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ORIGINAL PAPER

Resveratrol Improves Neuroimmune Dysregulation Through the Inhibition of Neuronal Toll-Like Receptors and COX-2 Signaling in BTBR T^{+} Itpr3^{tf}/J Mice

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Abstract
Autism is a neurodevelopmental disorder characterized by deficits in qualitative impairments in communication, repetitive and social interaction, restricted, and stereotyped patterns of behavior. Resveratrol has been extensively studied pharmacologically and biologically and has anti-inflammatory, antioxidant, and neuroprotective effects on neuronal damage in neurodegenerative disorders. The BTBR T^{+} Itpr3^{tf}/J (BTBR) autistic mouse model has been explored for treatment of autism, which shows low reciprocal social interactions, impaired juvenile play, and decreased social approach. Here, we explored whether resveratrol treatment decreases neuroimmune dysregulation mediated through toll-like receptor (TLR4) and nuclear factor-κB (NF-κB) signaling pathway in BTBR mice. We investigated the effect of resveratrol treatment on TLR2, TLR3, TLR4, NF-κB, and inducible nitric oxide synthase (iNOS or NOS2) levels in CD4 spleen cells. We also assessed the effect of resveratrol treatment on TLR2, TLR3, TLR4, NF-κB, iNOS, and cyclooxygenase (COX-2) mRNA expression levels in the brain tissue. We further explored TLR2, TLR4, NF-κB, iNOS, and COX-2 protein expression levels in the brain tissue. Resveratrol treatment on BTBR mice significantly decreased CD4^{+}TLR2^{+}, CD4^{+}TLR3^{+}, CD4^{+}TLR4^{+} CD4^{+}NF-κB^{+}, and CD4^{+}iNOS^{+} levels in spleen cells. Resveratrol treatment on BTBR mice decreased TLR2, TLR3, TLR4, NF-κB, iNOS, and COX-2 mRNA expression levels in brain tissue. Moreover, resveratrol treatment resulted in decreased protein expression of TLR2, TLR3, TLR4, NF-κB, iNOS, and COX-2 in brain tissue. Taken together, these results indicate that resveratrol treatment improves neuroimmune dysregulation through the inhibition of proinflammatory mediators and TLRs/NF-κB transcription factor signaling, which might be help devise future therapies for neuroimmune disorders.

Keywords Autism · Resveratrol · BTBR T^{+} Itpr3^{tf}/J and C57BL/6 · TLRs/NF-κB transcription factor · Spleen and brain

Introduction

Autism disorder is characterized by qualitative impairments in communication; restricted repetitive and stereotyped patterns of behavior activities and interests; and aberrant reciprocal social interactions Diagnostic and Statistical Manual of Mental Disorders (DSM-IV 2000; Lord et al. 2001). In addition, children with autism show a high dominance of various comorbid medical conditions (Ozsivadjian et al. 2014). The role of immune abnormalities in the development of autism is beginning to emerge. Numerous studies have reported markers of systemic neuroinflammation in autism (Rossignol and Frye 2014). Children with autism show immune dysfunction at a young age (Ashwood et al. 2011). The dysregulation of gene expression and function of immune cells is identified in autistic disorder (Enstrom et al. 2009). Chemokines and cytokines expression levels are elevated in autistic children (Al-Ayadhi and Mostafa 2013). Inflammatory mediators such as cytokines, chemokines, and their receptors are associated in the development of autistic disorder (Garbett et al. 2008). Recently, we reported the imbalance between anti- and proinflammatory cytokines in children with autism (Ahmad et al. 2017a). We also showed

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those children with autism show dysregulation in T helper (Th1, Th2, and Th17) and T regulatory cells transcription factor signaling, which are related with autism development (Ahmad et al. 2017b). Though, the exact molecular and cellular mechanism of autistic disorder remains unknown.

Toll-like receptors (TLRs) are widely expressed in various types of cells. TLRs are expressed in immune cells in the brain and might play an important role in neurophysiology (Bsibsi et al. 2002). Human neuronal cell lines express TLR2, TLR3, and TLR4 (Lafon et al. 2006). The distribution of TLRs expression in the brain during neurocysticercosis has been reported (Mishra et al. 2006). TLR2 expression has previously been detected in neurons, microglia, astrocytes, and ependymal cells (Mishra et al. 2006). TLR2 was expressed in neonatal mice brain (Stridh et al. 2011). TLR2 also mediates neuroinflammation and neuronal damage (Hoffmann et al. 2007). TLR3 expression has been previously reported in human neurons (Prehaud et al. 2005). TLR3 expression has been found in the human cerebellar cortex in herpes simplex encephalitis, rabies, and other neurological diseases (Jackson et al. 2006). TLR4 activates NF-κB signaling pathways linked to the transcription of many proinflammatory genes such as inducible nitric oxide synthase (iNOS or NOS2) and cyclooxygenase-2 (COX-2; Medzhitov et al. 1997). TLR4 is also expressed in microglia and astrocytes after inflammation in the central nervous system (Lehnardt et al. 2002).

Nuclear factor-κB (NF-κB) is an important transcription factor that regulates cellular responses in development, cell proliferation and inflammation (Chen et al. 2001). NF-κB regulates the transcription factor of several proinflammatory cytokines that play an important role in neuroinflammation-mediated neurodegeneration (Husain et al. 2017). NF-κB is a therapeutic target for neurodegenerative diseases (Camandola and Mattson 2007). Cyclooxygenases-2 (COX-2) is a critical marker of the complex process of neuroinflammation and actively involved in neuronal dysfunction (Yagami et al. 2016). COX-2 is constitutively expressed in several brain regions such as the hippocampus, cerebral cortex, and amygdala (Yagami et al. 2016). Overproduction of inducible nitric oxide synthase (iNOS) is associated with neuronal health, thereby leading to cell death (Broom et al. 2011). It is hypothesized that increased NF-κB, COX-2, and iNOS expression could play an important role in the development of autism. Therefore, decreasing these mediators may control neuroimmune dysfunction.

Resveratrol is found in several plants, fruits, nuts, red wine, and grapes. A recent study exhibited that resveratrol treatment prevents social deficit in animal models of autism, indicating a possible therapeutic approach toward autistic disorder (Bambini-Junior et al. 2014). A previous study also confirmed that resveratrol acts as an effective neuroprotective agent against cerebral ischemia (Ren et al. 2011). Resveratrol prevents neuronal apoptosis in an early brain injury model (Zhou et al. 2014). Administration of resveratrol produces protective responses against ischemia and improves neurological function in the brain (Ren et al. 2011; Pineda-Ramírez et al. 2017). Pretreatment with resveratrol also reduces injury and promotes proliferation of neural stem cells (Shen et al. 2016). In our recent studies, we revealed that resveratrol treatment attenuates chemokine receptor expression and improves immune dysregulation through regulation of Th1, Th2, Th17, and T reg cells transcription factor signaling in BTBR T^+ $Itpr3^{tf}$/J (BTBR) autistic mice model (Bakheet et al. 2016, 2017). Recently, we also indicated that resveratrol treatment mitigates IL-6 induced janus kinase 1 (JAK1) and signal transducer and activator of transcription-3 (STAT3) activation in BTBR mice (manuscript submitted). These activities might beneficial in protecting against neuroimmune dysfunction.

