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# Dysregulation of T cell immunoglobulin and mucin domain 3 (TIM-3) signaling in peripheral immune cells is associated with immune dysfunction in autistic children

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ABSTRACT

Evidence suggests that immune dysregulation is associated with autism spectrum disorder (ASD). T cell immunoglobulin and mucin domain-3 (TIM-3) has a critical role in several inflammatory disorders; however, the role of TIM-3 signaling has not been demonstrated in ASD. In the present study, we assessed the role of TIM-3 signaling in children with ASD. We expected that increased numbers of TIM-3$^+$ cells could alter immune function in children with ASD. We revealed production of TIM-3 on CD3$^+$, CD4$^+$, CD8$^+$, CD11a$^+$,b$^+$, CD14$^+$, CD62P$^+$, and CXCR5$^+$ PBMCs in children with ASD and typically developing (TD) controls using immunofluorescent staining. We further demonstrated the production of IL-1β, IFN-γ, IL-17 A, and Foxp3 in TIM-3$^+$ PBMCs of TD controls and individuals with ASD. We also observed the mRNA expression levels of *TIM-3, CD11a,b, CD14, IL-1β* and *IFN-γ* using RT-PCR. We further assessed the protein levels of TIM-3, IL-1β, CXCR5, and IFN-γ using western blotting. The results showed that children with ASD had increased numbers of CD3$^+$TIM-3$^+$, CD4$^+$TIM-3$^+$, CD8$^+$TIM-3$^+$, CD11a,b$^+$TIM-3$^+$, CD14$^+$TIM-3$^+$, CD62P$^+$TIM-3$^+$ and CXCR5$^+$TIM-3$^+$ cells compared with TD controls. Our results further showed that children with ASD had increased IL-1β$^+$TIM-3$^+$, IFN-γ$^+$TIM-3$^+$, and IL-17$^+$TIM-3$^+$, and decreased Foxp3$^+$TIM-3$^+$ production compared with that in TD controls. Our results indicated that children with ASD significantly induced TIM-3, CD11a,b, CD14, CXCR5, IL-1β and IFN-γ mRNA and protein expression levels compared with TD controls. The results suggested that detection of TIM-3 signaling could contribute to the early diagnoses of ASD.

## 1. Introduction

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders characterized by impairment in expressive communication deficits, reciprocal social interaction, and repetitive restricted behaviors, (American Psychiatric Association, 2015). The etiology of ASD is largely unknown; however, environmental, genetic, neurological, and immunological factors play important roles in the development of ASD (Cohen et al., 2005). Immune abnormalities causing a dysregulation of immune response that have been reported in children with autism, include abnormal cytokine levels, regulation of transcription factors, reduced lymphocyte numbers, imbalance of immunoglobulin levels, and altered T cell mitogen response (Ashwood et al., 2011a; Ahmad et al., 2017a, b; Goines et al., 2011). Recently, we revealed that immune alteration via an imbalance of anti- and pro-inflammatory cytokines, and regulation of transcription factor signaling are linked with the development of ASD (Ahmad et al., 2017a, b). We also showed that JAK/STAT activation and increased chemokine receptors expression have a critical role in immune dysfunction in children with autism (Ahmad et al., 2017c, 2018). However, the exact mechanism remains to be determined.

T cell immunoglobulin and mucin-domain-containing molecule 3 (TIM-3) is a regulatory factor in both innate and adaptive immunity (Yang et al., 2013). TIM-3 regulates the severity of autoimmune disease through macrophage activation (Monney et al., 2002). TIM-3 dysfunction has been associated with the development of several autoimmune

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diseases (Koguchi et al., 2006). A previous study demonstrated that TIM-3 aggravates brain inflammation (Xu et al., 2013), and is highly expressed in CNS tissues (Anderson et al., 2007). Previously, it was proposed that CD11a is associated in the migration of leukocytes into the CNS (Hamann et al., 2008) and that CD11b is highly expressed in microglia (Min et al., 2017). The elevated level of CD11b in microglia is connected with increased cytokine responses in the CNS (Christensen et al., 2014). Thus, TIM-3 and CD11a,b could represent therapeutic targets in several neuroimmune disorders, including ASD.

A previous study noted increased level of IFN-γ in mothers of children later diagnosed with ASD (Goines et al., 2011). In addition, IFN-γ signaling has been linked with immune regulation and social dysfunction (Filiano et al., 2016). Previously, increased levels of the cytokine IL-2 were observed in mid-gestational mothers of children with developmental delay (Jones et al., 2017). IL-17 A has been confirmed as important for the progression of neuroinflammation (Hu et al., 2014). Previous studies have also shown elevated IL-17 A cytokine production in autistic children (Al-Ayadhi and Mostafa, 2012; Akintunde et al., 2015) and confirmed that IL-17 A has a central role in the development of neurological disorders (Bai et al., 2008). Neurodevelopmental disorders are more severe in individuals with reduced Foxp3 expression (Yamano et al., 2005). Fewer $Foxp3^+$ cells were noted in patients with autism (Mostafa et al., 2010). These outcomes support the view that alterations in the immune responses occur in a significant proportion of children with ASD. Alterations in the cytokine network associated with changes in neuroimmune function suggest that it could serve as biomarkers of ASD. In this study, we hypothesized that TIM-3 signaling could be responsible for the neuroimmune dysfunction seen in children with ASD. Therefore, repairing TIM-3 signaling could be used as an approach to address the immune dysregulation in ASD.

