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The potent immunomodulatory compound VGX-1027 regulates inflammatory mediators in CD4$^+$ T cells, which are concomitant with the prevention of neuroimmune dysregulation in BTBR T$^+$ Itpr3^{tf}/J mice

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ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by symptoms that include social communication impairments, interaction deficits, and repetitive and stereotyped behaviors. Recent studies have suggested that imbalanced cytokine levels are associated with impaired behavioral outcomes in individuals with ASD. VGX-1027 is a potent immunomodulatory compound that has shown promise for the treatment of several neuroinflammatory disorders. Here, we studied the effects of VGX-1027 on BTBR T$^+$ Itpr3^{tf}/J (BTBR) mice, an animal model of autism. BTBR mice exhibit most of the core behavioral features of ASD, such as reduced sociability and increased repetitive behaviors. In this study, we investigated the effects of VGX-1027 on self-grooming, marble burying and sociability tests using BTBR mice. We further examined its effect on IL-1β, IL-6, IL-10, TNF-α, IFN-γ, and NF-κB p65 production in splenic CD4$^+$ cells and on IL-1β, IL-6, IL-10, TNF-α, IFN-γ, COX-2, and iNOS (NOS2) protein and mRNA expression in brain tissues. The administration of VGX-1027 was found to attenuate self-grooming and marble burying behaviors, and enhance social interactions in BTBR mice. Additionally, VGX-1027 treatment resulted in a substantial decrease in IL-1β, IL-6, TNF-α, IFN-γ, and NF-κB p65 production, but increased IL-10 production in CD4$^+$ T cells. Moreover, this agent was also found to significantly decrease IL-1β, IL-6, TNF-α, IFN-γ, COX-2, and NOS2 levels and increase IL-10 expression at the protein and mRNA level in brain tissues. Based on results using BTBR mice, our data provide the first evidence that VGX-1027 could potentially be used for the amelioration of autism-like symptoms.

1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by deficits in social and expressive communication and stereotypical behaviors [1]. Several biomarkers have been proposed for ASD, including immunological, biochemical, hormonal, neurophysiological, and neuropsychological [2]. It is known that a subtype of overall spectrum of ASD displays altered immune profiles that are linked to behavioral deficits [3]. Further, immune abnormalities including an imbalance in cytokine profiles are believed to have a significant role in ASD [4]. One previous study reported that children with ASD suffer from the development of neuroinflammation in different regions of the brain [5]. Previously, we also revealed that there are immune abnormalities such as imbalances in cytokine and transcription factor signaling in children with ASD and in the BTBR T$^+$ Itpr3tf/J (BTBR) mouse model of autism [6,7].

Cytokines act as immunomodulatory and endocrine messengers, and are involved in different processes during central nervous system (CNS) development [8]. Several studies have provided evidence that disrupted cytokine expression is a significant risk factor for ASD [9,10]. One recent study also showed that elevated mid-gestational levels of chemokines and inflammatory cytokines are highly associated with ASD in children [11]. Moreover, pro-inflammatory cytokines such as IL-1β and IL-6 were found to be significantly increased in children with ASD [3]. It was also shown that injection of the pro-inflammatory cytokine IL-6 into pregnant mice leads to an increase in autism-related behaviors in the adult offspring [12]. Further, higher levels of IL-1β, IL-6, IFN-γ, and TNF-α levels were observed in postmortem brain tissues of ASD patients [13,14]. A recent study also showed that higher levels of IFN-γ might be associated with severe neurodevelopmental disorders

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[15]. Moreover, increased IFN-γ and TNF-α expression was also demonstrated in the brains of people with autism [5]. These finding suggests that increased levels of proinflammatory cytokines could be critical for the development of ASD.

The activation of NF-κB upregulates the expression of proinflammatory mediators including cytokines, COX-2, and iNOS (NOS2), which play an important role in neuroinflammation-mediated neurodegeneration [16]. One previous study suggested that an exaggerated neuroinflammatory response could be due to aberrant NF-κB signaling during ASD [17]. It was also shown that NF-κB expression is significantly higher in the cerebellums of autistic children [18]. Further, COX-2 is an important mediator that is associated with the inflammatory cascade in the brain [19]. COX-2 expression levels are significantly higher in the cerebral cortex and hippocampus [20]. In addition, it was previously shown that increased iNOS signaling participates in the opening of glial cell hemichannels [21]. Our previous results also indicate that signaling via proinflammatory mediators and transcription factors is considerably increased in the BTBR T^+ $Itpr3^{tf}$/J (BTBR) mouse model of autism [22]. Accordingly, molecular evidence has suggested possible mechanisms through which the abnormal expression of NF-κB and COX2/iNOS mediators could affect brain development and lead to ASD.

Previously it was confirmed that IL-10 is a potent anti-inflammatory cytokine [23]. Specifically, activation of its receptor leads to the inhibition of proinflammatory cytokine production [24]. Further, IL-10 was reported to be an anti-inflammatory cytokine that is associated with decreasing inflammation [25]. Mechanistically, IL-10 attenuates NF-κB activation and also prevents the production of microglial proinflammatory mediators [26]. Previous results also suggested that IL-10-targeted therapeutics could be subjected to clinical trials for the treatment of neuroimmune diseases [27]. Therefore, the upregulation of IL-10 could represent a therapeutic strategy to inhibit the development of ASD.

VGX-1027 [(S,R)-3-phenyl-4,5-dihydro-5-isoxazole acetic acid] is a potent immunomodulatory compound that has shown efficacy against several human and animal immunoinflammatory diseases such as brain edema, diabetes, and rheumatoid arthritis [28–30]. The administration of VGX-1027 inhibits increased proinflammatory cytokine production and counteracts immunopathogenic pathways [31]. Previously, it was revealed that treatment with VGX-1027 reduces proinflammatory cytokine secretion and downregulates NF-κB and MAP kinase activation [30]. Moreover, a recent study also provided evidence that VGX-1027 treatment can attenuate uveitis, suggesting that it might have potential for the treatment of neuroinflammatory conditions [29].

BTBR mice display dysregulation in communication/social behaviors along with stereotypic behaviors, which are similar to the behavioral abnormalities observed in people with autism. BTBR mice have diminished sociability, high levels of repetitive self-grooming and minimal vocalization in social settings as compared to C57 mice which are considered social [30,31]. BTBR mice also have an altered inflammatory profile in several signaling pathways such as Th17, NF*k*B and oxidative stress which are also observed in subjects with autism [6,32–35]). Therefore, we explored the effects of VGX-1027 on various pro- and anti-inflammatory cytokines in brain and periphery of BTBR mice in the present work. Our data display that VGX-1027 redresses the imbalance of pro- and anti-inflammatory profile in BTBR mice.

2. Materials and methods

2.1. Chemicals and antibodies

The potent immunomodulatory compound VGX-1027, phorbol 12-myristate13-acetate, ionomycin, phosphate buffer saline, and RPMI 1640 medium were purchased from Sigma-Aldrich (St. Louis, USA). Primary antibodies specific for IL-1β, IL-6, IL-10, TNF-α, COX-2, iNOS, and β-actin, as well as anti-mouse, anti-rabbit, and anti-goat horseradish peroxidase (HRP) secondary antibodies were all purchased from Santa Cruz Biotech (Dallas, USA). Anti-CD4-PE, anti-CD4-FITC, anti-CD4-APC, anti-IL-1β-FITC, anti-IL-6-APC, anti-IL-10-PE, anti-TNF-α-FITC, anti–IFN–γ-PE, and anti–NF–κB p65-FITC, as well as RBC lysis and fixation buffers, permeabilization solution, and Golgiplug were purchased from BioLegend and BD Biosciences (San Diego, USA). Primers were purchased from GenScript (Piscataway, USA). Nitrocellulose membranes were purchased from Bio-Rad Laboratories (Hercules, USA). SYBR Green Master Mix and High-Capacity cDNA Reverse Transcription kits were purchased from Applied Biosystems (Foster City, USA). TRIzol reagent was purchased from Life Technologies (Paisley, UK). The chemiluminescence kit was purchased from Millipore (Billerica, USA).