The association between immune dysregulation and behavioral deficits in autistic disorder is not yet well understood; however, the BTBR T^+ $Itpr3^{tf}$/J (BTBR) mouse provides a well-established model to investigate several neurodevelopmental issues. BTBR mice exhibit low levels of sociability, high levels of self-grooming, and unusual pattern of ultrasonic vocalizations that may be homologous to communication deficits and repetitive behaviors observed in autistic disorders (McFarlane et al. 2008; Silverman et al. 2010). BTBR mice exhibit several immune abnormalities that are observed in children with autism (Li et al. 2009). In our previous studies, we revealed that BTBR mice increased expression levels of chemokine expression and dysregulates transcription factor signaling (Ansari et al. 2017a, b). BTBR mice are a promising model for understanding the molecular and cellular mechanisms that could be responsible for the development of autism. In the present study, we tested the effect of resveratrol treatment on TLRs, NF-κB, and COX-2 signaling pathways. Moreover, we assessed TLRs and NF-κB signaling-based mechanisms underlying the neuroprotective effects of resveratrol, which could offer a new approach for treating neuroimmunological disorders.

Materials and Methods

Chemicals and Antibodies

Male BTBR T^+ $Itpr3^{tf}$/J and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Resveratrol and Roswell Park Memorial Institute (RPMI) medium were purchased from Sigma-Aldrich (St Louis, MO, USA). Fluoroisothiocyanate (FITC), phycoerythrin (PE), and allophycocyanin (APC)-labeled CD4, TLR2, TLR3, TLR4, NFκB, and iNOS monoclonal antibodies, Golgistop, red blood cell (RBC) lysing, permeabilization, and fixation

buffers were obtained from BioLegend (San Diego, CA, USA), Miltenyi Biotech, (Bergisch Gladbach, Germany), BD Biosciences (San Jose, USA), and Santa Cruz Biotech (Santa Cruz, CA, USA). High-capacity cDNA kit, SYBR® Green PCR master mix, primers, and TRIzol reagent were purchased from Life Technologies (Renfrew, UK) and Applied Biosystems (Foster City, CA, USA). Nitrocellulose membrane and chemiluminescence western blotting detection kit were purchased from Bio-Rad Laboratories (Hercules, CA, USA) and GE Healthcare Life Sciences (Pittsburgh, PA, USA). Primary antibodies (TLR2, TLR4, NF-κB, iNOS, and COX-2) and secondary antibodies (anti-mouse, anti-goat, and anti-rabbit) were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA).

Mice

Male BTBR T^+ $Itpr3^{tf}$/J (BTBR) and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained at 22 ± 2 °C with a 12-h light/dark cycle, housed with water and food ad libitum. The studies were performed in accordance with the protocols approved by the Animal Ethics Committee of the King Saud University, College of Pharmacy (Saudi Arabia, Riyadh).

Drug Administration

The mice were acclimatized for 1 week and divided into four groups. C57BL/6 and BTBR mice were intraperitoneally (i.p.) injected with 1% of dimethyl sulfoxide (DMSO) in saline, which served as the control. C57BL/6 and BTBR mice were treated with resveratrol at 40 mg/kg, i.p., once a day for a week. The volume of drug injected into each mouse was administered based on its body weight. In our previous published studies, we used two different doses of resveratrol (20 and 40 mg/kg); these doses were selected as previously prescribed (Pang et al. 2015; Hong et al. 2016). However, in the current study we used only a single dose of resveratrol at 40 mg/kg in both C57BL/6 and BTBR mice because previous results showed that resveratrol at 20 mg/kg was not significantly effective treatment (Bakheet et al. 2016, 2017).

Flow Cytometric Analysis

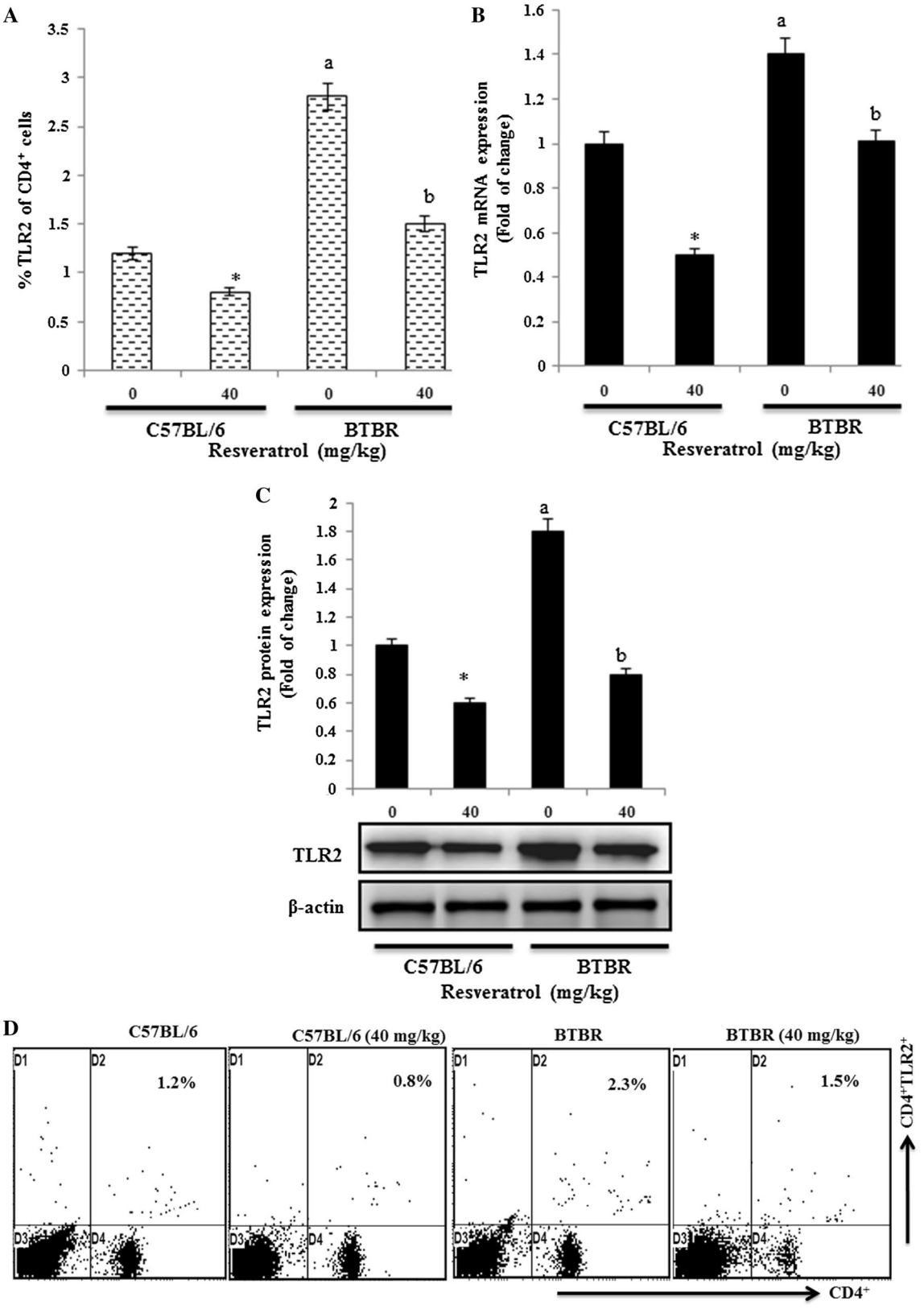
Spleens were removed from mice and prepared as described previously (Ahmad et al. 2015a). Estimation of TLRs and inflammatory mediators were assessed using the monoclonal antibodies against TLR2, TLR3, TLR4, NFκB, and iNOS, in CD4 cells. Cells were cultured in 24-well plate at a density of 2×10^6 cells/mL and activated with LPS at a concentration of 1.25 µg/mL, and incubated for 24 h. Cells were prepared for labeling with monoclonal antibodies against TLR2, TLR3, TLR4, NFκB, and iNOS as recommended by manufacturers (BD Bioscience and BioLegend, USA). The following monoclonal antibodies conjugates were used: TLR2-PE, TLR3-FITC, TLR4-PE, CD4-PE, CD4-FITC, NF-κB-FITC, and iNOS-PE (BioLegend and BD Biosciences, USA). The monocyte population was defined by gating of $CD4^+TLR2^+$, $CD4^+TLR3^+$, $CD4^+TLR4^+$, $CD4^+NF\text{-}κB^+$, and $CD4^+iNOS^+$ cells using the cell size (forward scatter—FSC) and cell granularity as parameters (side scatter—SSC) as described previously (Ahmad et al. 2017c). A total of 10,000 events were analyzed for each sample on FC500 flow cytometer (Beckman Coulter, USA).

