Psychoneuroendocrinology 94 (2018) 162–167

Contents lists available at ScienceDirect

Psychoneuroendocrinology

journal homepage: www.elsevier.com/locate/psyneuen

Immune aberrations in children with Autism Spectrum Disorder: a case-control study from a tertiary care neuropsychiatric hospital in India

Salah Basheer[a,e], Manjunatha M. Venkataswamy[b], Rita Christopher[c], Therese Van Amelsvoort[d], Shoba Srinath[e], Satish Chandra Girimaji[e], Vasanthapuram Ravi[b,*]

[a] Department of Clinical Neuroscience, National Institute of Mental Health and Neuro Sciences, Bangalore, India
[b] Department of Neurovirology, National Institute of Mental Health and Neuro Sciences, Bangalore, Karnataka, India
[c] Department of Neurochemistry, National Institute of Mental Health and Neuro Sciences, Bangalore, Karnataka, India
[d] Department of Psychiatry and Psychology, School of Mental Health and Neuroscience, Maastricht University, Maastricht, The Netherlands
[e] Department of Child and Adolescent Psychiatry, National Institute of Mental Health and Neuro Sciences, Bangalore, Karnataka, India

ARTICLE INFO

Keywords:
Autism spectrum disorder
Immunophenotyping
Cytokines
Immune dysfunction
Th17 cells
Myeloid dendritic cells
IL-6
IL-17

ABSTRACT

Multiple studies have identified the presence of peripheral immune aberrations in subjects with Autism Spectrum Disorder (ASD). However, comprehensive assessment of these peripheral immune aberrations, in the cellular and systemic compartments, in a single group of subjects with ASD is lacking. We assessed proportions of various subsets of immune cells in peripheral blood (T helper cells, T regulatory cells, B cells, monocytes, Natural Killer cells, dendritic cells) by multi-parametric flow cytometry in 50 children with ASD and compared it with thirty healthy controls matched for age, gender, socio-economic status and body mass index. There were no significant differences noted in the proportion of T regulatory cells, B cells, monocytes and Natural Killer cells, between ASD subjects and controls. On the contrary, the proportion of activated Th17 and myeloid dendritic cells were significantly higher in children with ASD. Based on these findings, group comparison of serum levels of Th17 cytokines (interleukin-6, interleukin-17A) was performed. Elevated serum levels of interleukin-6 and interleukin-17A in children with ASD corroborated our immunophenotyping findings. We did not find any significant differences among the pro-inflammatory (interleukin-1β), Th1 (interferon-γ) and Th2 (interleukin-4) cytokines. This is the first evidence with concurrent findings from immunophenotyping and cytokine data demonstrating activation of the Th17 pathway in subjects with ASD. This finding assumes significance in the light of recent maternal immune activation mouse model study that has highlighted the role of Th17 pathway in the pathophysiology of ASD. Future longitudinal studies are needed to clarify the role of this dysregulated immune pathway in the development of ASD.

1. Introduction

Autism spectrum disorders (ASD) is a group of heterogeneous neurodevelopmental disorders with persistent deficits in social interaction, language and communication abnormalities and stereotypical behavior (American Psychiatric Association 2013). Recent studies have reported an alarming rise in the prevalence of ASD (Christensen et al., 2016). The pathophysiology of ASD remains unclear and studies have reported association of several genetic and environmental factors (Mefford et al., 2012; Rossignol et al., 2014). Studies on alterations in peripheral immune components in subjects with ASD have implicated immune dysfunction in ASD (Estes and McAllister, 2015; Jyonouchi, 2013). Increased frequency of monocytes, myeloid dendritic cells, natural killer (NK) cells and aberrations in NK cell cytotoxicity have been reported in children with ASD (Ashwood et al., 2011; Breece et al., 2013; Enstrom et al., 2009; Vojdani et al., 2008). Variation in frequencies and response to *in vitro* stimulation among T cells; frequencies of both mature and activated B cells have also been reported (Ashwood et al., 2011; Mostafa et al., 2010; Saresella et al., 2009). Higher levels of pro-inflammatory cytokines including interleukin-1 beta (IL1- β), IL-6, IL-8, IL-17 and interferon-γ (IFN-γ) are reported in subjects with ASD (Masi et al., 2015). Cytokine mediators of Th17 cells have been suggested to have a role in ASD. For example, elevated levels of IL-17A, the predominant Th17 cytokine, has been detected in the serum of autistic children (Al-Ayadhi and Mostafa, 2012).

