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Downregulation in Helios transcription factor signaling is associated with immune dysfunction in blood leukocytes of autistic children

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ABSTRACT

Autism spectrum disorder (ASD) is a complex heterogeneous neurodevelopmental disorder in which immunological imbalance has been suggested to be a major etiological component. Helios, a transcription factor, has been studied extensively in the context of human T cell regulation in health and disease, yet the role of Helios signaling has not been examined in children with ASD. In the present study, we investigated the production of Helios in $CD4^+$, $CD8^+$, and $TIM\text{-}3^+$, $CXCR3^+$ cells in typically developing (TD) controls and children with ASD and in peripheral blood mononuclear cells (PBMCs). We assayed the production of $IFN\text{-}\gamma^+Helios^+$, $IL\text{-}21^+Helios^+$, $T\text{-}bet^+Helios^+$, and $Foxp3^+Helios^+$ cells, and determined Helios mRNA and protein expression levels in PBMCs, in TD controls and children with ASD. Our results revealed that children with ASD had lower numbers of $CD4^+Helios^+$ $CD8^+Helios^+$, $TIM\text{-}3^+Helios^+$, and $CXCR3^+Helios^+$ cells as compared to TD controls. Our results also showed that children with ASD had decreased $IFN\text{-}\gamma^+Helios^+$, $IL\text{-}21^+Helios^+$, $T\text{-}bet^+Helios^+$, and $Helios^+Foxp3^+$ production compared to that in TD controls. Moreover, our results indicated that children with ASD had lower Helios mRNA and protein expression levels compared to those in TD controls. These results suggest that the Helios transcription factor may be critical to immune alterations in children with ASD. Therefore, our results suggest that targeting Helios signaling might offer a strategy for developing ASD therapies.

1. Introduction

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder resulting in varying degrees of deficiencies in social skills, impaired communication, and stereotypic, repetitive and inflexible behaviors (American Psychiatric Association, 2015). Immune abnormalities as contributors to the development of ASD have been reported recently. In addition, ASD has been linked with immune dysfunction, autoimmunity, and neuroimmunity (Ashwood and Van de Water, 2004; Onore et al., 2012). Evidence also suggests that various types of maternal immune activation are associated with behavioral disorders, including ASD (Jonakait, 2007). Several other studies have indicated immune-mediated disorders that are common comorbidities in children with ASD (Mostafa and Al-Ayadhi, 2013; Zerbo et al., 2015). Moreover, neuroimmune dysregulation in ASD is complex and associated with the prenatal immune system (Bjorklund et al., 2016; Goines et al., 2011). There are strong indications that an imbalance in cytokine levels is a risk factor for several neurodevelopmental disorders, including ASD (Deverman and Patterson, 2009; Hsiao et al., 2013). We recently revealed that immune dysfunction through a cytokine imbalance, as well as altered transcription factor signaling, are associated with the development of ASD (Ahmad et al., 2017a, 2017b). We also showed that the Jak/Stat pathway and increased chemokine receptor expression play central roles in the immune dysfunction of children with ASD (Ahmad et al., 2017c). However, the exact mechanism underlying these associations remains a subject for further detailed investigations.

Helios is a transcription regulator that belongs to the Ikaros family and that has been studied extensively in the context of human T regulatory (Treg) cells in health and disease (Thornton et al., 2010; Gottschalk et al., 2012). Recently, Helios was shown to be selectively expressed by 70–80% of human $FoxP3^+$ T cells (Thornton et al., 2010). *Helios* was originally defined as a cell type-specific hematopoietic factor that participates in the development of lymphocytes (Quirion et al., 2009). Helios binds to the FoxP3 promoter and stimulates FoxP3

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synthesis (Getnet et al., 2010). Several other cell types lead to Helios expression (Narni-Mancinelli et al., 2012), and *Helios* has been associated with T cell activation and proliferation (Gottschalk et al., 2012). *Helios* is also expressed in ectodermal and neuroectodermal-derived tissues, and in Purkinje neurons (Martín-Ibáñez et al., 2012). However, little is known about the expression of *Helios* in the central nervous system (CNS). Further, the role of *Helios* signaling in children with ASD has not yet been studied.

Previously, abnormal immune responses, including increased T lymphocyte cell production, have been described in cases of ASD (Jyonouchi et al., 2001). Additionally, there have been several reports of dysregulated peripheral T cell activation, changes in lymphocyte subsets, and an imbalance in immunoglobulin levels (Enstrom et al., 2009; Wills et al., 2009). In a previous study, altered frequencies of T cell expression and co-stimulatory molecules have been reported in children with ASD (Ashwood et al., 2011). Brain specimens of children with ASD also show increased neuroinflammation and cytokine production (Li et al., 2009). Previously increased levels of pro-inflammatory cytokine in the cerebrospinal fluid of children with ASD were observed (Chez et al., 2007). In addition, pro-inflammatory cytokine production has been associated with immune responses on developmental regression in children ASD (Jyonouchi et al., 2001). Pro-inflammatory cytokines involved in pleiotropic effects in the CNS and neurodevelopment have been widely studied in children with ASD (Theoharides et al., 2016), and chemokine receptors have been associated with several behavioral impairments in individuals with ASD (Abdallah et al., 2012). A previous study showed increased chemokine receptor expression levels in children with ASD (Ashwood et al., 2011). A previous study also found that offspring of immune-activated mothers showed a systemic deficit in T regulatory cells (Hsiao et al., 2012). However, no underlying immune mechanism resulting in ASD dysfunction has been clarified. We hypothesized that downregulation of Helios signaling might be responsible for immune-mediated dysfunction in children with ASD. Hence, restoring Helios expression might be a therapeutic approach to address immune alterations in children with ASD.

