004), increased basal levels of NK cell activation but decreased response to stimulation (Enstrom et al., 2009c), skewed immunoglobulin profiles (Croonberghs, et al., 2002; Enstrom et al, 2009b) and alterations in the levels of complement components (Corbett et

al., 2007) have all been described in ASD and may provide pathogenic clues. The underlying mechanisms linking immune dysregulation and neuronal dysfunction is not clear, but there is evidence indicating that certain cytokines can impair neurodevelopment and behavior. Cytokines and their receptors are found in the healthy CNS during neural differentiation and plasticity and play important modulatory roles in these processes. For example, neuropoietic cytokines, such as IL-6, can directly alter neuron proliferation, survival, death, cortical neuron dendrite development, neural activity, long-term potentiation and neurodevelopment that may impact behavior (Gadient and Patterson 1999; Gilmore et al., 2005; Jutler et al., 2002; Mehler and Kessler 1998; Shi et al., 2003) while IL-1β and TNFα have been linked with oligodendrocyte toxicity, neurite growth and the regulation of homeostatic synaptic plasticity in the hippocampus (Barker et al., 2001; Cacci et al., 2008; Munoz-Fernandez and Fresno 1998). Thus cytokine dysregulation may have important biological effects on neuronal development and activity that adversely affect behavior.

In the present study, we report statistically significant elevation of IL-1β, IL-6, IL-8 and IL-12p40 cytokine levels in the plasma of ASD children compared with TD controls. The elevated cytokine levels seemed to be predominantly driven by the children with a regressive form of ASD who exhibited higher cytokine levels than those ASD children with no regression. These findings are compatible with altered levels of immunomodulatory factors in children with regressive ASD compared to non-regressive ASD children (Ashwood et al., 2008b; Bu et al., 2005; Enstrom et al., 2008a; Enstrom et al., 2008b). In addition, we found associations between the plasma levels of several cytokines and severity of certain core measures of ASD as assessed by ADI-R (IL-4), as well as aberrant behaviors as measured by ABC (IL-1β, IL-6, IL-8, IL-12p40), such that impairments in behavior were more pronounced as certain cytokine levels increased. In particular, several cytokines were observed to be associated with more impaired stereotypypical behaviors (IL-1, IL-6, IL-8 and IL-12p40). It is currently unclear how cytokines could affect sterotypical behaviors during childhood in ASD and these data should be treated with caution until further validation can be performed. Notably several other studies have shown increased severity of impairments in social interactions, communication and stereotypical behaviors, as well as aberrant behaviors, to be associated with altered levels of immune factors including: transforming growth factor-beta1 (Ashwood et al., 2008a), macrophage inhibitory factor (Grigorenko et al., 2008), platelet-endothelial adhesion molecule (Tsuchiya et al., 2007), immunoglobulin-G4 (Enstrom et al., 2009b), monocyte activation (Enstrom et al., 2010) and IL-23 (Onore et al., 2009). These observations and our own results may suggest that dysfunctional immune responses and/or activity may affect core features of ASD as well as associated behaviors in children with ASD. Another possibility is that dysfunction in common basic cellular processes, such as those involved in signaling, may be manifest as aberrations in both the immune and neuronal systems. As ASD may encompass several distinct phenotypes, the potential separation of ASD subgroups based on immunological parameters and/or associations with worsening behavior may have important implications for diagnosis, as well as the design and monitoring of therapeutic treatments of ASD. Further validation of the associations observed here between cytokine levels and behaviors is necessary, including analysis of other features often associated with ASD such as gastrointestinal problems, seizures, macrocephaly, cognitive impairments, and sleep disorders. Most critically, longitudinal studies beginning prior to diagnosis would provide the best means for understanding whether these changes play an etiologic role, have prognostic potential, or are purely phenomenological.

