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Activation of adenosine A2A receptor signaling regulates the expression of cytokines associated with immunologic dysfunction in BTBR T^+ $Itpr3^{tf}$/J mice

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ABSTRACT

Autism spectrum disorder (ASD) is neurodevelopmental disorders characterized by stereotypical repetitive behavior, impaired social interaction, and deficits in communication. The BTBR T^+ $Itpr3^{tf}$/J (BTBR) mice have been extensively used as an animal model of the ASD-like phenotype. Adenosine A2A receptors (A2ARs) are considered potential targets in the treatment of neurodegenerative diseases. In this study, we used the A2AR antagonist SCH 5826 (SCH) and the A2AR agonist CGS 21680 (CGS) to investigate the activation of A2AR signaling in immune cells. Further, we examined the effects of A2ARs on the expression of the cytokines interleukin 2 (IL-2), IL-6, IL-9, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and transforming growth factor β (TGF-β) in the spleen and in splenic $CD4^+$ T cells. In addition, we assessed the mRNA and protein expression levels of these cytokines in the brain tissue. Our results showed that the levels of IL-2^+, IL-6^+, IL-9^+, IFN-γ^+, and TNF-α^+ were significantly lower, whereas the levels of TGF-β^+ in the spleen and in splenic $CD4^+$ T cells were significantly higher in the CGS-treated mice than in the BTBR control and SCH-treated mice. In addition, reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis showed a decrease in the mRNA and protein expression levels of IL-2, IL-6, IL-9, IFN-γ^+, and TNF-α^+ and an increase in the mRNA and protein expression levels of TGF-β in the CGS-treated mice, while treatment with BTBR alone and SCH resulted in increased Th1 levels and decreased Th2 levels in the brain tissue. Our results suggest that treatment the A2AR agonist CGS may be a promising therapeutic option for neuroimmune dysfunction.

1. Introduction

Autism spectrum disorder (ASD) is neurodevelopmental disorder characterized by stereotypical repetitive behavior, impaired social interaction, and deficits in communication (Chevallier et al., 2012). Although the causes of ASD remain to be clarified, immune responses are believed to play a role in the pathogenesis of ASD (Ashwood and Wakefield, 2006). Numerous immune system abnormalities have been identified in individuals with ASD (Enstrom et al., 2009). These abnormalities include neuroinflammation, increased peripheral immune cell activation, decrease in the levels of anti-inflammatory cytokines, and increase in the levels of proinflammatory cytokines (Ashwood et al., 2011; Gupta et al., 2014). Children with ASD have high levels of neuroinflammatory cytokines (Garbett et al., 2008). An imbalance of Th1/Th2 subsets plays a role in the development of ASD (Ahmad et al., 2017a), which indicates an autoimmune phenomenon (Van Gent et al., 1997). Taken together these studies suggest that several cytokines and inflammatory mediators are involved in the pathogenesis of ASD. However, additional studies need to be performed to establish their exact cellular source or mechanism of action.

A previous study showed an abnormality in the cytokine profiles of patients with ASD (Suzuki et al., 2011). The expression of cytokines has been examined in the brain tissue of patients with ASD, and astroglial and microglial activation in the cerebellum is associated with an upregulation of cytokine expression (Vargas et al., 2005). Children with ASD have high levels of transcription factors (T-bet, GATA-3 and RORγt) and proinflammatory cytokines (Jyonouchi et al., 2001; Ahmad et al., 2016a). Interleukin 2 (IL-2) is a strong neuromodulator, which influences the survival of hippocampal cerebellar and cortical neurons and promotes neurite extension of septohippocampal neurons (Nisticò and De Sarro, 1991). In addition, IL-2 in the brain plays an