RNA Isolation and RT-PCR Analysis

Total RNA was isolated from the brains using TRIzol (Invitrogen) according to the previously described method (von Ziegler et al. 2013). RNA was quantified using NanoDrop (ThermoScientific). cDNA was synthesized using a high-capacity cDNA reverse transcription kit, followed by RT-PCR using SYBR® Green PCR master mix according to the manufacturer's instructions (Applied Biosystem, USA). The primer sequences were used as following: TLR2, forward: 5′-CAGTCTTCCTAGGCTGGTGC-3′ and reverse: 5′-AAGGAAACAGTCCGCACCTC-3′; TLR3, forward: 5′-CCAGCTATGTACGTGTGGGA-3′ and reverse: 5′-CCAGCAGAAGAGACACAACA-3′; TLR4, forward: 5′-CCATGCATTTGGCCTTAGCC-3′ and reverse: 5′-TGCAGCAGTCTACTGTGTGG-3′; iNOS, forward: 5′-AGGGCTATACTGCCCTCCAA-3′ and reverse: 5′-CTATGGCCGCTTTGATGTGC-3′; NF-κB, forward: 5′-TTTACTTTAGCGCGCCGTGG-3′ and reverse: 5′-GTTCCTGGTCCTGTGTAGCC-3′; COX-2, forward: 5′-CACTCATGAGCAGTCCCCTC-3′ and reverse: 5′-ACCCTGGTCGGTTTGATGTT-3′; iNOS, forward: 5′-AGGGCTATACTGCCCTCCAA-3′ and reverse: 5′-CTATGGCCGCTTTGATGTGC-3′; GAPDH, forward: 5′-ATCCCTCAAAGCTCAGCGTGTC-3′ and reverse: 5′-GGGTCTTCATTGCGGTGGAGAG-3′. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as reference gene for RT-PCR analysis. Data are expressed as levels of target mRNA expression relative to GAPDH mRNA expression (Ahmad et al. 2017d).

Western Blot Analysis

Protein extraction from brain tissue was performed as described previously (von Ziegler et al. 2013; Ansari et al. 2016). To detect target proteins, specific antibodies against TLR2, SC-10739; TLR4, SC-293072; NF-κB, SC-8008; iNOS, SC-7271; COX-2, SC-376861; and β-actin, SC-47778 (Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA) were used. The blots were then incubated with the corresponding conjugated m-IgGκ BP-HRP, SC-516102, and mouse anti-rabbit IgG-HRP, SC-2357, IgG-horseradish peroxidase



(HRP; Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA) secondary antibodies. Nitrocellulose membranes were visualized with C-Digital Imager (LI-COR Bioscience, Nebraska); images were analyzed using Luminata

◂**Fig. 1** **a** Flow cytometric analysis indicates the effect of resveratrol on TLR2$^+$ percentage by CD4$^+$ T cells in the spleen. **b** TLR2 gene expression was validated by RT-PCR in the brain tissue; GAPDH expression was used as an internal control. **c** TLR2 protein expression was evaluated through western blotting analysis and quantified relative to β-actin in the brain tissue. **d** Flow cytometric dot plots represent showing the percentage of CD4$^+$ T cells expressing TLR2$^+$ in the spleen cells from a mouse from each group: C57BL/6 and BTBR control mice, which received 1% DMSO in saline only [intraperitoneal (i.p.)]; C57BL/6 mice treated with resveratrol 40 mg/kg, i.p; BTBR mice treated with resveratrol 40 mg/kg, i.p. All treatments were administered for seven consecutive days. $^{*,a}P < 0.05$ compared to C57BL/6 control mice; $^{b}P < 0.05$ compared to BTBR control mice. All data were represented as mean ± SEM; the level of significance was set at $^{*}P < 0.05$

Forte Western HRP substrate (Millipore USA), quantified with relative to β-actin bands (Ahmad et al. 2015b).

Statistical Analysis

The results were initially tested for homogeneity of variance and normality and analyzed using a parametric test. All data were presented as mean ± SEM, for six mice in each group. The comparisons between treatments groups were conducted using a two-way repeated measures analysis of variance (ANOVA) by using statistical package GraphPad Prism, (GraphPad Software, La Jolla California, USA). The level of statistical significance was set at P value < 0.05.

Results

Effect of Resveratrol Treatment on Toll-Like Receptor Expression

Since BTBR mice are presently a promising model for understanding the mechanisms for the development of autism, we first investigated the effect of resveratrol administration on TLRs expression in BTBR and C57BL/6 mice. As shown in Fig. 1a, when resveratrol was administered in BTBR mice, a significant decrease in CD4$^+$TLR2$^+$ cells was observed as compared with BTBR control mice. Although increased numbers of CD4$^+$TLR2$^+$ cells were observed in spleen cells of C57BL/6 control mice, resveratrol treatment in C57BL/6 mice decreased CD4$^+$TLR2$^+$ cells. Next, to investigate whether resveratrol can decrease mRNA expression of TLR2, we treated BTBR mice with resveratrol. Our data in Fig. 1b show that TLR2 mRNA expression level was significantly lower in the brain tissue from resveratrol-treated BTBR mice than those from BTBR control. Furthermore, western blot studies were also conducted to examine TLR2 expression in the brain of BTBR mice. The results are shown in Fig. 1c; TLR2 protein expression was stronger in BTBR control mice than in resveratrol-treated mice (Fig. 1c). These observations show that resveratrol has a potent therapeutic effect in BTBR autistic mice and can protect against neuroimmune dysfunction.