The pattern in ASD of increased IL-6 and IL-1β cytokine levels, which are often described as archetypal pro-inflammatory cytokines, is consistent with *in vitro* evidence for higher production of these same cytokines by cultured monocytes from children with ASD when challenged with ligands from infectious agents (Enstrom et al., 2010) and may thus represent

an imbalance in the innate immune compartment favoring a breakdown of self-tolerance (Enstrom et al., 2009a). It should be noted that due to well documented sensitivity issues with the Luminex assay, baseline levels and detected levels of some cytokines are not the same as those observed by ELISA techniques, in particular this is true of the IL-1β and TNFα results; however, additional experiments were able to confirm between groups differences (data not shown). Moreover, increased expression of gene transcripts for IL-6 and IL-1 pathways are noted in postmortem specimens of the temporal cortex of the brain of individuals with ASD (Garbett et al., 2008) and increased protein levels of IL-6, and IL-1β have also been found in the brain and CSF of individuals with ASD (Vargas et al., 2005; Li et al., 2009). These cytokines appear to have been derived from microglial and astroglial cells and also implicate that disturbances of the innate immune system are relevant in ASD (Vargas et al., 2005). Few studies have analyzed plasma cytokines levels in children with ASD; however, consistent with our results is a previously reported finding of increased IL-12 in plasma from ASD individuals compared with that from controls (Singh et al., 1996) which may suggest a T_H1 plasma cytokine profile. In this study we also found increased IL-8 levels, a chemoattractant cytokine important in inflammatory process, a finding that corresponds to similar increases of IL-8 seen in the brain and CSF of individuals with ASD (Vargas et al., 2005; Li et al., 2009). Other studies have reported cytokine dysregulation in ASD including increased IFNγ, IL-2, TNFα, GM-CSF, IL-4, IL-5 in either the brain tissue, CSF, plasma, serum, cultured PBMC or cultured NK-cells from individuals with ASD (Croonenberghs et al., 2002; Enstrom et al., 2009c; Jyonouchi et al., 2005; Li et al., 2009; Molloy et al., 2006; Vargas et al., 2005). However, other studies have failed to demonstrate any differences in plasma IFNγ or TNFα levels (Singh et al., 1996; Sweeten et al., 2004), or have demonstrated decreased IFNγ or IL-2 responses to stimulation in mononuclear cell cultures in individuals with ASD (Gupta et al., 1998). These discrepancies are most likely due to characteristics of the patient populations under investigation and/or mismatching of the age of the cases and controls - with several studies comparing adult controls with ASD children - as well as to the analytical techniques, power of the statistical analysis and tissue / specimens used. Often further complicating these studies has been the use of siblings of ASD children as controls. As ASD is highly heritable, similar findings in analyses of the immune system in ASD children and their siblings most likely reflects shared immune susceptibilities that are upstream of the CNS pathology (Ashwood et al., 2008b; Sarasella et al., 2009). A further issue highlighted by the current study is the difference in the cytokine profile between those children with no regression and those with regression. Although it is hard to ascertain from previous studies how many study subjects with regression were included, it may be differences in the subgroups of ASD that lead to the variable reports for plasma cytokine levels reported in the literature thus far. Whether increased cytokine production occurs in all children with regression or that the regressive group has a higher number of individuals with high cytokine production – a putative “high-immune responder” group – needs further investigation, as do the apparent associations between cytokines and more impaired behaviors.

In summary, using a large number of participants from a population based case-control study, we demonstrate that there are significant increases in plasma IL-1β, IL-6, IL-8 and IL-12p40 levels in young children who have confirmed ASD as compared with confirmed and age-matched typically developing children. These levels were predominantly driven by increases in cytokines in CHARGE study children who experienced loss of language or social skills. Furthermore, elevations in these cytokine levels were associated with greater severity in the core domains of ASD and with greater impairments in aberrant behaviors. These findings suggest that ongoing inflammatory responses may be linked to disturbances in behavior, a conjecture that requires confirmation in a larger study, and most critically, in longitudinal investigations of the changes in cytokine levels prior to the diagnosis or development of ASD symptoms and over the natural history of the disorder. The biological

impact of increased cytokines in ASD children and their association with more impaired behaviors is intriguing and warrants further consideration.