2.2. Animal groups and VGX-1027 treatment

All animal experiments and procedures were conducted in accordance with accepted standards of animal care and the legal requirements for animals in the King Saud University, Kingdom of Saudi Arabia. Male C57BL/6 (C57) and BTBR T^+Itpr3^{tf}/J (BTBR) mice ranging from 6 to 7 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME, USA), and used for this study. To evaluate the effect of VGX-1027 treatment on the production or expression of pro- and anti-inflammatory mediators, C57 and BTBR mice (nine mice per group) were treated with VGX-1027 intraperitoneally (i.p. at 0.5 mg/mouse) once daily for 7 consecutive days. C57 and BTBR mice were also treated with saline, as control groups, for comparison. This dose of VGX-1027 was selected based on the results of a previous study [36]. Treatment regimen commenced on evening of day 1 and continued until evening of day 7 followed by behavioral testing on the morning of day 8. Marble burying test was conducted first, followed by self-grooming test and then tissues were collected from the same animals for molecular analyses. Brain was immediately isolated and divided into hemispheres, half was stored in RNAlater for RNA isolation and another half was stored at −70C for western blot analysis after behavioral tests on day 8. Spleen was utilized for preparation of single cell suspension for flow cytometry experiment on day 8. In a separate batch of mice, open field test and sociability test were conducted in chronological order on the morning of day 8 with similar dosing regimen as stated above.

2.3. Self-grooming

Mice were scored for spontaneous repetitive self-grooming behavior. Briefly, each mouse was placed individually into clean, empty cages (30 × 40 cm) without bedding and left undisturbed to freely explore the area. Following a 10-min habituation, a trained observer for 10 min who was unaware of the treatment strategy manually scored self-grooming activity. The trained investigator sat approximately 2 m from the cage and recorded with a stopwatch. Grooming behavior included head washing, body grooming, genital/tail grooming, scratching, and paw and leg licking behaviors [37]. Cages were cleaned by a tissue paper soaked in 70% ethanol followed by wiping with a dry tissue paper before testing the next mouse.

2.4. Marble burying

Twenty marbles were prepared in a 5 × 4 grid in a cage filled with 5 cm of clean bedding. Each mouse was individually placed into this cage and allowed to freely explore for 30 min. At the end of the session, the mouse was gently removed and the number of buried marbles (< 2/3 the depth at which the marbles were buried) was counted by the tester who was unaware of the treatment strategy [38]. Bedding was changed before testing a new mouse.

Table 1
Primers sequence. IL, Interleukin; TNF-α, Tumor necrosis factor-α; IFN-γ, Interferon gamma; NF-κB, Nuclear factor κB; COX-2, Cyclooxygenase-2; iNOS, Inducible nitric oxide synthase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

Targeted gene	Direction and Sequence
IL-1β	F: 5′-ACCTAGCTGTCAACGTGTGG-3′ R: 5′- TCAAAGCAATGTGCTGGTGC-3′
IL-10	F: 5′-CAGAGAAGCATGGCCCAGAA-3′ R: 5′-AGGACACCATAGCAAAGGGC -3′
TNF-α	F: 5′-GGACTAGCCAGGAGGGAGAA-3′ R: 5′-CGCGGATCATGCTTTCTGTG-3′
IFN-γ	F: 5′-AGGAAGCGGAAAAGGAGTCG-3′ R: 5′-GGGTCACTGCAGCTCTGAAT-3′
NF-κB p65	F: 5′-TGCAGAGAGACTGATCGGGA-3′ R: 5′-GCCTGGTCCCGTGAAATACA-3′
COX-2 iNOS	F: 5′-GCCGGGTAAGCATTGTTCCT-3′ R: 5′-ACCTAAAACCCTTCCTAAACCTTGT-3′
GAPDH	F: 5′-CTATGGCCGCTTTGATGTGC-3′ R: 5′-CAACCTTGGTGTTGAAGGCG-3′
	F: 5′-CCCAGCAAGGACACTGAGCAAG-3′R: 5′-GGTCTGGGATGGAAATTGTGAGGG-3′

2.5. Three-chambered sociability test

This test is utilized to assess sociability as described earlier [31]. This test was conducted in a three-chambered acrylic rectangular box (22 cm × 60 cm x 22 cm) in which test mouse is placed to explore three chambers for 10 min by lifting left and right retractable doors at the same time. Both sides of the chambers (left and right) have inverted metal wire mesh pencil cups, only difference being that one cup is empty and other cup has a novel mouse (Balb/c, 8–10 weeks old, male). Before carrying out the actual test, test mouse is allowed to explore three chambered box for 10 min during which time it does not have pencil cups/novel mouse. Moreover, the novel mouse is also acclimated to pencil cups a day before the actual test. Two independent observers blinded to the treatment groups were employed for recording social interactions (described as head/body moves near the cup of novel mouse/empty cup) and time spent in each chamber. Standard social behavior is usually specified by greater time engagement of test mouse in novel mouse chamber as well more interactions with novel mouse cup. Social domain exploration (%) is quantified as the time elapsed by the test mouse in novel mouse chamber/total amount of time elapsed in all chambers x100, whereas social proximity index (%) is quantified as the time elapsed by test mouse near the novel mouse cup/total amount of time elapsed near both cups ×100 as described earlier by us and others [31,35,39].

2.6. Open field test

Locomotor activity was determined to ascertain whether the test drug had any motor regulatory effects. The test was conducted in a 50 cm × 50 cm open field box having 16 equal squares and 22 cm high walls as detailed earlier [35]. Locomotion was ascertained by exploration of each mouse in a 10-min test session during which a scientist blinded to different treatment groups quantified time number of squares traversed by each mouse.

2.7. Flow cytometric analysis

Flow cytometry was performed to evaluate intracellular IL-1β, IL-6, IL-10, TNF-α, IFN-γ, and NF-κB p65 levels in $CD4^+$ T cells from the spleen. Briefly, to detect the production of inflammatory mediators, spleen cells were incubated with PMA/ionomycin (Sigma-Aldrich) and treated with GolgiStop (BD Biosciences), prior to intracellular staining [7]. Spleen cells were washed and surface staining of $CD4^+$ T cell receptors was performed (BioLegend; San Diego, USA). Spleen cells were fixed and permeabilized and then stained with anti-IL-1β, anti-IL-6, and anti-IL-10, anti-TNF-α, anti–IFN–γ, and anti–NF–κB p65 antibodies (BioLegend; San Diego, USA), as previously described [40]. In total, 10,000 cells were acquired for flow cytometric analysis and were analyzed using CXP software (Beckman Coulter, USA).

2.8. Western blot analysis

Western blot analysis was conducted as previously described [41]. Briefly, protein was extracted from the brain and quantification was carried out using the Direct Detect spectrometer (Millipore, Billerica, USA) [7,42]. Then, for each group, 30 μg of protein was separated by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to PVDF membrane (Millipore, USA). Membranes were blocked overnight at 4 °C in blocking solution, which was followed by incubation with primary antibodies against IL-1β, IL-6, IL-10, TNF-α, COX-2, and iNOS. After a 1-h incubation with peroxidase-conjugated secondary antibodies (Santa Cruz, USA) at room temperature, bands corresponding to IL-1β, IL-6, IL-10, TNF-α, COX-2, and iNOS were visualized using a Chemiluminescence kit (Millipore, USA), and band intensity was detected and presented relative to that of β-actin.