Next, we determined whether resveratrol could decrease the induction of TLR3 in BTBR mice. Following resveratrol treatment on BTBR mice, CD4$^+$TLR3$^+$ cells were significantly decreased as compared with BTBR control mice (Fig. 2a). In contrast, a low number of CD4$^+$TLR3$^+$ cells was observed in resveratrol-treated C57BL/6 mice, which was significantly greater in spleen cells in C57BL/6 control mice (Fig. 2a). We next examined whether resveratrol downregulates TLR3 expression levels. Treatment with resveratrol in BTBR mice resulted in inhibition of TLR3 mRNA expression in the brain tissue (Fig. 2b). We also found that resveratrol treatment in C57BL/6 mice decreased TLR3 mRNA levels (Fig. 2b). As shown in Fig. 2c, the TLR3 protein expression level was induced in BTBR control mice, and this expression was significantly reduced in the brain tissue of resveratrol-treated BTBR mice (Fig. 2c). These results indicate that resveratrol is important to reduce TLR3 expression in BTBR autistic mice. Taken together, these results show that, resveratrol reduced TLR3, thus effectively suppressing neuroimmune dysregulation and can also enhance neurological recovery.

To elucidate the mechanism of action of the neuroprotective effect of resveratrol on TLRs activity, TLR4 was examined. In the presence of resveratrol, BTBR mice showed inhibitory effect on CD4$^+$TLR4$^+$ in spleen cells as compared to BTBR control mice (Fig. 3a). To examine the further inhibitory effect of resveratrol on TLR4 activity, the mRNA and protein expression of TLR4 were investigated in brain tissue. As shown in Fig. 3b, c, the brain tissue expressed extremely high level of TLR4 in BTBR control mice. However, TLR4 mRNA and protein expression were markedly decreased in response to resveratrol treatment. Treatment with resveratrol on C57BL/6 mice decreased expression of TLR4 in brain tissue. These results show that resveratrol inhibits TLR4 expression, suggesting that resveratrol treatment may have role in preventing neuroimmune dysfunction.

Effect of Resveratrol on NF-κB, iNOS, and COX-2 Expression

NF-κB is a key transcription factor regulating neuroinflammatory factors; hence, it is an attractive potential target for neuroprotection therapy. We evaluated NF-κB expression levels in both spleen and brain tissues in C57BL/6 and BTBR mice. In spleen cells, the production of NF-κB$^+$ in CD4 cells was significantly lower in resveratrol-treated BTBR mice, whereas BTBR control mice showed a marked increase in CD4$^+$NFκB$^+$ cells (Fig. 4a). Based on these results, we next examined whether the regulation of NF-κB expression in BTBR mice was mediated by resveratrol.

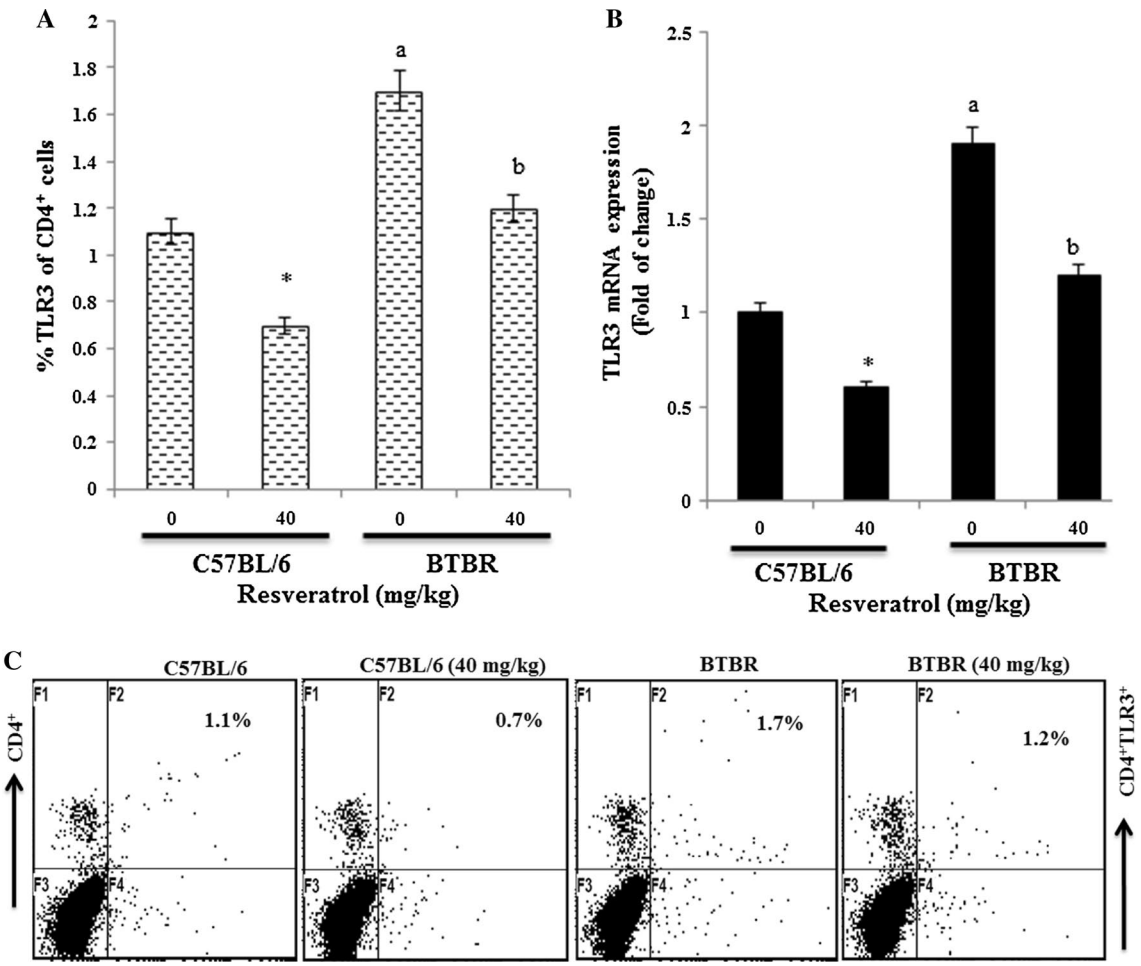


Fig. 2 **a** Flow cytometric analysis indicates the effect of resveratrol on TLR3$^+$ percentage by CD4$^+$ T cells in the spleen. **b** TLR3 gene expression was validated by RT-PCR in the brain tissue; GAPDH expression was used as an internal control. **c** Flow cytometric dot plots represent showing the percentage of CD4$^+$ T cells expressing TLR3$^+$ in the spleen cells from a mouse from each group: C57BL/6 and BTBR control mice, which received 1% DMSO in saline only [intraperitoneal (i.p.)]; C57BL/6 mice treated with resveratrol 40 mg/kg, i.p; BTBR mice treated with resveratrol 40 mg/kg, i.p. All treatments were administered for seven consecutive days. $^{*,a}P < 0.05$ compared to C57BL/6 control mice; $^{b}P < 0.05$ compared to BTBR control mice. All data were represented as mean ± SEM; the level of significance was set at $^{*}P < 0.05$

The significant decrease in NF-κB mRNA expression was observed in the brain tissue of BTBR mice treated with resveratrol as compared with BTBR control mice (Fig. 4b). The resveratrol treatment in BTBR mice caused a significant decrease in NF-κB protein expression as compared to BTBR control mice in the brain tissue (Fig. 4c). The lower level of NF-κB expression was also found in resveratrol-treated C57BL/6 mice as compared to C57BL/6 control mice. These results suggest that resveratrol has the potential to inhibit NF-κB activation. Thus, more study of resveratrol as a possible drug target for neuroimmune system-related diseases is warranted.

