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Elevated immune response in the brain of autistic patients

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ABSTRACT

This study determined immune activities in the brain of ASD patients and matched normal subjects by examining cytokines in the brain tissue. Our results showed that proinflammatory cytokines (TNF-α, IL-6 and GM-CSF), Th1 cytokine (IFN-γ) and chemokine (IL-8) were significantly increased in the brains of ASD patients compared with the controls. However the Th2 cytokines (IL-4, IL-5 and IL-10) showed no significant difference. The Th1/Th2 ratio was also significantly increased in ASD patients. Conclusion: ASD patients displayed an increased innate and adaptive immune response through the Th1 pathway, suggesting that localized brain inflammation and autoimmune disorder may be involved in the pathogenesis of ASD.

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1. Introduction

Autistic spectrum disorders (ASD) are complex, pervasive developmental disorders of childhood characterized by impairments in social interaction, deficits in verbal and non-verbal communication, and restricted repetitive and stereotyped patterns of behavior and interests (DSMIV criteria, American Psychiatric Association, 1994). The prevalence of ASD is currently estimated to be 6–7 per 1000, affecting 4 times more boys than girls (Rice, 2007). Susceptibility to ASD is clearly attributable to genetic factors (Folstein and Rosen-Sheidley, 2001), but the etiology of the disorder is unknown, and no biomarkers have yet been identified as characteristic of ASD. Recent reports suggest that a combination of environmental or perhaps in utero risk factors, autoimmune risk factors and localized inflammation of the central nervous system may contribute to the pathogenesis of ASD (Nelson et al., 2001; Vargas et al., 2005; Zimmerman et al., 2005; Chez et al., 2007). Jyonouchi et al. (2001, 2002) demonstrated that elevated levels of the proinflammatory cytokines tumor necrosis factor (TNF)-α and IL-1β were produced by peripheral blood mononuclear cells from children with ASD. Singh (1996) reported increased plasma levels of the Th1 cytokines IL-12 and IFN-γ, and Croonenberghs et al. (2002) found increased levels of IFN- γ in the supernatant of whole blood cultures from children with ASD. In addition, Molloy et al. (2006) reported that children with ASD had increased activation of both Th2 and Th1 arms of the adaptive immune response in blood mononuclear cells, with a Th2 predominance, and without the compensatory increase in the regulatory cytokine IL-10. In both peripheral blood and intestinal mucosa, Ashwood and Wakefield (2006) found that CD3+TNF-α and CD3+IFNγ were increased and CD3+IL-10 were markedly decreased in ASD children with GI symptoms. However, it is difficult to interpret these findings with respect to the pathogenesis of autism since it is not clear that the immune findings in peripheral blood mononuclear cells in autistic children correlate with immune-mediated pathology within the central nervous system. Recently, one study investigated the nature of inflammatory responses in the brain of autistic patients by examining 79 proteins including cytokines, chemokines and growth factors using protein array methods (Vargas et al., 2005). In this study, Vargas et al. demonstrated that transforming growth factor (TGF)-β1, derived from neuroglia, was significantly increased in the middle frontal gyrus (MFG) of autistic patients, while macrophage chemoattractant protein (MCP)-1, IL-6 and IL-10 were increased in the anterior cingulated gyrus (ACG). In addition, using protein array approach, Vargas et al. (2005) also found that MCP-1, IL-6, IL-8 and IFNγ were significantly increased in the cerebrospinal fluid (CSF). One of the advantage of protein array is the ability to detect a whole category of proteins in a very efficient way using very small quantity of tissues. But it is also less specific comparing with enzyme-linked immunosorbent assay (ELISA). (Simpson, 2003). To further investigate whether immune-mediated mechanisms are involved in the pathogenesis of autism and gain a clearer picture of cytokine activities in the brain of autistic patients, we carried out the study to

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determine the activities of a set of cytokines including the pro-inflammatory cytokines (IL-6, IL-1β, TNF-α, GM-CSF), Th1 cytokines (IL-2, IFN-γ), Th2 cytokines (IL-4, IL-5, IL-10) and chemokine (IL-8) in the brain of ASD patients using Multiplex Bead Immunoassays. We focused on using the brain tissue other than blood and serum samples, so that the results can directly reflect the immune response in the brain. The reason we chosen the above set of cytokines to be measured is because that they represent cytokines from different category and therefore both innate and adaptive immune responses can be determined. Our results showed that TNF-α, IL-6, GM-CSF, IFN-γ and IL-8 were significantly increased in the brains of ASD patients compared with the control subjects. Th1/Th2 ratio was also significantly elevated in ASD patients. In addition, we found that the regulatory IL-10 was not compensatory increased in response to the Th1 cytokines increase in ASD patients.