2.9. RT-PCR analysis for mRNA expression

Total RNA was isolated from brain tissues using TRIzol reagent (Invitrogen) as previously described [7,43]. cDNA was synthesized using a high capacity cDNA reverse transcription kit (Applied Biosystems) and subjected to quantitative PCR, which was performed using the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, USA) and SYBR® Green PCR master mix. The primers used in this study are listed in Table 1. The data were presented as the fold-change in mRNA expression normalized to levels of the endogenous reference gene, β-actin. The quantity of mRNA encoding IL-1β, IL-10, TNF-α, IFN-γ, NF-κB p65, COX-2, and iNOS was normalized to that of GAPDH.

2.10. Statistical analysis

All results are reported as the mean ± SEM, and analyzed by performing a two-way ANOVA. If there were significant differences, Tukey's post-hoc test corrected for multiple comparisons was utilized. The results were considered significant at $p < 0.05$. All analyses were performed using GraphPad Prism 8 software.

3. Results

3.1. VGX-1027 treatment prevents self-grooming and marble burying behaviors

Several tests pertaining to autism-like behavior such as self-grooming, marble burying and three-chambered social interaction test were conducted to evaluate the efficacy of VGX-1027 in BTBR and C57 mice (Fig. 1A–D). In BTBR mice treated with saline, self-grooming times were increased as compared to that in C57 mice treated with saline (Fig. 1A). However, as shown in Fig. 1A, VGX-1027 treatment resulted in a significant reduction in self-grooming scores in BTBR mice compared to that in BTBR mice treated with saline (Fig. 1A). BTBR mice treated with saline also buried more marbles than C57 mice treated

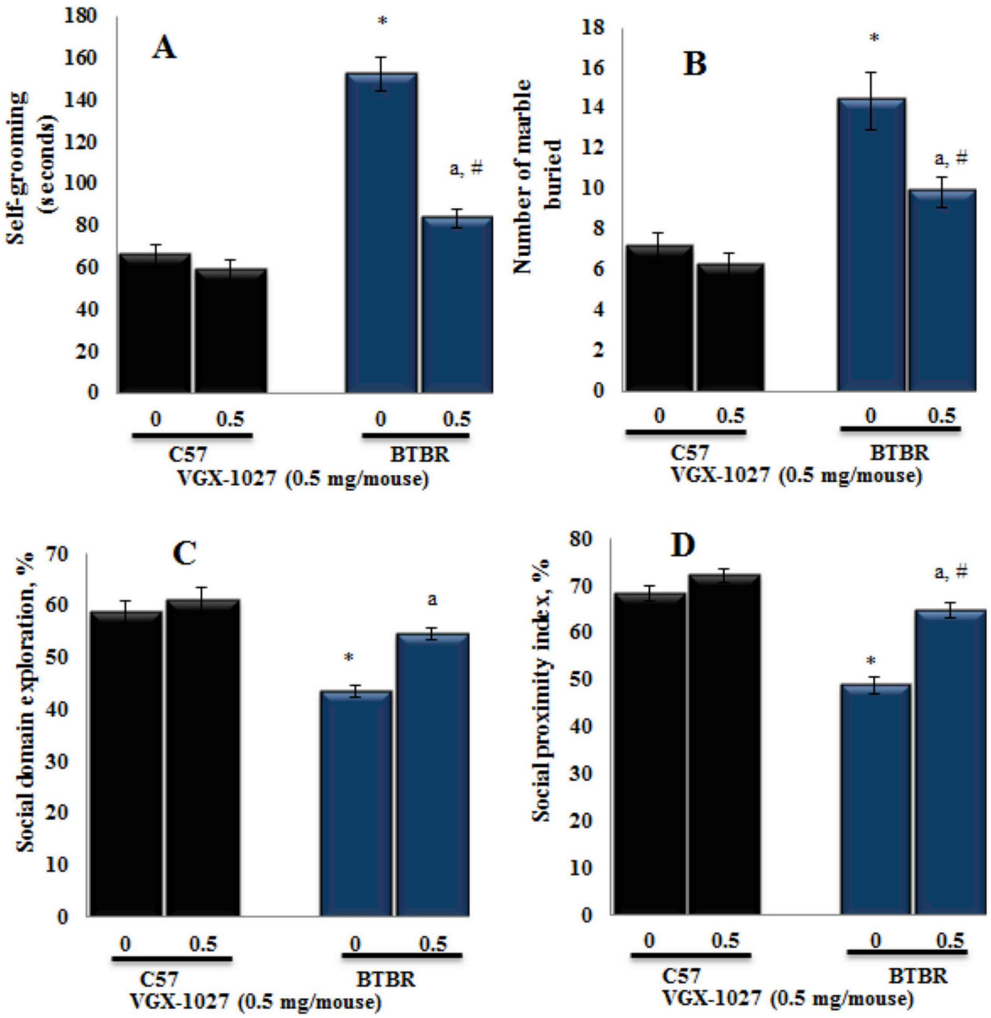

Fig. 1. Effects of VGX-1027 on autism-like behavioral tests in C57 and BTBR mice. A) Self-grooming test (Strain effect, $F_{(1,32)} = 138$, $P < 0.0001$; Treatment effect, $F_{(1,32)} = 65.23$, $P < 0.0001$, Treatment x Strain effect, $F_{(1,32)} = 42.24$, $P < 0.0001$), **B**) Marble burying test (Strain effect, $F_{(1,32)} = 50.61$, $P < 0.0001$; Treatment effect, $F_{(1,32)} = 12.40$, $P = 0.0013$, Treatment x Strain effect, $F_{(1,32)} = 5.63$, $P = 0.023$), **C**) Social domain exploration (Strain effect, $F_{(1,32)} = 33.40$, $P < 0.0001$; Treatment effect, $F_{(1,32)} = 12.55$, $P = 0.0012$, Treatment x Strain effect, $F_{(1,32)} = 5.37$, $P = 0.027$), and **D**) Social proximity index (Strain effect, $F_{(1,32)} = 69$, $P < 0.0001$; Treatment effect, $F_{(1,32)} = 37$, $P < 0.0001$, Treatment x Strain effect, $F_{(1,32)} = 13.50$, $P = 0.0009$). Control C57 and BTBR mice were treated with saline by intraperitoneal (i.p) injection. C57 and BTBR mice were also treated with VGX-1027 (0.5 mg/mouse, i.p.) daily for 7 days. Statistical analysis was performed by two-way ANOVA followed by Tukey's *post-hoc* test corrected for multiple comparisons. Values are indicated as the mean ± SEM, n = 9. $^{*}P < 0.05$ compared to saline-treated C57 mice; $^{a}P < 0.05$ compared to saline-treated BTBR mice; $^{\#}P < 0.05$ compared to VGX-1027 treated C57 mice.

with saline (Fig. 1B). However, the number of marbles buried by BTBR mice treated with VGX-1027 (0.5 mg/mouse) was significantly lower than that in BTBR mice treated with saline (Fig. 1B). Furthermore, in terms of sociability, saline treated BTBR mice showed decreased social domain exploration (Fig. 1C) as well as social proximity index (Fig. 1D) as compared to saline treated C57 mice. However, VGX-1027 was able to enhance sociability (social interactions with novel mouse as well as social domain exploration) towards normality (Fig. 1C and D). Effects of VGX-1027 was also determined to rule out any effect on motor function in all the studied groups. In terms of locomotor activity, the number of squares crossed by the mice in four groups were similar (data not shown) and there were no statistical differences among differences whatsoever as assessed by two-way ANOVA and Tukey's post-hoc test. Therefore, VGX-1027 treatment has the potential to restore normal social behavior and diminish stereotypic behaviors in BTBR mice without affecting their ambulatory activity.