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important role in facilitating proinflammatory and anti-inflammatory responses within the central nervous systems (CNS) (Vargas et al., 2005). IL-6 is one of the most important neuroimmune factors involved in physiological brain development and in several neurological disorders (Schaper and Rose-John, 2015; Spooren et al., 2011). A previous study showed high levels of IL-6 in children with ASD (Jyonouchi et al., 2001). Further, dendritic spine development and impairments in synapse formation on neuronal circuit balance indicate that increased levels of IL-6 play an important role in modulating autism-like behaviors (Wie et al., 2012). IL-9 plays an important role in T-cell differentiation and activation in autoimmune inflammation of the CNS (Li et al., 2011). An experimental model of autoimmune encephalomyelitis showed that IL-9 mediated Th17-cell differentiation triggers the complex Stat signaling pathway (Li et al., 2011). Results from previous studies indicate that individuals with ASD have an atypical immune response, with alterations in the expression levels of IL-2, IL-6, interferon gamma (IFN-γ), and tumor necrosis factor α (TNF-α) (Goines and Ashwood, 2013; Lyte et al., 2011). The expression levels of the proinflammatory cytokines such as IL-6 and TNF-α, which activate the hypothalamic-pituitary-adrenal axis, are increased in patients with ASD (Ashwood et al., 2011; Dunn, 2006). TGF-β levels are low in children and adults with ASD, and low levels of TGF-β are associated with a severe degree of ASD (Ashwood et al., 2008; Okada et al., 2007).

The four different types of adenosine receptors, namely A1R, A2AR, A2BR, and A3R (Ohta and Sitkovsky, 2001) are expressed in several cell types at many levels, and they control various physiological functions, including cardiovascular, respiratory, and CNS functions (Chen et al., 2013; Fredholm et al., 2001). Among the different types of adenosine receptors, the adenosine A2A receptor (A2AR) is expressed at high levels (Chen et al., 2013; Eltzschig et al., 2012). In addition, animal studies have shown that the A2AR plays a role in locomotion, anxiety, inhibition of excitatory neuronal activity, and sleep regulation (Moreau and Huber, 1999). Therapeutic agents targeting the A2ARs have been developed for autoimmune and neurological disorders (Li et al., 2012; Ramlackhansingh et al., 2011). The selective A2AR agonist CGS 21680 (CGS) reduces the levels of proinflammatory cytokines and inflammatory mediators (Mazzon et al., 2011). In a previous study, we showed that A2ARs antagonist/agonist effectively regulated the prominent repetitive behavior in BTBR mice; moreover, we showed the effects of A2ARs on Th17/Treg cells-related transcription factors (manuscript submitted). In this study, we examined the effects of the A2AR antagonist SCH 5826 and the A2AR agonist CGS on Th1/Th2 cytokine profile.

BTBR T^{+} $Itpr3^{tf}$/J (BTBR) mice have been used extensively as an animal model to study a range of autism-related behaviors, including deficiencies in unusual ultrasonic vocalizations, increased repetitive and self-grooming social behaviors (Chadman and Guariglia, 2012). The abnormal immune responses seen in BTBR mice with behavioral disorders are also seen in children with ASD (Heo et al., 2011). Previous studies have shown therapeutic strategies targeting the behavioral phenotypes of autism in BTBR mice (Meyza et al., 2013; Silverman et al., 2010). A previous study showed increased levels of proinflammatory cytokines in the whole brain and specific brain regions of BTBR mice (Heo et al., 2011). In this study, we examined the hypothesis that A2ARs regulate excessive production of cytokines by modulating the signaling pathways, and our findings may provide insights in treating the behavioral deficits in autism. Further, we have identified molecular targets relevant to the therapeutic aspects of autism to explore novel strategies for the prevention and treatment of ASD.