2. Materials and methods

2.1. Study material

Frozen human brain tissue (frontal cerebral cortex) of 8 autistic patients and 8 age and gender matched normal subjects were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders. Donors with autism fit the diagnostic criteria of the Diagnostic and Statistical Manual—IV, as confirmed by the Autism Diagnostic Interview—Revised. This study was approved by the Institutional Review Board of the NY State Institute of Basic Research and the subjects' information were summarized in Table 1.

2.2. Preparation of brain tissue homogenate

Frozen frontal cortex tissue was homogenized (10% W/V) in cold buffer containing 50 mM Tris–HCl (pH 7.4), 8.5% sucrose, 2 mM EDTA, 10 mM β-mercaptoethanol and protease inhibitor cocktail (Sigma-Aldrich). The protein concentration was assayed by the Bradford method (Bradford, 1976).

2.3. Multiplexed analyses of cytokines in brain cortex with the Bio-Plex system

A standard capture sandwich assay was used to determine the levels of different cytokines in brain cortex. Each captured antibody was coupled to a different bead set (Invitrogen's Multiplex Bead Immunoassays). The system used a liquid suspension array of 10 sets of beads (Invitrogen, Human Cytokine 10-plex) internally dyed with different ratios of two spectrally distinct fluorochromes to assign a unique spectral address. Each set of beads was combined with a monoclonal antibody raised against GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, and TNF-α. Beads were incubated first (2 h, at room temperature) with diluted standards (serial dilutions from 1.95 to 32,000 pg/ml) or the brain cortex sample (soluble fraction of homogenate), and then with biotinylated detector antibodies (30 min, at room temperature). They were washed twice in phosphate-buffered saline, and incubated for 30 min at room temperature with phycoerythrin-conjugated streptavidin. Cytokine levels were measured with a Luminex 200™ system (Bio-Rad Laboratories). Each measurement was taken in duplicate. Standard curves were generated by using the reference cytokine concentrations supplied by the manufacturer. Raw data (mean fluorescent intensities) were analyzed by Bio-Plex Manager Software 4.1 version (Bio-Rad Laboratories) to obtain concentration values.

Table 1
Study subject information

Case no.	Age (year)	Sex	Group	PMI (h)	Cause of death
797	9	M	Autism	13	Drowning
4671	4	F	Autism	13	Multiple injuries
4849	7	M	Autism	20	Drowning
4899	14	M	Autism	9	Drowning
1174	7	F	Autism	14	Multiple system organ failure
1638	20	F	Autism	50	Seizure disorder
1349	5	M	Autism	39	Drowning
5027	37	M	Autism	26	Obstruction of bowl due to adhesion
1185	4	M	Control	17	Drowning
1407	9	F	Control	20	Asthma
1500	6	M	Control	18	Multiple injuries
1706	8	F	Control	20	Rejection of cardiac allograft
1708	8	F	Control	20	Compressional asphyxia
4670	4	M	Control	17	Commotio coedis
4722	14	M	Control	16	Multiple injuries
4645	39	M	Control	12	HASCVD

Note: HASCVD stands for Hypertensive Arteriosclerotic Cardiovascular Disease.

2.4. Statistical analysis

Since our sample numbers are small, we used nonparametric tests to increase the robustness of the results. Group differences between autistic cases and controls in the brain cytokine levels were compared using the Mann–Whitney *U* test. Significance was assessed at the 0.05 level.

3. Results

The mean age of autistic group and the control group is 12.8 years and 12.5 years respectively. PMI (post-mortem index) is the time elapsed until brain tissue obtainment post-mortem. The mean values of PMI in autistic group and the control group were 23±11 and 18±5 respectively. There was no significant differences between the two groups ($P>0.05$). In addition, there was no significant regressional relationship found between the length of PMI and the values of cytokines in these samples. We consider that the variation of the PMI in the samples did not affect the studies. The reason of death for each study subject and other morbidities including seizure disorders and other medical conditions is also documented in Table 1.