In the present study, we examined the effect of resveratrol on iNOS expression in BTBR and C57BL/6 mice. Our results indicated that BTBR control mice increased percentage of CD4$^+$iNOS$^+$ as compared to C57BL/6 control mice (Fig. 5a). In contrast, BTBR mice treated with resveratrol showed a marked decrease in iNOS gene expression as compared to BTBR control mice (Fig. 5a). BTBR mice treated with resveratrol showed that the protein expression level of iNOS was inhibited as compared with BTBR control mice (Fig. 5b). As illustrated in Fig. 5, there was also significant difference between C57BL/6 and BTBR mice in iNOS mRNA and protein expression levels. Resveratrol-treated C57BL/6 mice also showed decreased iNOS as compared with C57BL/6 control mice. Thus, these results recommend that resveratrol can improve the outcome, as well as the development, of autism by modulating iNOS involved in neuroimmune dysfunction.

Since resveratrol inhibits iNOS activation, we also tested the effect of resveratrol on COX-2 expression. We observed the protein and mRNA expression levels of COX-2 in the

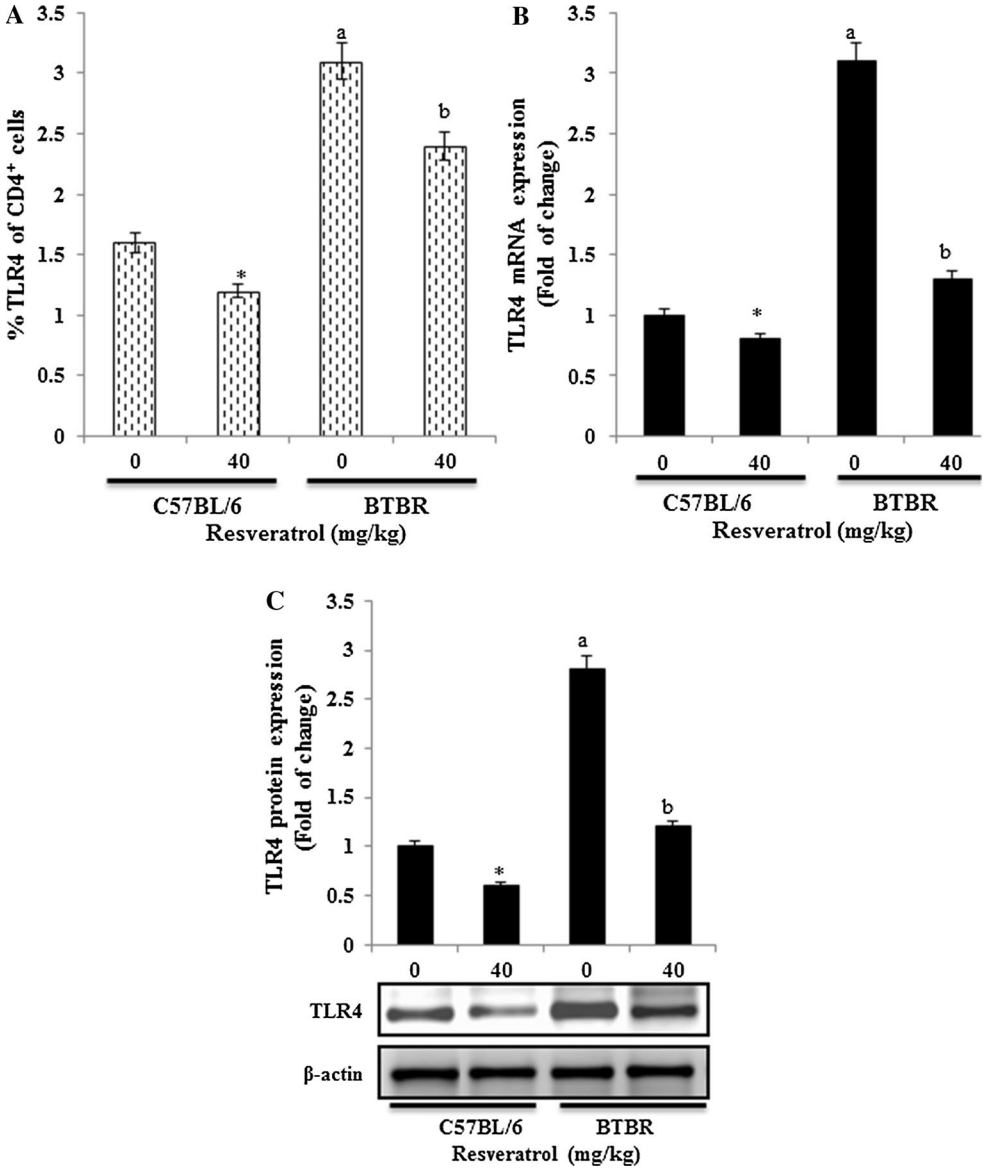
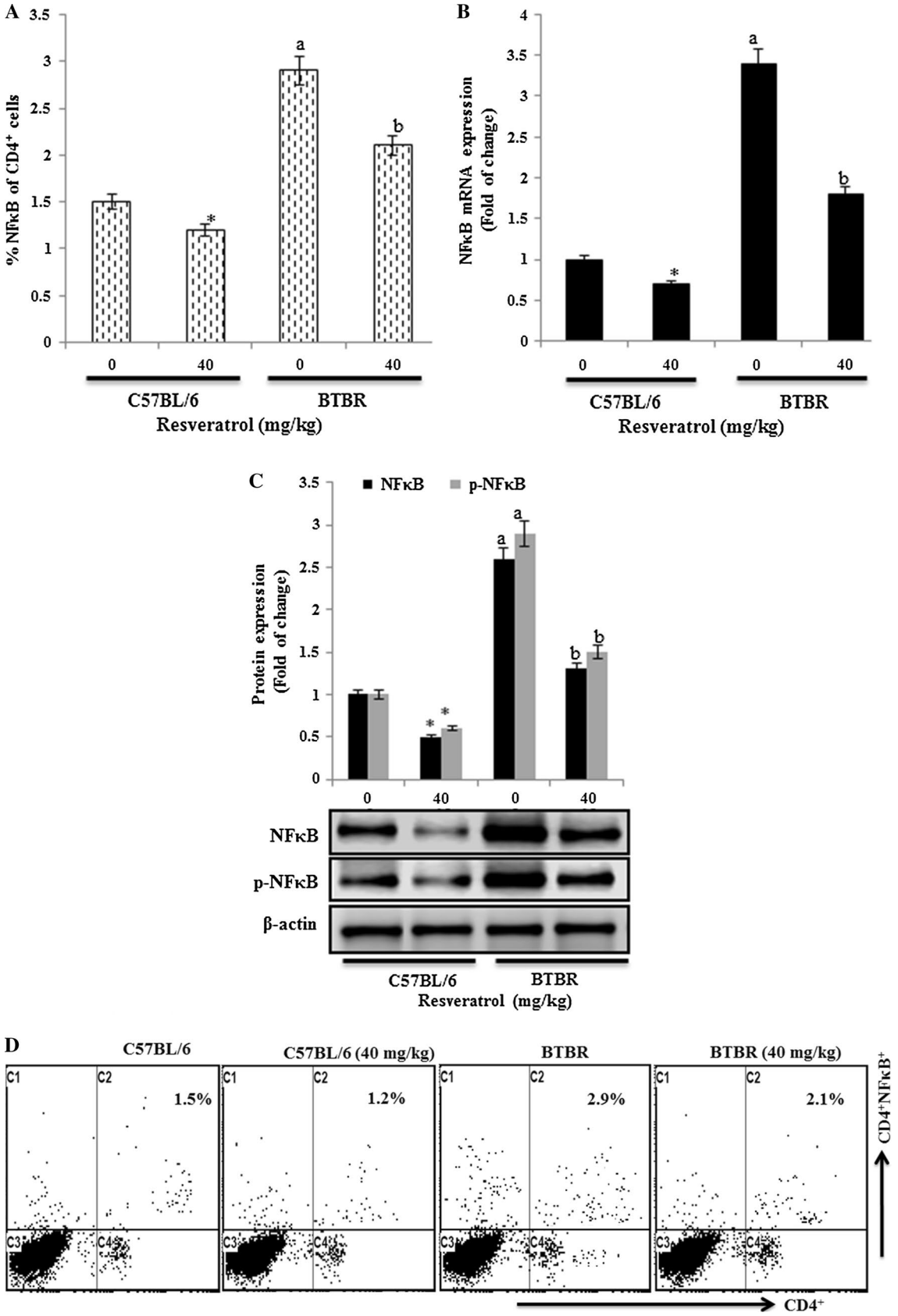