Alterations in the prenatal immune environment are suggested to contribute to the risk of developing ASD (Krakowiak et al., 2017). In mouse models of ASD, maternal immune activation (MIA) induced by

* Corresponding author.
E-mail address: vravi@nimhans.ac.in (V. Ravi).

https://doi.org/10.1016/j.psyneuen.2018.05.002
Received 7 February 2018; Received in revised form 28 March 2018; Accepted 1 May 2018

inflammatory stimuli and microbial agents are shown to alter the levels of inflammatory cytokines in the fetal and neonatal brains (Garay et al., 2013). These immune alterations lead to abnormal CNS development in the off-springs resulting in ASD like behavior. Further, the cytokine alterations were found to persist long after birth (Depino, 2013; Garay et al., 2013). In another study on the mouse MIA model, higher proportion of Th17 cells and elevated levels of plasma cytokine IL-17A in the mother during gestation were shown to induce an abnormal cortical development and ASD like behavior in the off-spring (Choi et al., 2016).

In this context, comprehensive assessment of the peripheral immune aberrations in a single group of subjects with ASD is lacking. Environmental factors are known to play a significant role in immune function (Brodin et al., 2015), and therefore immune studies from various ethnicities are essential. Further, there are no studies investigating immune dysfunction in this disorder from an Indian patient population. The present study was therefore designed to first explore the alterations in peripheral immune cell subpopulations in children with ASD in comparison to healthy controls. Following which, based on findings from immunophenotyping experiments, the relevant serum cytokines were compared between the two groups.

2. Methods

2.1. Study subjects

This prospective, cross-sectional, case-control study was conducted between November 2014 to January 2017 at the National Institute of Mental Health and Neuro Sciences (NIMHANS), Bangalore, India. The study was approved by Institute Ethics Committee of NIMHANS. Written informed consent was obtained from the legal guardians of the study participants and informed assent was taken from participants wherever applicable.

Fifty children (3–12 years of age) fulfilling criteria of diagnosis for ASD (Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2013), with Childhood Autism Rating Scale (CARS) (Schopler et al., 1980) 2nd edition score greater or equal to 30 were recruited into the study as cases. The cases were recruited in a consecutive manner from Department of Child and Adolescent Psychiatry, NIMHANS. Diagnosis of ASD was corroborated by 1) Autism Diagnostic Interview- Revised (ADI-R) (Lord et al., 1994) 2) INCLEN Diagnostic Tool for ASD (INDT-ASD) (Juneja et al., 2014) and 3) clinical judgement of two of the authors (SS and SCG). Children with known clinical syndromes like Fragile X syndrome, Rett's syndrome and Tuberous Sclerosis were excluded. Thirty typically developing children (3–12 years of age) with Social Quotient (SQ) based on Vineland Social Maturity Scale (VSMS) (Sparrow, 2011), greater than 85 and Indian Scale for Assessment of Autism (ISAA) (Ministry of Social Justice and Empowerment: Government of India 2009) score less than 70 (cut-off score for ASD) were recruited as controls. Controls were recruited by word of mouth from children of hospital staff and colleagues as well as from nearby schools. Children with neurological or psychiatric disorders or family history of ASD were excluded from control group. Participants on drugs that affect immune function like corticosteroids and Vitamin D during the 3 months prior to inclusion in the study were excluded from both the groups. Children with recent history (< 2 weeks) of infection were also excluded from the study.

Following recruitment, study participants underwent baseline data collection and physical assessments. Modified Kuppuswamy Scale, which is a standard measure widely used in India, was used for assessment of socioeconomic status (SEC) in the study participants (Kumar et al., 2007).