3.1. Pro-inflammatory cytokines profile in the brain cortex of ASD patients

Pro-inflammatory cytokines (IL-6, IL-1β, TNF-α, GM-CSF) are predominantly produced by activated immune cells and involved in the amplification of inflammatory reactions. This set of cytokines was analyzed in the brain cortex from both ASD patients and the control subjects. The mean values (ASD versus control) of IL-6 and IL-1β were 2441.89±267.34 pg/mg protein versus 920.81±107.92 pg/mg protein ($P<0.001$), and 44.21±21.33 pg/mg protein versus 18.54±15.8 pg/mg protein ($P=0.11$) respectively. The mean values (ASD versus control) of TNF-α and GM-CSF were 51.81±6.72 pg/mg protein versus 29.85±4.31 pg/mg protein ($P<0.05$), and 354.04±46.38 pg/mg protein versus 193.64±26.43 pg/mg protein ($P<0.01$) (Fig. 1). Our results indicate that pro-inflammatory cytokines TNF-α, IL-6 and GM-CSF were significantly increased in the brain of ASD patients compared with the control, although IL-1β was not significantly different in ASD patients.

3.2. Th-1 cytokines profile in the brain cortex of ASD patients

IL-2 and IFN-γ, secreted by Th-1 cells (IL-2 is also produced by naïve T cells), were analyzed in the brain cortex from both ASD patients and normal subjects. The mean value of IL-2 (ASD versus control) was 18.34±4.28 pg/mg protein versus 20.87±4.13 pg/mg protein ($P>0.05$). The mean value of IFN-γ (ASD versus control) was 2.51±0.34 pg/mg protein versus 1.45±0.18 pg/mg protein ($P<0.05$)