2. Materials and methods

2.1. Chemicals and antibodies

The A2AR antagonist SCH 58261 (SCH) was purchased from Sigma Aldrich, USA. The A2AR agonist CGS 21680 (CGS), primary antibodies IL-2, IL-6, IL-9, IFN-γ, TNF-α, TGF-β, and β-actin and secondary anti-goat, anti-rabbit, anti-mouse, and horseradish peroxidase-conjugated antibodies were purchased from Santa Cruz Biotechnology, USA. FcR blocking reagent, phycoerythrin (PE), IL-2, IL-6, IL-9, IFN-γ, TNF-α, and TGF-β anti-mouse monoclonal antibodies, fluorescein isothiocyanate (FITC)-labeled CD4, and buffers for lysing, permeabilizing, and fixing RBCs were purchased from BD Biosciences, USA, Miltenyi Biotech, Germany and Bio Legend, USA. Nitrocellulose membranes were purchased from Bio-Rad Laboratories, Hercules, USA. Primers, high-capacity cDNA reverse transcription kit and SYBR® Green and PCR Master Mix were purchased from Applied Bio systems, Paisley, UK and Genscript Piscataway, USA. TRIzol and RPMI reagents were purchased from Life Technologies, Paisley, UK. Chemiluminescence reagent for western blot analysis was obtained from GE Healthcare Life Sciences, USA.

2.2. Animals

We purchased 6–8-week-old male BTBR T^{+} $Itpr3^{tf}$/J (BTBR) mice and 6–8-week-old male adult C57BL/6 (B6) mice from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained at 25 ± 2 °C with a 12-h light/12-h dark cycle, housed in a specific pathogen-free environment, and given Purina standard rodent chow diet (manufactured by Grain Silos and Flour Mills Organization, Riyadh, Saudi Arabia) and water ad libitum. All procedures were performed in accordance with the guidelines of the King Saud University, Institutional Animal Care and Use Committee.

2.3. Experimental design and drug administration

The mice were acclimatized for two weeks and divided into five groups of six mice each, as follows: B6 and BTBR control mice received intraperitoneal (i.p.) administration of 1% dimethyl sulfoxide (DMSO) in saline only, BTBR + SCH group received a single dose of SCH (0.03 mg/kg, i.p.) for 7 days, BTBR + CGS group received a single dose of CGS (0.03 mg/kg, i.p.) for 7 days, SCH + CGS group received a single dose of both CGS and SCH together (0.03 mg/kg, i.p.) for 7 consecutive days. The volume of drug administered to each mouse was dependent on its body weight. The doses of SCH and CGS were selected based on results of a previous study (Kermanian et al., 2013). All mice were killed on day 8 after treatment for the spleen and brain tissues were isolated.

2.4. Assessment of the intracellular levels of IL-2, IL-6, IL-9, IFN-γ, TNF-α, and TGF-β in the spleen cells

Spleen cell preparations were made as described previously (Bakheet et al., 2016a). We examined the intracellular levels of IL-2, IL-4, IL-6, IL-9, TNF-α, and IFN-γ expression in CD4 T cells of the mice. The splenocytes were cultured in 12-well plates (2×10^6 cells/mL), activated with anti-CD28/CD3 (1 μg/mL), and incubated for 24 h. GolgiPlug (1 μL/mL) was added to each well for the last 4 h of incubation, and then, the cells were collected, washed, and then resuspended in staining buffer. The cells were incubated with monoclonal antibodies against the CD4 T cell surface receptor for 30 min at 4 °C, washed twice with staining buffer, and incubated with IL-2, IL-4, IL-6, IL-9, TNF-α, IFN-γ, and TGF-β monoclonal antibodies for 30 min at 4 °C. Events were collected and analyzed using the CXP software (Beckman Coulter, USA) (Ahmad et al., 2016b).

2.5. Gene expression analysis using quantitative RT-PCR

Total RNA was isolated from the brain tissues using the TRIzol reagent, and then, cDNA was synthesized by using a high-capacity cDNA reverse transcription kit with a thermal cycler, followed by real-time PCR using an SYBR® Green PCR master mix according to the manufacturer's instructions. The samples were matched to a standard curve generated by amplifying serially diluted products using the same real-time PCR conditions. The primers used in the assay were selected from PubMed and other