2. Materials and methods

2.1. Study population

This cross-sectional study was conducted with 40 children. They were recruited from the Autism Research and Treatment Center, College of Medicine, King Saud University, Riyadh, Saudi Arabia, during their follow-up visits. Subjects met the criteria for the diagnosis of ASD according to the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (The American Psychiatric Association, 2015). The ASD group comprised 29 males and 11 females, with ages ranging between 4 and 11 years (mean ± SD = 7.5 ± 2.1 years). All participating subjects were excluded from the investigation if they had dysmorphic features, tuberous sclerosis, Angelman syndrome, or other serious neurological (for example, seizures), psychiatric (for example, bipolar disorder) or known medical conditions. All participants were screened via parental interview for current and past physical illness. Children with known endocrine, cardiovascular, pulmonary, liver, kidney or other medical disease were excluded from the study.

The control group comprised 35 age- and sex (27 males, 8 females)-matched healthy children. Their ages also ranged between 4 and 11 years (mean ± SD = 8.1 ± 2.2 years). They were recruited from the Well Baby Clinic, King Khalid University Hospital, King Saud University. They were the siblings of children attending this clinic because of a minor illness (e.g., tonsillitis, common cold, and acute bronchitis). The control children were not related to the children with ASD, and no clinical findings suggested allergic manifestations, infections, or neuropsychiatric or immunological disorders. This study was approved by the local Ethical Committee of the Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia. Written consent for participation in the study were obtained and signed by the parents or the legal guardians of the subjects.

2.2. Clinical evaluation

Clinical evaluation of patients with ASD was based on clinical histories taken from caregivers, a clinical examination, and a neuropsychiatric assessment. Moreover, the degree of ASD severity was measured using the Childhood Autism Rating Scale (CARS) (Schopler et al., 1986), which rates a child on a scale from one to four in each of the fifteen areas: relating to people; imitation; emotional response; object use; body use; fear or nervousness; listening response; verbal and non-verbal communications; activity level; level and consistency of intellectual response; adaptation to change; visual response; smell, taste, and touch response; and general impressions. According to this scale, children whose scores ranged from 30 to 36 had mild to moderate ASD, whereas those scoring between 37 and 60 points had severe ASD.

2.3. Flow cytometric analysis

PBMCs were separated by density gradient centrifugation as described previously (Ahmad et al., 2017b). Flow cytometric analysis was performed to assess Helios production in $CD4^+$, $CD8^+$, TIM-3^+, and $CXCR3^+$ cells. We also assessed IFN-γ^+Helios$^+$, IL-21^+Helios$^+$, T-bet$^+$Helios$^+$, and Helios$^+$Foxp3^+ production. In brief, PBMCs were stimulated for 6 h with PMA and ionomycin (Sigma-Aldrich, St. Louis, USA), in the presence of GolgiStop (BD Biosciences, San Diego, USA), as previously described (Ahmad et al., 2017b; Noster et al., 2014). PBMCs were washed and surface stained for CD4, CD8, TIM-3, and CXCR3 (BioLegend, San Diego, USA). Cells were fixed and permeabilized (BioLegend), for staining of anti-Helios, anti-IFN-γ, anti-IL-21, anti-T-bet, and anti-Foxp3 (BioLegend) to detect cytokines and transcription factors. Forward scatter/side scatter and single-cell gating were used to exclude dead cells from all analyses. All data were acquired and analyzed with a FC 500 flow cytometer (Beckman Coulter, USA) and CXP software (Beckman Coulter).

2.4. Gene expression analysis

Total RNA was isolated from PBMCs using TRIzol reagent (Life Technologies, Paisley, UK) as previously described (Ahmad et al., 2018; Noster et al., 2014). Reverse transcription was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, USA), and relative gene expression was determined by real-time PCR (RT-PCR) using SYBR Green (Applied Biosystems, USA). Target gene expression was normalized to that of GAPDH. The following primers from Genscript (Piscataway, USA) were used: GAPDH F, 5′-CCCAGCAAGGACACTGAGCAAG-3′ and GAPDH R: 5′-GGTCTGGGATGGAAATTGTGAGGG-3′; and Helios F: 5′-TGGCCCCATCTCTCTCATCA-3′ and R: 5′-ACTGGTGGTCATCATGGCTG-3′.

2.5. Western blotting analysis

Total protein was extracted from PBMCs as previously described (Chen et al., 2007). In brief, 25 μg protein was separated by 10% SDS-PAGE according to molecular weight, followed by electrophoretically transfer to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, USA). Protein blots were blocked overnight at 4 °C with primary antibody against Helios (sc-390357) and 2 h or incubation at room temperature with peroxidase-conjugated secondary antibody (sc-516102; Santa Cruz Biotech, Dallas, USA), bands were detected using Luminata Forte Western HRP Substrate (Millipore, Billerica, USA) and quantified relative to β-actin. Images were obtained using a C-Digit Chemiluminescent Western Blot Scanner (LI-COR, Lincoln, USA).