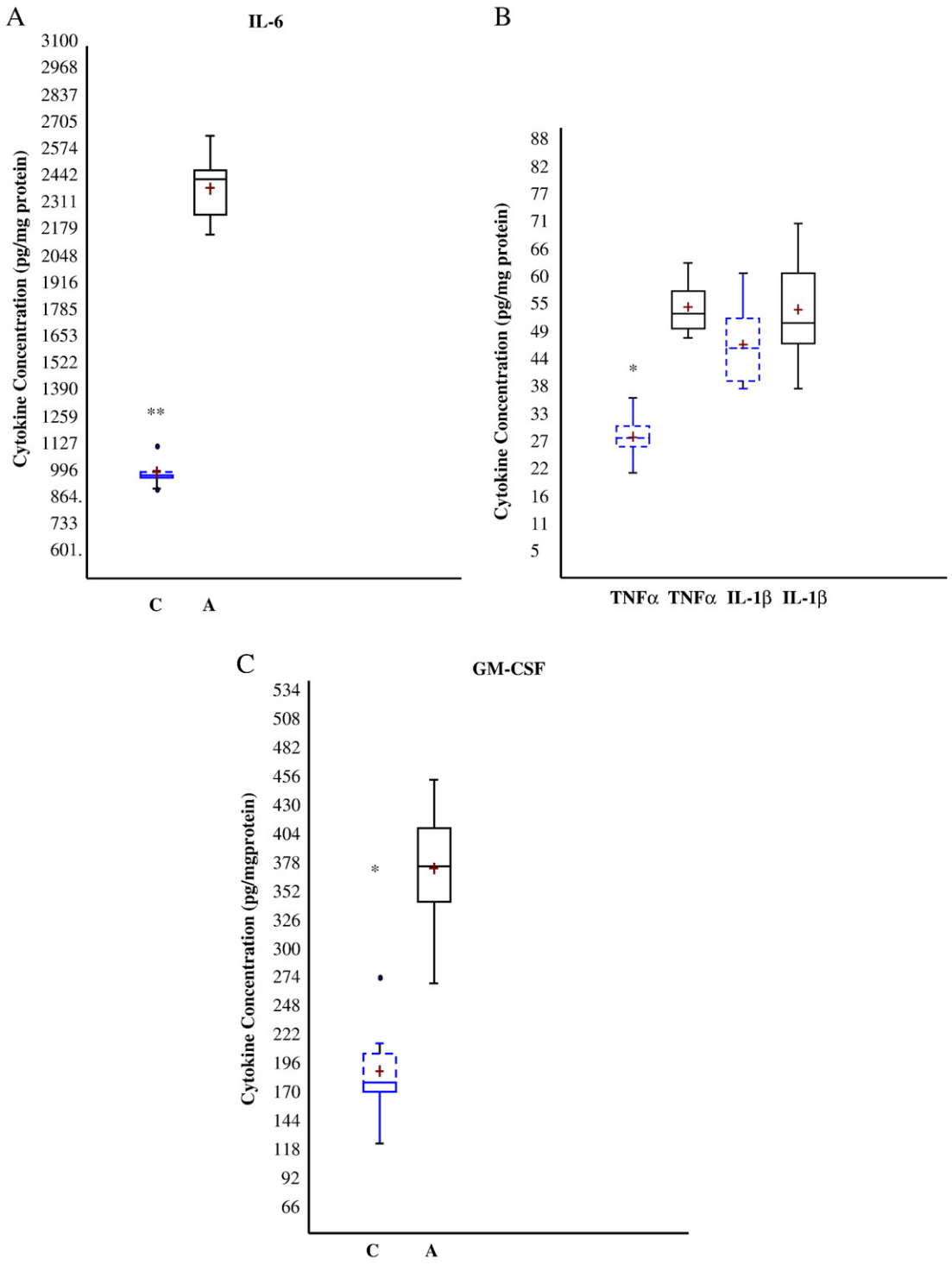

Fig. 1. Pro-inflammatory cytokines profile in the brain cortex of ASD patients. A: This figure presents the concentration of IL-6. The difference between autistic group shown as solid-line box plot and control group shown as dashed-line box plot was significant (**=$P<0.01$, $n=8$). B: This figure presents the concentration of TNF-α and IL-1β. Both TNF-α was significantly different (*=$P<0.05$, $n=8$) between the autistic group (solid-line box plot) and the control group (dashed-line box plot). IL-1β showed no significant difference between the two groups ($P>0.05$, $n=8$). C: This figure presents the concentration of GM-CSF. The difference between autistic group shown as solid-line box plot and control group shown as dashed-line box plot was significant (*=$P<0.05$, $n=8$).

(Fig. 2). Our results showed that IFN-γ was significantly increased in the brains of ASD patients.

3.3. Th-2 type cytokines profile in the brain cortex of ASD patients

IL-4, IL-5 and IL-10, secreted by Th-2 cells, were analyzed in the brain cortex from both ASD patients and normal subjects. It is worthy to point out that IL-10 is also produced by multiple lineage cells including regulatory T cells. The mean values of IL-4 and IL-5 (ASD versus control) were 11.98±1.59 pg/mg protein versus 12.33±1.87 pg/mg protein ($P>0.05$), and 30.51±4.13 pg/mg protein versus 25.8±3.46 pg/mg protein ($P>0.05$) respectively. The mean value of IL-10 (ASD versus control) was 11.13±2.18 pg/mg protein versus 13.45±2.59 pg/mg protein ($P>0.05$) (Fig. 3). Our results indicated that IL-4, IL-5 and IL-10 were not

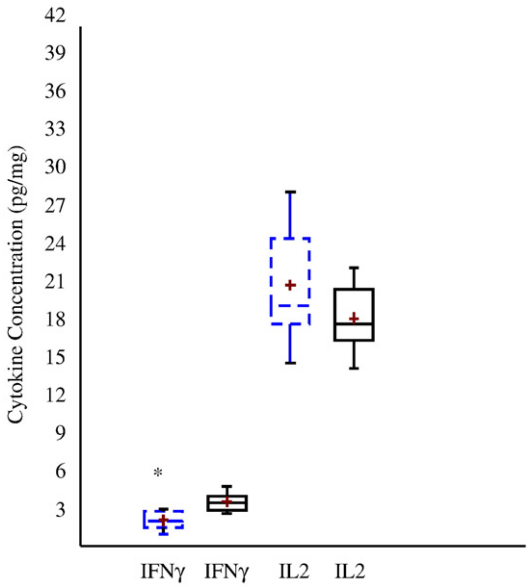

Fig. 2. Th-1 cytokines profile in the brain cortex of ASD patients. This figure presents the concentration of IFN-γ and IL-2. IFN-γ production is significantly elevated (*=$P<0.05$, $n=8$) in the autistic group (solid-line box plot) by compared with the control group (dashed-line box plot). IL-2 showed no significant difference between the two groups ($P>0.05$, $n=8$).

significantly changed in the brains of ASD patients in comparison with their matched control subjects.