3.2. VGX-1027 treatment regulates inflammatory mediators

We further evaluated the effects of VGX-1027 administration on IL-1β production in $CD4^+$ T cells in control C57 and BTBR mice. In BTBR mice treated with VGX-1027, the percentage of IL-1β-producing splenic $CD4^+$ T cells was significantly decreased compared to that in BTBR mice treated with saline (Fig. 2A). Western blotting and quantitative PCR analyses also demonstrated a decrease in IL-1β protein and mRNA levels in the brain tissues of BTBR mice treated with VGX-1027 as compared to that in BTBR mice treated with saline (Fig. 2B and C, respectively).

As shown in Fig. 3A, the number of IL-6-producing splenic $CD4^+$ T cells was significantly increased in BTBR mice treated with saline as compared that in control C57 mice; however, this was suppressed in BTBR mice treated with VGX-1027 (Fig. 3A). Further, protein and mRNA levels of the proinflammatory cytokine IL-6 in the brain were also decreased in BTBR mice treated with VGX-1027, as compared to those in BTBR mice treated with saline (Fig. 3B and C, respectively).

Treating BTBR mice with VGX-1027 also resulted in a significant reduction in the percentage of TNF-α producing splenic $CD4^+$ T cells as compared to that in BTBR mice treated with saline (https://www.sciencedirect.com/science/article/pii/S0041008X18304186?via%3Dihub Fig. 4A). We next evaluated intracellular IFN-γ cytokine production by $CD4^+$ T cells. The percentage of IFN-γ-producing splenic $CD4^+$ T cells was significantly decreased in VGX-1027-treated BTBR mice, but was increased in BTBR mice treated with saline compared to that in control C57 mice (Fig. 4A). Consistent with these findings, treating BTBR mice with VGX-1027 also significantly decreased TNF-α protein levels in the brain as compared to those in BTBR mice treated with saline (Fig. 4B). To further explain the mechanism associated with the effect of VGX-1027 administration on BTBR mice, we assessed *TNF-α* and *IFN-γ* mRNA expression in brain tissues. For this, *TNF-α* and *IFN-γ* mRNA expression was significantly attenuated in VGX-1027-treated BTBR mice as compared to that in saline-treated BTBR mice (Fig. 4C). Thus, our results showed that VGX-1027 can improve immune dysfunction by downregulating proinflammatory cytokines in BTBR mice.

We further explored whether VGX-1027 affects the production of

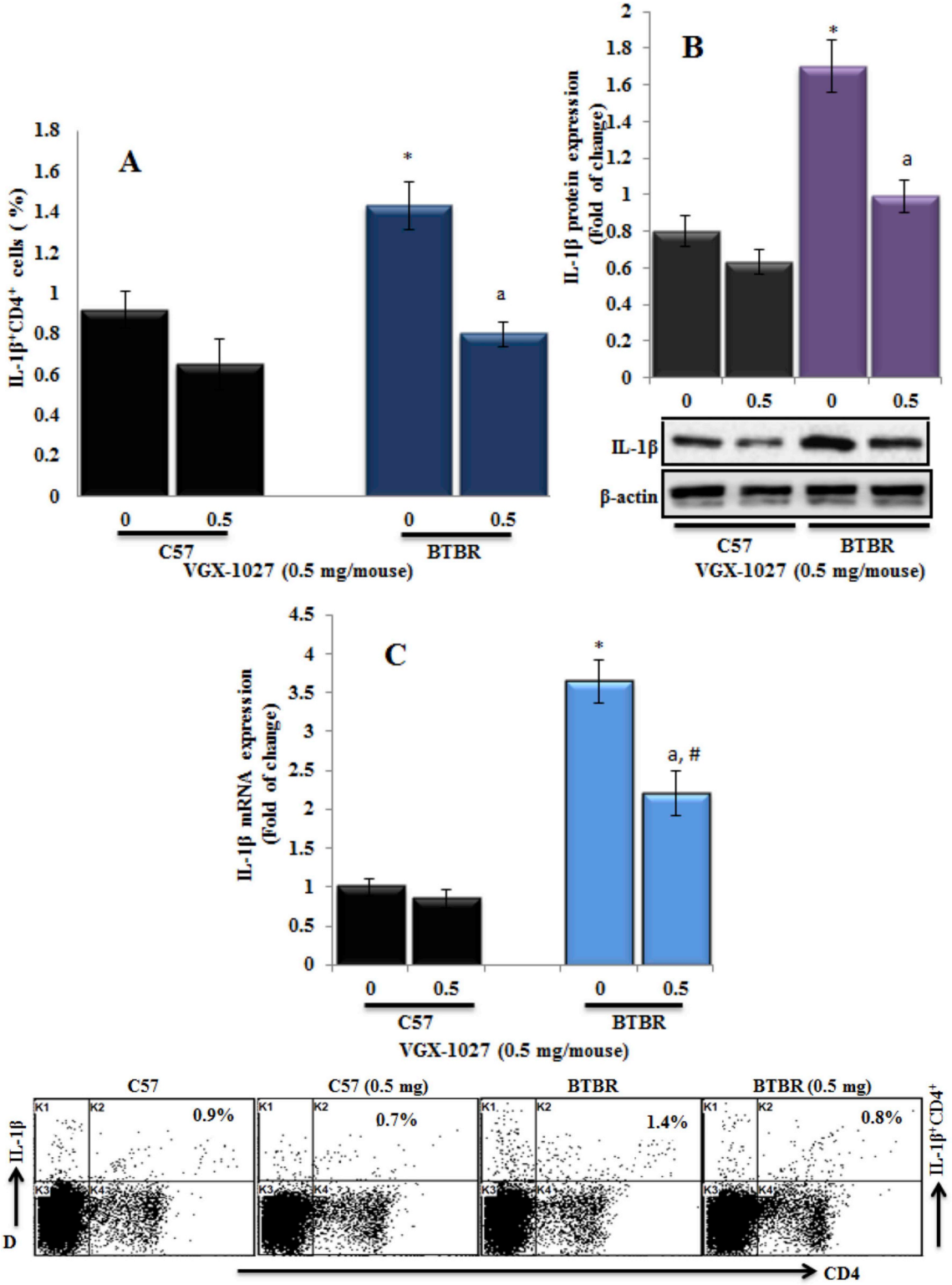

Fig. 2. VGX-1027 treatment regulates IL-1β expression in BTBR mice. A) Effects of VGX-1027 on intracellular IL-1β-producing $CD4^+$ T cells were analyzed by flow cytometry using spleen cells (Strain effect, $F_{(1,20)} = 10.60$, $P = 0.004$; Treatment effect, $F_{(1,20)} = 19.67$, $P = 0.0003$, Treatment x Strain effect, $F_{(1,20)} = 3.14$, $P = 0.091$). **B)** IL-1β protein expression in brain tissues was measured by western blotting and normalized to β-actin expression in the brain tissues (Strain effect, $F_{(1,20)} = 39$, $P < 0.0001$; Treatment effect, $F_{(1,20)} = 19$, $P = 0.0003$, Treatment x Strain effect, $F_{(1,20)} = 7.17$, $P = 0.014$). **C)** *IL-1β* mRNA expression was measured in brain tissues by RT-PCR and normalized to *GAPDH* levels (Strain effect, $F_{(1,20)} = 88.60$, $P < 0.0001$; Treatment effect, $F_{(1,20)} = 114$, $P = 0.0013$, Treatment x Strain effect, $F_{(1,20)} = 9.26$, $P = 0.0064$). **D)** Representative flow cytometric dot plots for one mouse from each group. Control C57 and BTBR mice were treated with saline via intraperitoneal (i.p) injection. C57 and BTBR mice were treated with VGX-1027 (0.5 mg/mouse, i.p.) daily for 7 days. Statistical analysis was performed by two-way ANOVA followed by Tukey's *post-hoc* test corrected for multiple comparisons. Values are indicated as the mean ± SEM, n = 6, $^*P < 0.05$ compared to saline-treated C57 mice; $^aP < 0.05$ compared to saline-treated BTBR mice; $^\#P < 0.05$ compared to VGX-1027 treated C57 mice.