Fig. 3 **a** Flow cytometric analysis indicates the effect of resveratrol on TLR4$^+$ percentage by CD4$^+$ T cells in the spleen. **b** TLR4 gene expression was validated by RT-PCR in the brain tissue; GAPDH expression was used as an internal control. **c** TLR4 protein expression was evaluated through western blotting analysis and quantified relative to β-actin in the brain tissue in C57BL/6 and BTBR control mice, which received 1% DMSO in saline only [intraperitoneal (i.p.)]; C57BL/6 mice treated with resveratrol 40 mg/kg, i.p; BTBR mice treated with resveratrol 40 mg/kg, i.p. All treatments were administered for seven consecutive days. $^{*,a}P < 0.05$ compared to C57BL/6 control mice; $^{b}P < 0.05$ compared to BTBR control mice. All data were represented as mean ± SEM; the level of significance was set at $^{*}P < 0.05$

brain tissue of BTBR and C57BL/6 mice. Our results showed COX-2 mRNA expression was reduced in resveratrol-treated BTBR mice, as compared with BTBR control mice (Fig. 6a). In contrast, resveratrol-treated BTBR mice showed attenuated induction of COX-2 protein expression. Similarly, COX-2 protein level was increased in C57BL/6 control mice, whereas resveratrol treatment significantly decreased COX-2 protein level in brain tissue (Fig. 6b). These results propose that resveratrol administration has a protective effect on BTBR mice and could have possible beneficial effects for the treatment of autism.

Discussion

Several effects have been defined for resveratrol treatment; the mechanisms responsible for its neuroprotective effects are not yet clear. Resveratrol treatment prevents in social deficits and improves hippocampal atrophy in autistic animals (Moriya et al. 2011; Bambini-Junior et al. 2014). Resveratrol also reduces the neuronal damage in multiple sclerosis disorder (Shindler et al. 2010). Previous


A
% NFκB of CD4+ cells
3.5
3
2.5
2
1.5
1
0.5
0
a
b
*
0
40
0
40
C57BL/6
BTBR
Resveratrol (mg/kg)
B
NFκB mRNA expression
(Fold of change)
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
*
0
40
0
40
C57BL/6
BTBR
Resveratrol (mg/kg)
C
NFκB
p-NFκB
Protein expression
(Fold of change)
3.5
3
2.5
2
1.5
1
0.5
0
a
a
b
b
*
*
0
40
0
40
NFκB
p-NFκB
β-actin
C57BL/6
BTBR
Resveratrol (mg/kg)
D
C57BL/6
C57BL/6 (40 mg/kg)
BTBR
BTBR (40 mg/kg)
C1
C2
C3
C4
1.5%
1.2%
2.9%
2.1%
CD4+NFκB+
CD4+

◂**Fig. 4** **a** Flow cytometric analysis indicates the effect of resveratrol on NF-κB$^+$ percentage by CD4$^+$ T cells in the spleen. **b** NF-κB gene expression was validated by RT-PCR in the brain tissue; GAPDH expression was used as an internal control. **c** NF-κB protein expression was evaluated through western blotting analysis and quantified relative to β-actin in the brain tissue. **d** Flow cytometric dot plots represent showing the percentage of CD4$^+$ T cells expressing NFκB$^+$ in the spleen cells from a mouse from each group: C57BL/6 and BTBR control mice, which received 1% DMSO in saline only [intraperitoneal (i.p.)]; C57BL/6 mice treated with resveratrol 40 mg/kg, i.p; BTBR mice treated with resveratrol 40 mg/kg, i.p. All treatments were administered for seven consecutive days. $^{*,a}P < 0.05$ compared to C57BL/6 control mice; $^{b}P < 0.05$ compared to BTBR control mice. All data were represented as mean ± SEM; the level of significance was set at $^{*}P < 0.05$

studies indicated that resveratrol treatment improved memory dysfunction from hippocampal neuron loss in Alzheimer's disease (Huang et al. 2012). Resveratrol has been shown to decrease COX-2 and iNOS expression by blocking improper NF-κB activation (Surh et al. 2001). Resveratrol blocks IL-1β and TNF-α induced activation of NF-κB (Martinez and Moreno 2000). Possible neuroprotective role of resveratrol might be via disruption of the inflammatory mediators signaling pathway. In our previous studies, we revealed that resveratrol effectively reduces behavior activity, decreases chemokine receptor expression, and regulates Th1, Th2, Th17, and T regulatory cell-mediated transcription factors signaling in BTBR mice (Bakheet et al. 2016, 2017). The purposes of this study were to further document the neuroprotective effect of resveratrol in BTBR mice and to describe a molecular basis of its action.

BTBR mice show social abnormalities and high level of repetitive behaviors grooming to the first diagnostic symptoms of autism (Bolivar et al. 2007). Stereotypy and behavior rigidity are widely known in BTBR mice and are defining features of autism (Pearson et al. 2011). In our recent studies, we revealed that BTBR mice regulate transcription factors pathway and alter chemokine and cytokine expression (Bakheet et al. 2016, 2017). We also showed that BTBR mice modulate neuroimmune development through Th17/RORγt pathways (Ansari et al. 2017a). The BTBR autistic mice model is essential not only in understanding the etiology of neuroimmune function but also in discovering early diagnostic markers for neurodevelopmental and autistic disorders.