3.4. Th1/Th2 cytokine ratio increases in the brains of ASD patients

The Th1/Th2 ratio determined by IFNγ/IL-4 was also analyzed in the brain cortex from both ASD patients and normal subjects. The mean value of Th1/Th2 was 0.21±0.03 in ASD patients versus 0.12±0.02 in normal subjects ($P<0.05$). This result demonstrated that the Th1/Th2 ratio was significantly increased in the brains of ASD patients (Fig. 4).

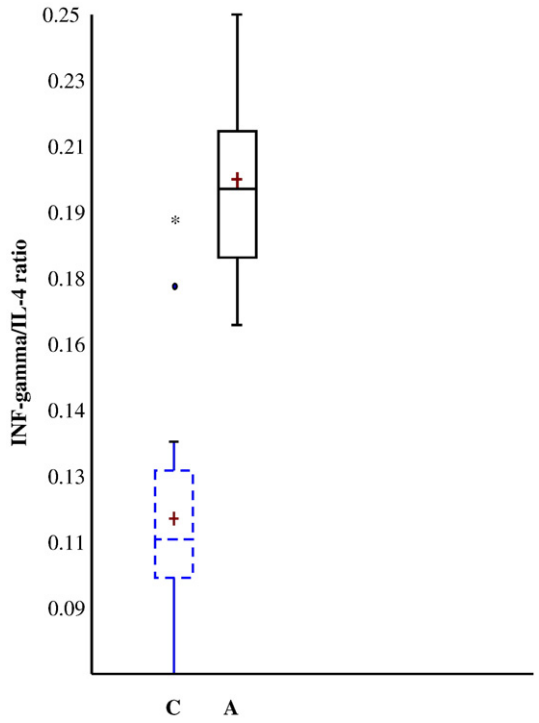

Fig. 4. Th1/Th2 cytokine ratio increases in the brain of ASD patients. This figure presents the ratio of IFN-γ concentration versus IL-4 concentration. IFN-γ/IL-4 ratio was significantly increased ($P<0.05$, $n=8$) in the autistic group (solid-line box plot) by compared with the control group (dashed-line box plot).

3.5. Chemokine profile in the brain cortex of ASD patients

Chemokine IL-8 was also analyzed in the brain cortex from both ASD patients and normal subjects. The mean value of IL-8 was 936.42±