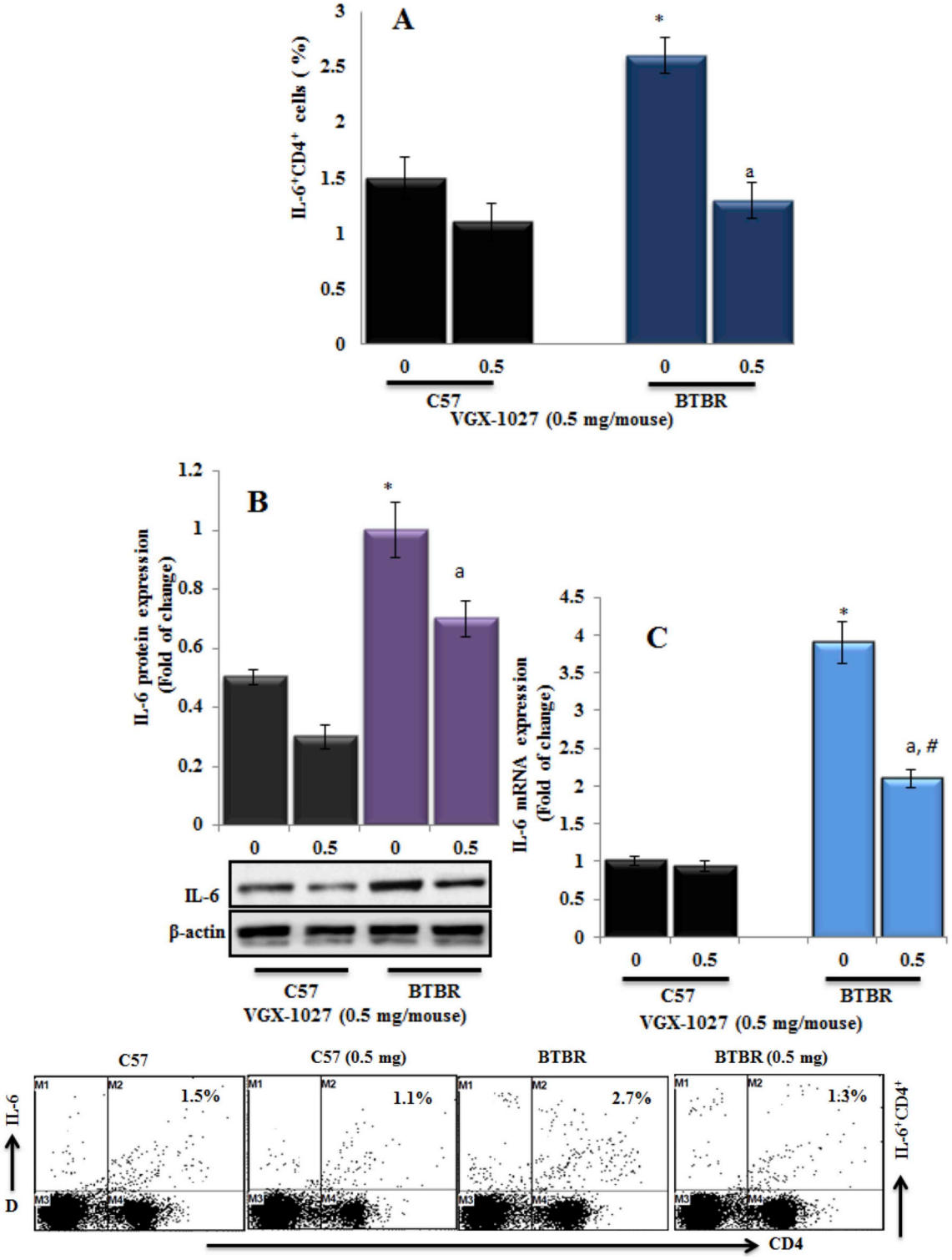

Fig. 3. Effect of VGX-1027 treatment on IL-6 production in BTBR mice. A) Effects of VGX-1027 on intracellular IL-6-producing $CD4^+$ T cells were analyzed by flow cytometry using spleen cells (Strain effect, $F_{(1,20)} = 14.80$, $P = 0.001$; Treatment effect, $F_{(1,20)} = 25.40$, $P < 0.0001$, Treatment x Strain effect, $F_{(1,20)} = 7.12$, $P = 0.014$). **B)** IL-6 protein expression was measured in brain tissues by western blotting and normalized to β-actin levels (Strain effect, $F_{(1,20)} = 76.20$, $P < 0.0001$; Treatment effect, $F_{(1,20)} = 23.50$, $P < 0.0001$, Treatment x Strain effect, $F_{(1,20)} = 0.94$, $P = 0.34$). **C)** *IL*-6 mRNA levels were measured in brain tissues by RT-PCR and normalized to *GAPDH* expression (Strain effect, $F_{(1,20)} = 173$, $P < 0.0001$; Treatment effect, $F_{(1,20)} = 36.50$, $P < 0.0001$, Treatment x Strain effect, $F_{(1,20)} = 31.20$, $P < 0.0001$). **D)** Representative flow cytometric dot plots for one mouse from each group. Control C57 and BTBR mice were treated with saline via intraperitoneal (i.p) injection. C57 and BTBR mice were treated with VGX-1027 (0.5 mg/mouse, i.p.) daily for 7 days. Statistical analysis was performed by two-way ANOVA followed by Tukey's *post-hoc* test corrected for multiple comparisons. Values are indicated as the mean ± SEM, n = 6. $^*P < 0.05$ compared to saline-treated C57 mice; $^aP < 0.05$ compared to saline-treated BTBR mice; $^{\#}P < 0.05$ compared to VGX-1027 treated C57 mice.

anti-inflammatory cytokines that plays an important role in regulating neuroinflammatory disorders. We first measured IL-10-producing $CD4^+$ T cells in the spleen. Results revealed that the proportion of $CD4^+IL\text{-}10^+$ cells increased in VGX-1027-treated BTBR mice, as compared to that in BTBR mice treated with saline (Fig. 5A). Next, we further confirmed the effect of VGX-1027 on IL-10 expression in brain tissues. In