2.2. Immunophenotyping

Peripheral blood (5 ml) was collected for separation of peripheral blood mononuclear cells (PBMCs) for immunophenotyping from all the study participants. Serum was separated and stored at −80 °C for cytokine analysis. Isolation of peripheral blood mononuclear cells for immunophenotyping was carried out within six hours after blood collection by using density gradient separation method. Briefly, the blood was diluted with equal volume of RPMI 1640 (Sigma Aldrich, St. Louis, USA) and overlaid on 10 ml of Histopaque (Sigma Aldrich). PBMCs were separated by centrifuging at 400 g at room temperature for 25 min and washed. Cells were re-suspended in 1 ml freezing medium containing 90% heat-inactivated fetal calf serum and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) in cryovials and placed in a cryo-safe container at −80 °C overnight and transferred into liquid nitrogen for long-term storage.

Immunophenotyping was performed based on the multi-color antibody panels recommended by Human Immunology Project Consortium (HIPC) (Maecker et al., 2012). The frozen PBMCs were thawed and re-suspended in pre-warmed RPMI medium with 10% Fetal Calf Serum (FCS). The cells were stained with the various antibody panels for identifying several subpopulations of cells of the immune system such as activated and memory T cells, regulatory T cell (Tregs), B cells, natural killer cells (NK cells), monocytes and dendritic cells (DCs). The antibodies included CCR7-PE, CD4-V510, CD45RA-PE-Cy7, CD38-APC, CD8-APC-H7 and CD3-V450, CD25-PE, CD4-V510, CD127-APC, CD45RO-APC-H7 and CD3-V450, CD56-PE, CD123-PerCP-Cy5.5, CD11c-PE-Cy7, CD16-APC, CD3/19/20-APC-H7, CD14-V450 and HLA-DR-V500and HLA-DR-V500, CD24-PE, CD19-PerCP, CD27-PECy7, CD20-APC-H7 and IgD-V500 (BD Biosciences, USA). The stained samples were acquired on FACS Verse flow cytometer (BD Biosciences, USA). Dead cells were gated out by staining with the BD Horizon Fixable Viability Stain 520 (BD Biosciences, USA). The data was analysed using the Flow Jo software (Treestar Inc, USA). Gating strategies for all the immune cell populations analyzed are shown in Supplemental Figures A-D. The standard method to reduce the effects of long term storage and thawing on the various immune cell subsets were followed in this study (Kumar et al., 2017; Ramachandran et al., 2012).

2.3. Serum cytokine estimation

The serum levels of human cytokines were measured using the Cytometric Bead Array (CBA) Flex Enhanced Sensitivity kits from BD Biosciences (CA, USA). Briefly, 50 µl of a mixture of bead population with distinct fluorescence intensities, coated with specific antibodies for capturing different cytokines was incubated with 50 µl of serum for 1 h at room temperature. These cytokine-captured beads were then mixed with 50 µl of phycoerythrin-conjugated detection antibodies to form sandwich complexes. After incubation for 2 h at room temperature in dark followed by washing, the samples were subjected to flow cytometry analysis using FACS Verse machine (BD, USA). Data was analysed on the BD FCAP array software and standard curves were generated for each of the cytokine measured. The data from the standard curve was used to extrapolate the level of cytokines in the clinical samples.

2.4. Statistical analysis

Statistical analyses were conducted to assess differences in proportions of various immune cell populations and cytokine level between children with ASD and typically developing children. A univariate mean comparison test that was either parametric or non-parametric depending on the normality of the data was done. If the data were normally distributed, then parametric statistics were used (*t*-test); if not, the nonparametric rank test (Mann Whitney *U* test) was applied. Goodness-of-fit statistics (Shapiro-Wilk) was performed to assess normality. Unadjusted P values are presented for the immunophenotyping data since it was the exploratory part of the study. Moreover, the use of correction for multiple comparisons in this area is debated (Ashwood et al., 2011; Rothman, 1990). The cytokines to be analyzed were selected based on the cellular immunophenotyping findings. Correlation