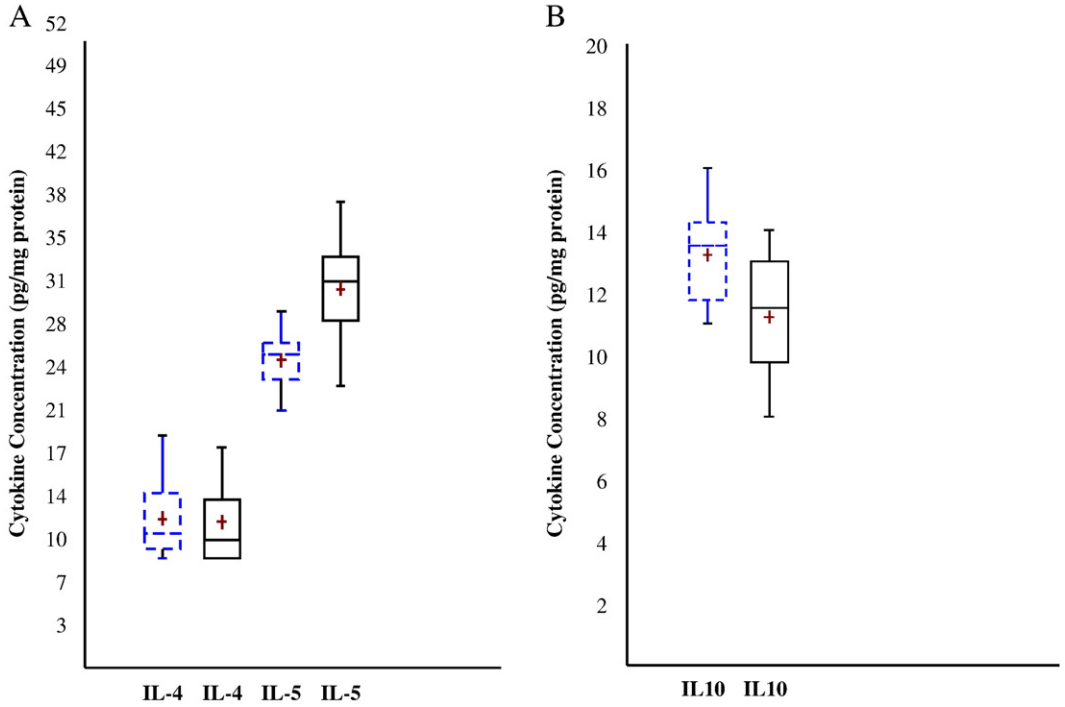

Fig. 3. Th-2 type cytokines profile in the brain cortex of ASD patients. The figures present the concentration of IL-4, IL-5 (A) and IL-10 (B). These three cytokines were not significantly different was ($P>0.05$, $n=8$) between the autistic group (solid-line box plot) and the control group (dashed-line box plot).

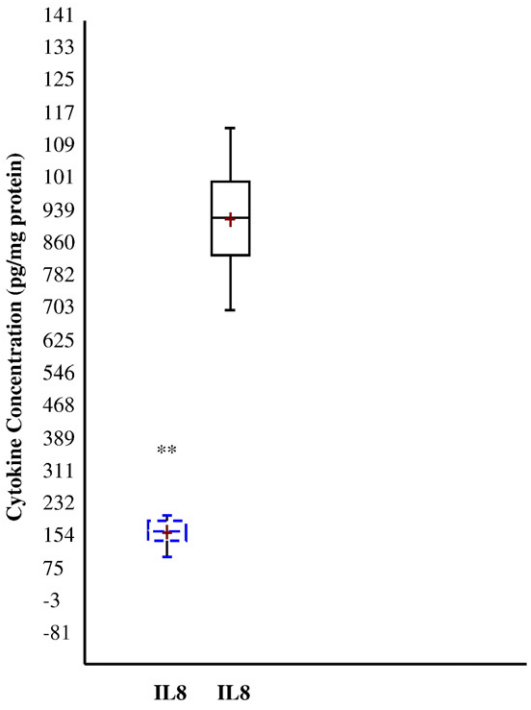

Fig. 5. Chemokine profile in the brain cortex of ASD patients. This figure presents the concentration of IL-8. IL-8 was remarkably increased ($P<0.01$, $n=8$) in the autistic group (solid-line box plot) by compared with the control group (dashed-line box plot).

111.33 pg/mg protein in ASD patients versus 157.71±21.43 pg/mg protein in normal subjects ($P<0.01$), demonstrating a dramatic increase of IL-8 in ASD patients compared with the controls (Fig. 5).

4. Discussion

Although the pathogenesis of ASD is not well understood, recent studies have suggested that localized inflammation of the central nervous system may contribute to the pathogenesis of ASD. A number of studies have shown that TNFα, IFNγ, IL-1β and IL-12 were increased in the peripheral blood of ASD patients (Zimmerman et al., 2005; Molloy et al., 2006; Ashwood and Wakefield, 2006). TNFα was also shown to be increased in the cerebral spinal fluid of autistic patients (Vargas et al., 2005; Chez, 2007). However, there is only one study that has been conducted to investigate the inflammatory cytokine profile in the brain of autistic patients using protein arrays (Vargas et al., 2005). To further characterize the nature of the inflammatory responses in autistic brains, we used a recently developed flow cytometry method (multiplexed bead analysis) to measure cytokine levels in the brain (frontal cerebral cortex) homogenate. This method provides many advantages over the traditional ELISA methods, and with better results for characterization of cytokines (Khan et al., 2004; DuPont et al., 2005). This method also makes it possible to measure several cytokines in a single sample to establish a profile of the inflammatory reaction. Our results showed that pro-inflammatory cytokines (TNF-α, IL-6 and GM-CSF) were significantly increased in the brains of ASD patients in comparison with the control subjects. TNF-α and IL-6 are cytokines involved in cell-mediated immune response and their production has been shown to be associated with tissue inflammation and necrosis (Beutler and Cerami, 1989). IL-6 has a longer half life and exerts regulatory actions in chronic inflammatory condition. The role of TNF-α as a neuromodulating agent has also been described in brain development, and it may play a role in neurons and neuroglial cells modulating glutaminergic transmission (Pickering et al., 2005). Excessive glutamate excitotoxic effects acting on NMDA receptors could occur in the presence of excess TNF-α (Pickering et al., 2005). This occurrence can lead to effects on microglial activation, as well as on nuclear factor kappa-B (NF-kB). Such changes have also been seen in models of inflammation inducing epileptic activity in which neuroglial inflammation has caused epileptic spikes (Pickering et al., 2005). Thus elevations of TNF-α, IL-6 and GM-CSF in the brain of ASD patients suggest that children with ASD have a heightened immune response that may be associated with chronic brain inflammation and tissue necrosis although etiology is unknown.