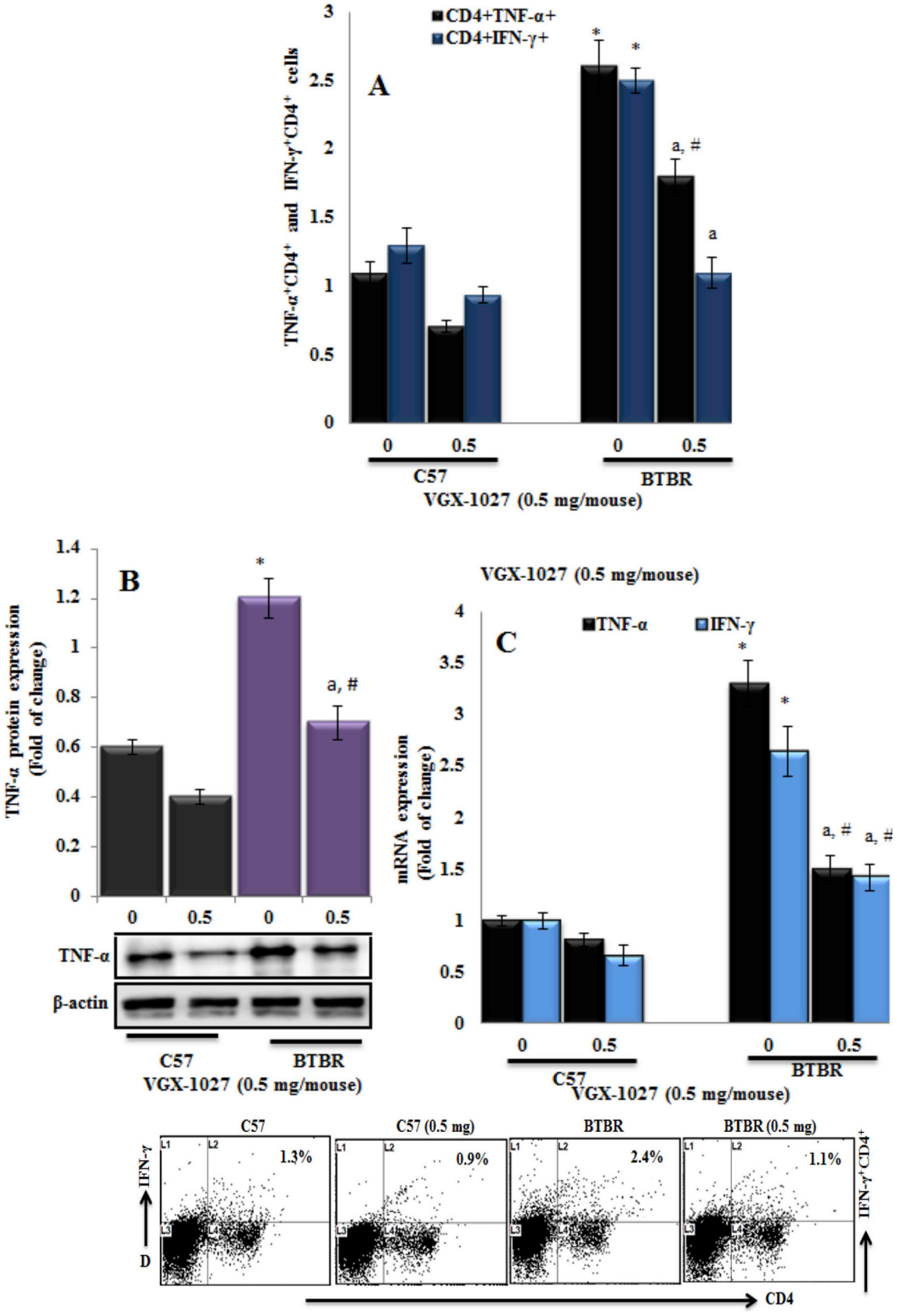

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Fig. 4. VGX-1027 suppresses TNF-α and IFN-γ expression in BTBR mice. A) Effects of VGX-1027 on intracellular TNF-α (Strain effect, $F_{(1,20)} = 102$, $P < 0.0001$; Treatment effect, $F_{(1,20)} = 21.50$, $P = 0.0002$, Treatment x Strain effect, $F_{(1,20)} = 2.55$, $P = 0.125$) and IFN-γ-producing (Strain effect, $F_{(1,20)} = 46$, $P < 0.0001$; Treatment effect, $F_{(1,20)} = 77$, $P < 0.0001$, Treatment x Strain effect, $F_{(1,20)} = 26.90$, $P < 0.0001$) $CD4^+$ T cells were analyzed by flow cytometry using spleen cells. **B)** TNF-α protein expression in brain tissues was measured by western blotting and levels were normalized to β-actin expression (Strain effect, $F_{(1,20)} = 66.40$, $P < 0.0001$; Treatment effect, $F_{(1,20)} = 40.10$, $P < 0.0001$, Treatment x Strain effect, $F_{(1,20)} = 7.37$, $P = 0.013$). **C)** *TNF-α* (Strain effect, $F_{(1,20)} = 116$, $P < 0.0001$; Treatment effect, $F_{(1,20)} = 51.60$, $P < 0.0001$, Treatment x Strain effect, $F_{(1,20)} = 33.80$, $P < 0.0001$) and *IFN-γ* (Strain effect, $F_{(1,20)} = 63.60$, $P < 0.0001$; Treatment effect, $F_{(1,20)} = 26.80$, $P < 0.0001$, Treatment x Strain effect, $F_{(1,20)} = 8.55$, $P = 0.0084$) mRNA expression levels in brain tissue were measured by RT-PCR and normalized to *GAPDH* levels. **D)** Representative flow cytometric dot plots for one mouse from each group. Control C57 and BTBR mice were treated with saline via intraperitoneal (i.p) injection. C57 and BTBR mice were treated with VGX-1027 (0.5 mg/mouse, i.p.) daily for 7 days. Statistical analysis was performed by two-way ANOVA followed by Tukey's *post-hoc* test corrected for multiple comparisons. Values are indicated as the mean ± SEM, n = 6. $^*P < 0.05$ compared to saline-treated C57 mice; $^aP < 0.05$ compared to saline-treated BTBR mice; $^\#P < 0.05$ compared to VGX-1027 treated C57 mice.