In the present study, we showed the effect of resveratrol on TLRs signaling. TLRs are involved in higher brain functions (Okun et al. 2011). Our finding of the effect of resveratrol on TLRs pathway is still limited in BTBR autistic mice and could be a target for therapeutic strategies for neurological disorders and autism. TLRs have been known to play important roles in the pathogenesis of brain diseases (Hanke and Kielian 2011). TLR2 activation is associated with neurogenesis (Rolls et al. 2007). TLR2 also mediates neuroinflammation and neuronal damage (Hoffmann et al. 2007). TLR2 ligands stimulate astrocytic and microglial cells leading to an upregulation in the production of cytokines and chemokines (Jack et al. 2005). TLR2 signaling increase in the brain is actively associated in neuroinflammation in the CNS (Zekki et al. 2002). We confirmed that resveratrol downregulated both mRNA and protein TLR2 expression levels; these effects might help explain the neuroprotective ability of resveratrol. Our results suggested that resveratrol has an inhibitory effect on TLR2 production and could play an important role in the treatment of autistic disorders.

In order to investigate TLR3 involvement in the neuroimmune dysfunction, we evaluated the effect of resveratrol in BTBR mice. We demonstrated here that resveratrol administration significantly declined the number of CD4 expressing TLR3 cells. To further elucidate the mechanism of action of resveratrol, we evaluated gene and protein expression of TLR3 in the brain tissue and found that TLR3 was significantly increased in BTBR control mice, whereas treatment with resveratrol considerably decreased its expression. Activation of TLR3 signaling at initial developmental stages induces schizophrenia- and autism-like behavior (Patterson 2011). Neuronal TLR3 activation alters brain and neuronal function (Liu et al. 2014). TLR3 activation might be involved in neurodegenerative, psychiatric, and pain disorders (Field et al. 2010). TLR3 also initiates the stimulation of signal transduction through interferon regulatory factor 3 (IRF3) and NF-κB (Field et al. 2010). Our results showed that resveratrol effectively decreases the expression of TLR3. These results support our hypothesis that resveratrol could be a therapeutic target for neuroimmune disorders, and that resveratrol has anti-neuroinflammatory effects and could have therapeutic potential for the treatment of autism.

We studied whether resveratrol treatment could change TLR4 expression. TLR4 cells significant increase in BTBR control mice; however, resveratrol administration decreases TLR4 mRNA and protein levels. Our results indicated that resveratrol regulates immune dysfunction in autistic disorder by decreasing TLR4 expression. TLR4 has been associated with the regulation of altered behaviors (Okun et al. 2011). Recently, we revealed that TLR4 signaling is associated with children with autism (Nadeem et al. 2017). TLR4 is distributed in the brain and has a significant role in the CNS through the regulation of neuroinflammation (Jin et al. 2008). TLR4 also plays a critical role in the initiation of the neuroinflammatory process leading to the transcription of multiple proinflammatory genes in several neuroinflammatory disorders (Trotta et al. 2014). Neuroinflammation is a key component of various neurological diseases. TLR4 is predominantly expressed in microglia and the resident immune cells in the brain (Laflamme et al. 2003). TLR4

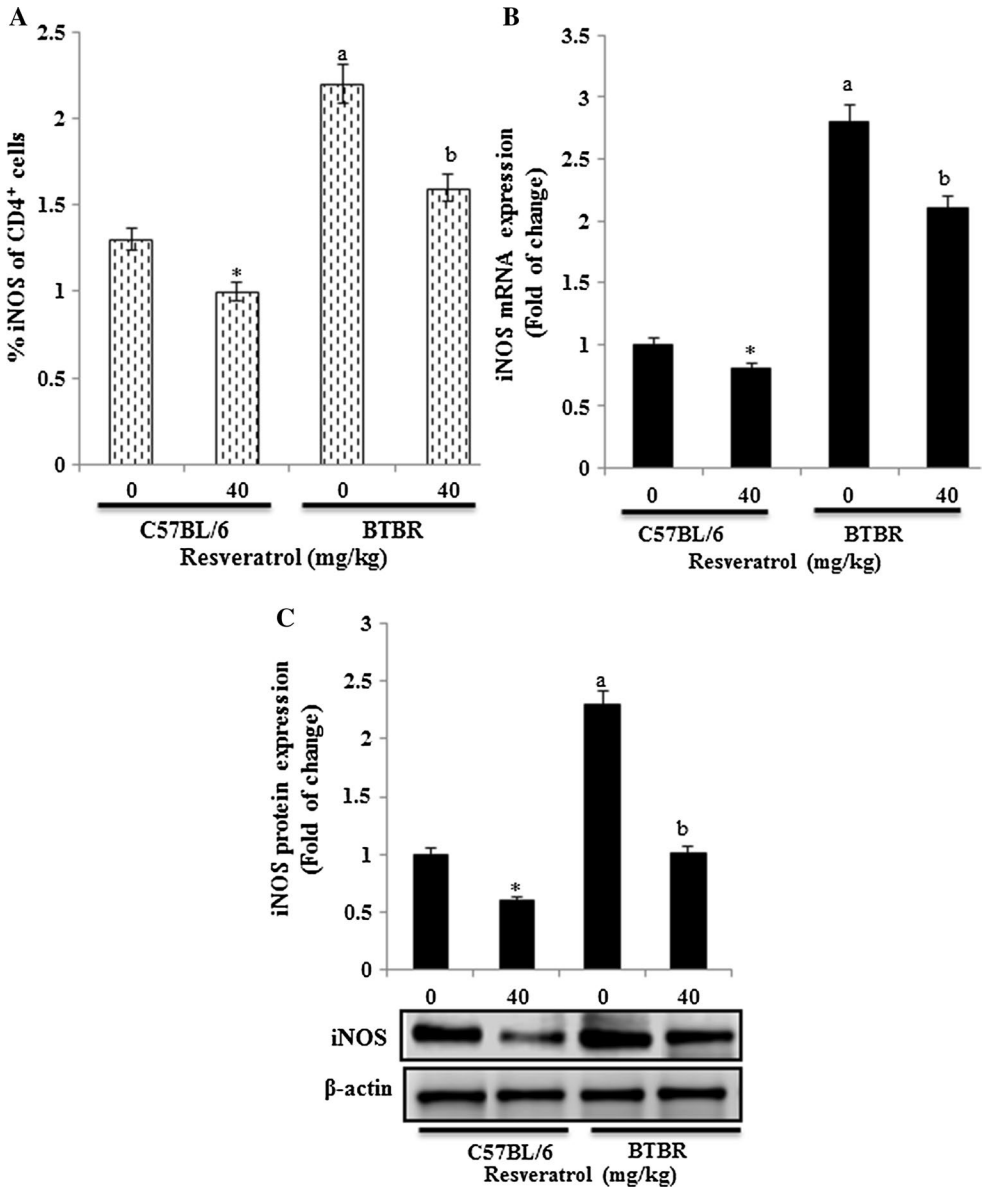


Fig. 5 **a** Flow cytometric analysis indicates the effect of resveratrol on iNOS$^+$ percentage by CD4$^+$ T cells in the spleen. **b** RT-PCR analysis indicates the effect of resveratrol on iNOS gene expression in the brain tissue; GAPDH expression was used as an internal control. **c** iNOS protein expression was evaluated through western blotting analysis and quantified relative to β-actin in the brain tissue in C57BL/6 and BTBR control mice, which received 1% DMSO in saline only [intraperitoneal (i.p.)]; C57BL/6 mice treated with resveratrol 40 mg/kg, i.p; BTBR mice treated with resveratrol 40 mg/kg, i.p. All treatments were administered for seven consecutive days. $^{*,a}P < 0.05$ compared to C57BL/6 control mice; $^{b}P < 0.05$ compared to BTBR control mice. All data were represented as mean ± SEM; the level of significance was set at $^{*}P < 0.05$