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Table 1

Comparison of plasma cytokine levels in children with autism spectrum disorders (n=97), typically developing (n=87) controls and children with developmental disabilities other than autism (n=39). Data are presented as median and interquartile ranges.

	Typically Developing	Developmental Delay	Autism spectrum disorders
IL-1β	62.8 (15-148.8)	46.1 (15-153.8)	**110.6*** (25.7-245.8)
IL-2	8 (6-83.9)	7.3 (6-97.8)	22.9 (6-89.3)
IL-4	36.7 (16.8-83.3)	39.9 (14.1-58.2)	35.5 (18.2-66.2)
IL-5	9.8 (3-17.1)	8.5 (3-14.5)	99 (3-14.9)
IL-6	11.8 (3-29.2)	3 (3-18.8)	**20.3***# (9.5-43.5)
IL-8	3.9 (3-10.6)	4.9 (3-19.6)	**14.1*** (3.3-22.1)
IL-10	16.4 (5-38.8)	12.4 (5-24.1)	11.9 (5-33.8)
IL-12 p40	171.7 (125.6-241.7)	169.7 (119.9-221.9)	**196.2***# (162.4-277.9)
IL-13	20.9 (11.5-36.1)	18 (10-36.4)	20 (10-38.7)
GM-CSF	54.2 (15-92.8)	52.3 (15-123.8)	63 (32.7-118.6)
IFNγ	62.8 (23.4-172.5)	84.3 (16.8-142.6)	70.2 (25.9-135.8)
TNFα	63.9 (20.7-128.1)	51.6 (29.1-144.4)	81.8 (34.5-204.7)

* $p<0.05$ compared with typically developing controls;

\# $p<0.05$ compared with developmentally delay controls.

Table 2

Comparison of plasma cytokine levels in children with autism spectrum disorders (ASD) based on onset of symptoms. Children who were ASD and regressed (n=40) are compared with ASD children with no regression (n=53) as based on definitions determined using ADI-R. (Note in n=4 subjects clinical regression could not be determined). Data are presented as median and interquartile ranges.

		Autism spectrum disorders	
	Typically Developing	**No-Regression**	**Regression**
IL-1β	62.8 (15-148.8)	61 (15-241.8)	**144.3***‡ (64.6-353.3)
IL-2	8 (6-83.9)	17.7 (6-63.7)	19.3 (6-114.4)
IL-4	36.7 (16.8-83.3)	28.8 (14.9-65.3)	39.4 (27.3-79.3)
IL-5	9.8 (3-17.1)	9.2 (3-12.5)	11.5 (5.1-19.4)
IL-6	11.8 (3-29.2)	15.1 (3-34.7)	**32.6*** (14.2-62.5)
IL-8	3.9 (3-10.6)	**6.8***(3-23.7)	**14.5*** (3.7-21.7)
IL-10	16.4 (5-38.8)	7.5 (5-25.9)	15.6 (5-34.66)
IL-12 p40	171.7 (125.6-241.7)	**192.3*** (160.4-272.7)	**198.6*** (155-255.7)
IL-13	20.9 (11.5-36.1)	14.1 (10-40.1)	29.4 (13.4-40.1)
GM-CSF	54.2 (15-92.8)	51.3 (15.4-81.2)	**101.2***‡ (45.8-133.4)
IFNγ	62.8 (23.4-172.5)	51.2 (16.8-135.9)	94.1 (45.4-194.6)
TNFα	63.9 (20.7-128.1)	56.2 (26.3-136.8)	111.1 (43.2-208.8)

* $p<0.05$ compared with typically developing controls;

‡ $p<0.05$ compared with no-regression autism spectrum disorder children.