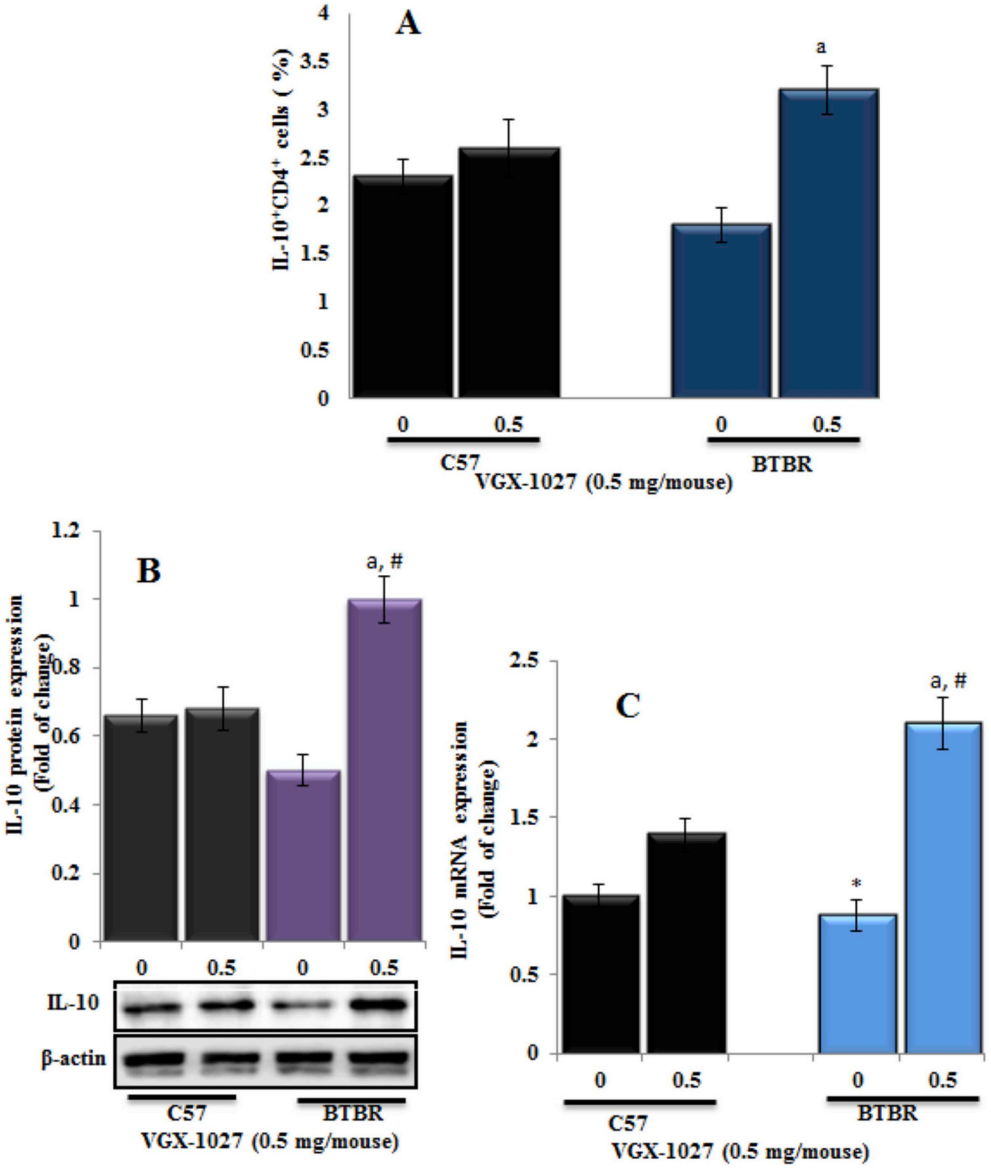

Fig. 5. VGX-1027 rescues the decrease in IL-10 production in BTBR mice. A) Effects of VGX-1027 on intracellular IL-10-producing $CD4^+$ T cells were analyzed by flow cytometry using spleen cells (Strain effect, $F_{(1,20)} = 0.047$, $P = 0.82$; Treatment effect, $F_{(1,20)} = 13.70$, $P = 0.0014$, Treatment x Strain effect, $F_{(1,20)} = 5.70$, $P = 0.026$). **B)** IL-10 protein expression in brain tissues was measured by western blotting and normalized to β-actin levels (Strain effect, $F_{(1,20)} = 1.93$, $P = 0.18$; Treatment effect, $F_{(1,20)} = 20.40$, $P = 0.0002$, Treatment x Strain effect, $F_{(1,20)} = 17.40$, $P = 0.0005$). **C)** *IL*-10 mRNA expression levels in brain tissues were measured by RT-PCR and normalized to *GAPDH* expression (Strain effect, $F_{(1,20)} = 6.70$, $P = 0.017$; Treatment effect, $F_{(1,20)} = 50$, $P < 0.0001$, Treatment x Strain effect, $F_{(1,20)} = 13.40$, $P = 0.0015$). Control C57 and BTBR mice were treated with saline by intraperitoneal (i.p) injection. C57 and BTBR mice were treated with VGX-1027 (0.5 mg/mouse, i.p.) daily for 7 days. Statistical analysis was performed by two-way ANOVA followed by Tukey's *post-hoc* test corrected for multiple comparisons. Values are indicated as the mean ± SEM, n = 6. $^*P < 0.05$ compared to saline-treated C57 mice; $^aP < 0.05$ compared to saline-treated BTBR mice; $^\#P < 0.05$ compared to VGX-1027 treated C57 mice.

VGX-1027-treated BTBR mice, IL-10 protein levels were increased compared to those in BTBR mice treated with saline (Fig. 5B). To further understand the molecular mechanism through which anti-inflammatory cytokine production was increased, mRNA expression was assessed in brain tissues. Treating BTBR mice with VGX-1027 significantly increased in *IL*-10 mRNA expression level as compared to those in BTBR mice treated with saline (Fig. 5C). Our results showed that VGX-1027 could significantly increase the anti-inflammatory cytokine response.

We next confirmed the effect of VGX-1027 on inflammatory signaling. Results showed that VGX-1027-treated BTBR mice exhibited a significant decrease in NF-κB p65-producing splenic $CD4^+$ T cells as compared to that in BTBR mice treated with saline (Fig. 6A). The potential anti-inflammatory effects of VGX-1027 on COX-2 and iNOS expression were then further investigated. Treating BTBR mice with VGX-1027 significantly reduced COX-2 and iNOS protein/mRNA levels in the brain as compared to saline-treated in BTBR mice (Fig. 6B and C, respectively). Taken together, these results showed that VGX-1027 can inhibit pro-inflammatory cytokine production and induce the expression of anti-inflammatory mediators.