activation persuades proinflammatory atmosphere between MAPK and Jak/Stat signaling in brain astrocyte cultures (Gorina et al. 2011). Our results enhance the understanding of resveratrol treatment in modulation of neuroimmune processes by affecting TLR4 expression, which may have significant associations for the treatment of neuroinflammatory disorders including autism.

In our study, we have found that there was a significant increase in the expression of NF-κB in BTBR control mice when compared with C57BL/6 control mice. Resveratrol treatment in BTBR mice significantly decreased NF-κB expression as compared with BTBR control mice; treatment with resveratrol on C57BL/6 also inhibited NF-κB expression levels. The mechanism of resveratrol action on the inhibition of NF-κB signaling remains to be determined. In our recent study, we revealed higher expression of TLR4 and NF-κB of autistic children (Nadeem et al. 2017). NF-κB activation plays very important role in many neurodevelopmental disorders (Glass et al. 2010). Children with autism show significantly increase in NF-κB activation (Naik et al. 2011). Resveratrol has been shown to exhibit neuroprotection through inhibition of NF-κB activation (Rege et al. 2014). Our results support the effect of resveratrol in NF-κB in BTBR autistic mice and suggest that resveratrol is an effective inhibitor of NF-κB, which may explain its neuroimmune immunomodulatory effects.

We found that resveratrol administration significantly inhibits iNOS expression; thus, suppression of iNOS by resveratrol could be due to inhibition of NF-κB activation. Our results deliver new evidence indicating that iNOS may

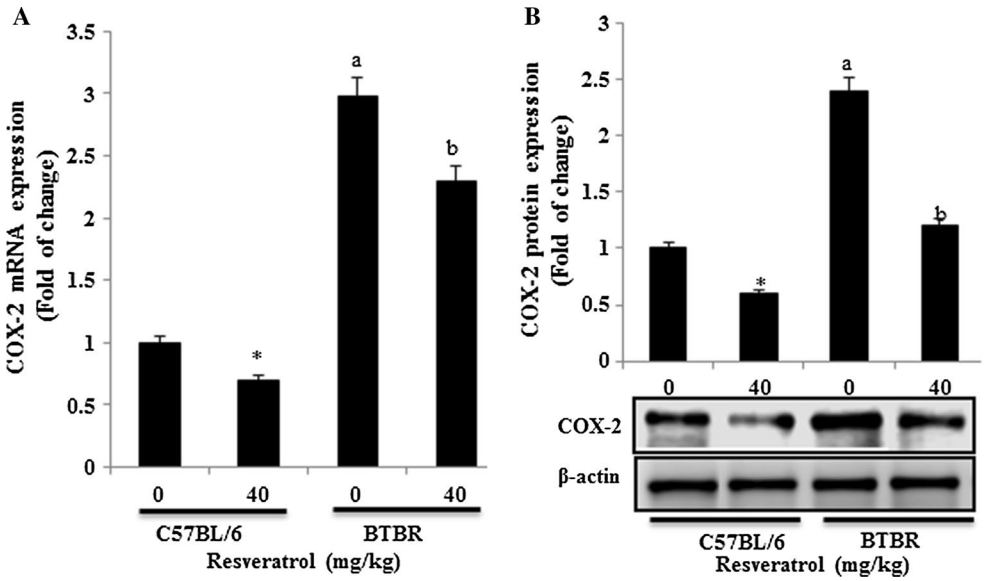


Fig. 6 **a** RT-PCR analysis indicates the effect of resveratrol on COX-2 gene expression in the brain tissue; GAPDH expression was used as an internal control. **b** COX-2 protein expression was evaluated through western blotting analysis and quantified relative to β-actin in the brain tissue in C57BL/6 and BTBR control mice, which received 1% DMSO in saline only [intraperitoneal (i.p.)]; C57BL/6 mice treated with resveratrol 40 mg/kg, i.p; BTBR mice treated with resveratrol 40 mg/kg, i.p. All treatments were administered for seven consecutive days. $^{*,a}P < 0.05$ compared to C57BL/6 control mice; $^{b}P < 0.05$ compared to BTBR control mice. All data were represented as mean ± SEM; the level of significance was set at $^{*}P < 0.05$

be a promising therapeutic target for treatment of autism. Moreover, a broad understanding of the specific effect of resveratrol on iNOS may help further explain the mechanism of action of resveratrol in immune disorders. Previously, it was demonstrated that there was a significant increase in gene expression of NOS2 in individuals with autism as compared to controls (Chana et al. 2015). iNOS expression was triggered in microglia upon inflammation (Bechade et al. 2014). Our previous results also showed that iNOS was significantly aggravated in children with autism (Nadeem et al. 2017). Taken together, these results suggest that resveratrol attenuates neurodevelopment disorder by reducing iNOS expression in BTBR mice. Resveratrol effectively decreased COX2 expression that can be important for neuroimmune protection, suggesting it could play a significant role in the regulation of autistic dysfunction. We found that resveratrol blocked COX2 expression, which is known to be regulated through NF-κB (Shishodia and Aggarwal 2002). The neuroprotective role of resveratrol on BTBR mice supported our observations. Our results indicate that resveratrol could be one of the novel therapeutic candidates that can modulate neuroimmune dysfunction through inhibition of TLRs, iNOS, and COX2 expression, thereby laying the foundation for therapeutic benefit for neuroinflammation and autistic disorders in the future.

It is to be noted here that our study did not measure the expression of different TLRs expression in specific neuronal cells such as microglial or neuronal cortical cells which requires cell culture techniques. This might give better picture of different pathways associated with TLR signaling and the effect of resveratrol on these TLRs associated pathways. This could be an interesting area for future research.

Conclusion

Taken together, our results show that resveratrol administration significantly attenuated neuroimmune development through the inhibition of neuronal TLRs expression, suggesting that resveratrol has a protective effect on autism. Our results also provide evidence that the resveratrol mechanism of action may be through the inhibition of the NF-κB, iNOS, and COX-2 signaling pathway. This neuroprotection may be due to the downregulation of neuroimmune dysfunction. These results provide evidence that resveratrol may be effective in treating autistic disorder. Future work on specific neuronal cells via cell culture techniques could help to elucidate the role of resveratrol in different neurodevelopment disorders including autism.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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