Immune responses that are stimulated by exposure to specific antigens are considered adaptive responses and are often driven by CD4+ T helper (Th) cells. Th cell clones have been classified into distinct functional types on the basis of the cytokines they secrete (Abbas et al., 1996). The most clearly defined of these subsets are Th1, which is characterized by the production of interferon (IFN)-γ and IL-2, and Th2, which is characterized by the production of IL-4, IL-5 and IL-10. The main effect or function of Th1 cytokines is phagocyte mediated defense, especially against intracellular microbes, while Th2 cytokines function to affect IgE and eosinophil/mast cell-mediated immune responses. An imbalance of these cytokines, skewed toward Th2, is seen in allergic responses (Ngoc et al., 2005) and some systemic autoimmune responses such as systemic lupus erythematosus (Spadaro et al., 2003). A skewing toward Th1 cytokines is seen in some organ specific autoimmune disorders such as insulin-dependent diabetes mellitus and multiple sclerosis (Liblau et al., 1995). In our study, we detected significant elevations in IFN-γ (Th1 origin), but not in IL-4, IL-5 and IL-10 (Th2 origin), suggesting that the brain of ASD patients have an excess adaptive response through activation of the Th1 pathway, rather than activation of the Th2 pathway. The increased activation of Th1 cytokines, but not Th2 cytokines in the ASD brain also suggests that an autoimmune disorder may be involved in the pathogenesis of ASD, but not an allergic response. Molloy et al. (2006) reported that children with ASD had increased activation of both Th2 and Th1 immune response in blood mononuclear cells, with Th2 predominance. This finding is different from our results, suggesting that immune responses in the brain and blood mononuclear cells in autistic patients could be different. The difference between Molloy's finding and our results could also be caused by different examining methods and different study subjects. In addition, the ratio of IFN-γ to IL-10 was significantly higher in ASD patients than in controls, showing an absence of a compensatory increase in IL-10. This supports the concept of a dysregulation of the adaptive immune responses in ASD patients. These results are also consistent with those reported by Jyonouchi et al. (2002) involving heightened and dysregulated innate immune responses in children with ASD as evidenced by elevated proinflammatory cytokine production from their peripheral blood mononuclear cells.

In addition, our study demonstrated that chemokine IL-8 was also significantly increased in the brain of ASD patients in comparison to the control group. IL-8 has powerful chemotactic effects on T cells and neutrophils. Elaboration of IL-8 by resident tissues is an important mechanism for directing leukocytes to migrate, especially through tissues without blood vessels. In recent years, increasing attention has been focused on chemokines as inflammatory mediators in the CNS. The limited number of studies that have investigated chemokine and chemokine receptor expression in Alzheimer's disease (AD) brains and in cell culture models seem to support a role for inflammation in AD pathogenesis (Grammas et al., 2006). Studies have also suggested a possible role of chemokines as communication molecules between neurons and microglia (Biber et al., 2008). The significant increase of IL-8 in the brain of ASD patients implicated a chemokine mediated inflammation and this excess activation of IL-8 may be a potent signal to recruit T lymphocytes that leads to the damage in the brain of ASD patients.

There are certain limitations in this investigation. The sample sizes are relatively small. In addition, only 10 cytokines were analyzed. Despite these limitations, this is the first study to our knowledge to

investigate various inflammatory cytokine expression levels in the brain of ASD patients using the most recently developed cytometry methods.

In summary, we found that various inflammatory cytokines including TNF-α, IL-6, GM-CSF, IFN-γ and chemokine IL-8 were significantly increased in the brains of ASD patients compared with the control subjects. In addition, we detected significant elevations in IFN-γ (Th1 origin), but not in IL-4, IL-5 and IL-10 (Th2 origin), suggesting that the brain of ASD patients have an excess adaptive response through activation of the Th1 pathway, rather than activation of the Th2 pathway. These findings serve as further evidence that inflammation may be an important part of the pathogenesis of ASD. Based on these findings, further investigation directed at cytokine modulation as a therapeutic approach to ASD is a potential next step.

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