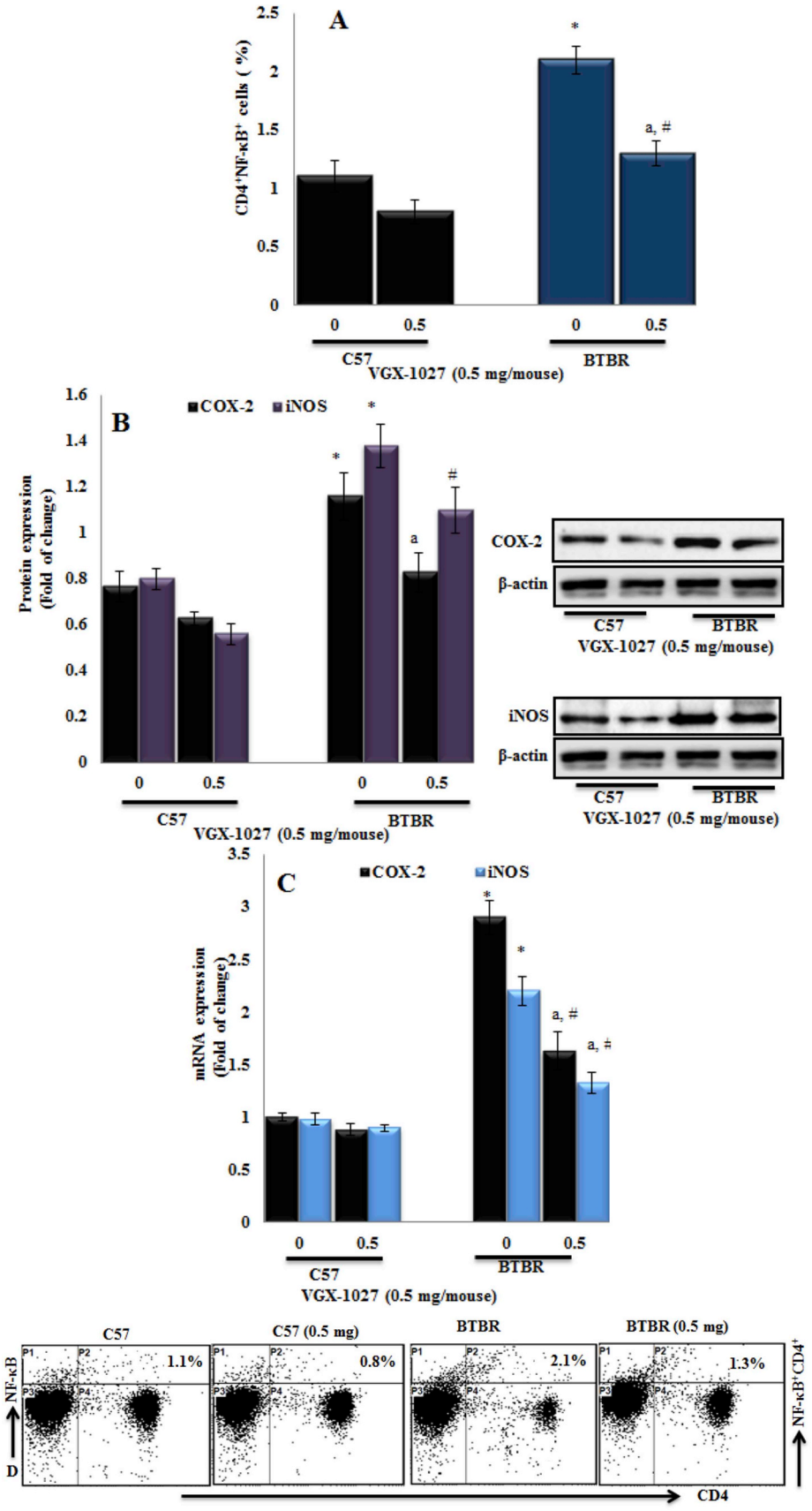

Fig. 6. Modulation of NF-κB p65, COX-2, and iNOS expression in BTBR mice by VGX-1027 administration. **A)** Effects of VGX-1027 on intracellular NF-κB p65-producing $CD4^+$ T cells were analyzed by flow cytometry using spleen cells (Strain effect, $F_{(1,20)} = 42.60$, $P < 0.0001$; Treatment effect, $F_{(1,20)} = 23$, $P = 0.0001$, Treatment x Strain effect, $F_{(1,20)} = 4.70$, $P = 0.041$). **B)** COX-2 (Strain effect, $F_{(1,20)} = 15.30$, $P = 0.0009$; Treatment effect, $F_{(1,20)} = 9.70$, $P = 0.0054$, Treatment x Strain effect, $F_{(1,20)} = 1.60$, $P = 0.22$) and iNOS (Strain effect, $F_{(1,20)} = 53.70$, $P < 0.0001$; Treatment effect, $F_{(1,20)} = 11.60$, $P = 0.0028$, Treatment x Strain effect, $F_{(1,20)} = 0.68$, $P = 0.79$) protein levels in brain tissues were measured by western blotting and normalized to β-actin expression. **C)** *COX-2* (Strain effect, $F_{(1,20)} = 112$, $P < 0.0001$; Treatment effect, $F_{(1,20)} = 31$, $P < 0.0001$, Treatment x Strain effect, $F_{(1,20)} = 21$, $P = 0.0002$) and *iNOS* (Strain effect, $F_{(1,20)} = 76.20$, $P < 0.0001$; Treatment effect, $F_{(1,20)} = 28.80$, $P < 0.0001$, Treatment x Strain effect, $F_{(1,20)} = 16.50$, $P = 0.0006$) mRNA expression levels in brain tissues were measured by RT-PCR and normalized to *GAPDH* levels. **D)** Representative flow cytometric dot plots of one mouse from each group. Control C57 and BTBR mice were treated with saline via intraperitoneal (i.p) injection. C57 and BTBR mice were treated with VGX-1027 (0.5 mg/mouse, i.p.) daily for 7 days. Statistical analysis was performed by two-way ANOVA followed by Tukey's *post-hoc* test corrected for multiple comparisons. Values are indicated as the mean ± SEM, n = 6. $^*P < 0.05$ compared to saline-treated C57 mice; $^aP < 0.05$ compared to saline-treated BTBR mice; $^\#P < 0.05$ compared to VGX-1027 treated C57 mice.

4. Discussion

The etiology and development of ASD remain unclear. It has been suggested that immune dysfunction contributes to behavioral effects during several neurodevelopmental disorders including ASD [44]. There have been several reports of immune aberrations in a subgroup of individuals with ASD [45]. Previously, it was reported that immune abnormalities associated with ASD subjects include abnormal Th1/Th2 cytokine production [46]. Maternal immune activation has also been associated with the development of autism-like behaviors [47]. Previously, it was reported that the BTBR mice exhibit reduced social interactions that resemble the symptoms of ASD [48]. In a previous study, it was also observed that BTBR mice display increased repetitive behaviors, which involve higher self-grooming and marble-burying behaviors [37]. Moreover, the expression of various pro-inflammatory mediators was also found to be significantly increased in BTBR mice, which was associated with the severity of behavioral abnormalities [33,49]. Our recent study also suggested that BTBR mice have distinct immune profiles due to an imbalance in cytokine and transcription factor signaling [22,41].

In this study, we investigated whether VGX-1027 could reduce repetitive behaviors and increase sociability in BTBR mice. Our findings suggest that VGX-1027 treatment has the potential to ameliorate behavioral deficits observed in BTBR mice. It has also been observed that VGX-1027 can inhibit clinical and histological signs of inflammation and also suppress the production of pro-inflammatory mediators [29]. Administration of VGX-1027 was found to exert beneficial effects on colitis, pleurisy, and lethal endotoxemia [30]. Therapeutic treatment with this drug also diminished the neuroinflammatory response and limited post-traumatic edema [28]. Previously, it was also shown that VGX-1027 could counteract immunopathogenic pathways that lead to autoimmune disease through inhibition in pro-inflammatory cytokine production [30].

In this study, we further explored whether VGX-1027 could exert inhibitory effects on IL-1β- and IL-6-producing $CD4^+$ T cells in BTBR mice. We confirmed that it could inhibit immunoregulatory responses based on the observed reductions in IL-1β and IL-6 protein and mRNA expression in brain tissues. Recent studies revealed an association between increases in pro-inflammatory cytokines and abnormal behaviors. IL-1β is a potent pro-inflammatory mediator that is increased in the hypothalamic–pituitary–adrenal axis, and can cause major depression [51]. Elevated levels of IL-1β are also associated with the regressive form of ASD and stereotypical behaviors [52]. One previous study provides evidence that IL-1β is associated with impaired behavioral outcome in ASD [53]. Moreover, increased IL-6 levels were reported to be associated with intelligence and socialization in children with ASD [54]. Another study also showed that both IL-1β and IL-6 are significantly increased in the brains of children with ASD [55]. Similarly, a recent study revealed that blocking IL-6 leads to decreased sociability in BTBR mice [56]. Accordingly, there are several drugs under investigation as anti-IL-6 therapeutics for numerous conditions including brain disorders [57]. Our results suggest that the anti-inflammatory effects of VGX-1027 could be due to the suppression of IL-1β and IL-6 responses. However, we need to be cautious in interpreting our preclinical findings as they may not correlate with subjects with ASD. Clinical trial with proper intervention protocol needs to conducted to answer this question.

We also wanted to assess the effects of VGX-1027 on TNF-α- and IFN-γ-producing $CD4^+$ T cells in the spleen. We observed that both TNF-α and IFN-γ were increased in saline-treated BTBR mice, but the VGX-1027 treatment could ameliorate these effects. A previous study demonstrated that a disturbance in cytokine signaling could induce ASD-like social and memory deficits [53]. Further, increased TNF-α expression is associated with neuromodulatory effects and its role has been defined in brain development, as it was found that it can play a significant role in glutamatergic synaptic transmission [58]. Further, increased levels of IFN-γ and TNF-α were reported to be associated with the pathophysiology of ASD [5,13,59,60]. Administration of VGX-1027 could downregulate IFN-γ and TNF-α in BTBR mice. These results suggest that the suppression of IFN-γ and TNF-α by VGX-1027 could be responsible for amelioration of autism-like symptoms in BTBR mice.

In this study, we further explored the therapeutic efficacy of VGX-1027 by examining its effect on IL-10 in $CD4^+$ T cells of BTBR mice. It has been shown that anti-inflammatory IL-10 signaling during prenatal development mitigates behavioral dysfunction [61]. In addition, increased expression of IL-10 was also found to diminish the fetal brain proinflammatory cytokine response [62], which could lead to normalization of several behavioral dysfunctions. One recent study also showed that IL-10 is significantly decreased in children with autism [63]. Our results suggest that VGX-1027 can greatly enhance the IL-10 response, which could help to explain its therapeutic effect in BTBR mice.

Our results indicate that VGX-1027 administration to BTBR mice leads to the suppression of NF-κB, COX-2, and iNOS signaling; therefore, this could also contribute to amelioration of behavioral dysfunction in BTBR mice.Previous reports also showed augmented expression of iNOS/COX2/NF-κB in BTBR mice and of ASD patients [17,34,64,65]. Microglial inflammation is triggered in response to increased NOS2/COX2 expression [66]. In addition, it has been suggested that NF-κB activation could be a therapeutic target for several neurodegenerative disorders [65]. Thus, anti-inflammatory effects of by VGX-1027 in BTBR micecan be explained by its ability to downregulate pro-inflammatory signaling.

In short, our results suggest that treatment with VGX-1027 effectively downregulates neuroinflammation which is coupled with amelioration of autism-like symptoms in BTBR mice. Efficacy of VGX-1027 is dependent on restoration of balance between anti-inflammatory and pro-inflammatory signaling in BTBR mice.

Declaration of competing interest

The authors declare no conflict of interest.

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