## 2. Materials and methods

### 2.1. Participants

This cross-sectional study was conducted on children with classic-onset autism, over a period of 8 months from the beginning of January 2017 to the end of August 2017. The autistic group involved 40 children (29 males and 11 females) enrolled from the Autism Research and Treatment Center, College of Medicine, King Saud University, Riyadh, Saudi Arabia. Subjects achieved the criteria for the diagnosis of autism according to the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (The American Psychiatric Association, 2015). Their ages ranged between 3 and 11 years (mean ± SD = 7.65 ± 2.1 years). Subjects included in this study had no related neurological disorders and were not receiving any medication.

The control group comprised 40 age- and sex-matched healthy children (29 males and 11 females). They were selected from healthy older siblings of healthy infants who attended the Well Baby Clinic, King Khalid University Hospital for routine following up of their growth parameters. The control children were not related to the children with autism, and had no proven clinical findings suggestive of immunological or neuropsychiatric disorders. Their ages ranged from 3 to 11 years (mean ± SD = 7.91 ± 2.22 years). The local Ethical Committee of the King Khalid University Hospital approved this study. In addition, informed written agreement for participation in the study was assigned by the parents or the legal guardians of the studied subjects.

### 2.2. Study measurements

Clinical assessment of autistic subjects was based on their clinical history taken from caregivers, a clinical examination, and a neuropsychiatric assessment. The severity of disease were evaluated by using the Childhood Autism Rating Scale (Schopler et al., 1986) which rates the child on a scale from one to four in each of sixteen areas (emotional response; relating to people; body use; imitation; listening response; object use; verbal communication; fear or nervousness; activity level; non-verbal communication; adaptation to change; level and consistency of intellectual response; visual response; smell and touch response, taste, and general impressions). According to this scale, children whose scores range from 30 to 36 points have mild to moderate autism (n = 25), while those with scores ranging from 37 to 60 points have severe autism (n = 15). The control group was not related to the children with ASD, and showed no clinical findings of neuropsychiatric or immunological disorders

### 2.3. Isolation of PBMCs

PMBCs were isolated by density gradient centrifugation as described previously (Ahmad et al., 2017b). Mononuclear cells were harvested from children with ASD and typically developing (TD) controls and utilized for flow cytometry, RT-PCR, and western blotting analysis.

### 2.4. Flow cytometry analysis

Flow cytometry analysis was performed to assess the TIM-3 production in $CD3^+$, $CD4^+$, $CD8^+$, $CD11a^+$,$b^+$, $CD14^+$, $CD62L^+$, and $CXCR5^+$ cells. We also assessed IL-2, IFN-γ, IL-17 A, and Foxp3 production in TIM-$3^+$ cells. Briefly, PBMCs were stimulated for 4 h with PMA and ionomycin Sigma-Aldrich (St. Louis, USA), in presence of brefeldin BD Biosciences (San Diego, USA), as previously described (Ahmad et al., 2017a, b; Noster et al., 2014). PBMCs were washed and surface stained for TIM-3, CD3, CD4, CD8, CD11a,b, CD14, CD62 P, and CXCR5 BioLegend (San Diego, USA). For cytokines and transcription factor staining, cells were fixed and permeabilized (BioLegend); for staining with anti-IL-1β, anti-IFN-γ, anti-IL-17 A, and anti-Foxp3 antibodies (BioLegend). Flow cytometry data were analyzed using CXP software (Beckman Coulter, USA).

### 2.5. Gene expression analysis

Total RNA was extracted using the Trizol reagent Life Technologies (Paisley, UK) according to **a** previously described method (Ahmad et al., 2017a, b; Noster et al., 2014). Briefly, cDNA was synthesized using a high-capacity cDNA reverse transcription kit Applied Biosystems (Foster City, USA). The following primers were used for RT-PCR analysis. TIM-3 F: 5′-GAATACAGAGCGGAGGTCGG-3′; R: 5′-CATTGCA AAGCGACAACCCA-3′; CD11a F: 5′-ACTGTAAGAGGCCAAAGGGC-3′; R: 5′-CTGGTCACACGTTCGAGACA-3′; CD11b F: 5′-ATATCAGCACATCGG CCTGG-3′; R: 5′-TCACACTGCCACCGAGC-3′; CD14 F: 5′-GAAGGCGAA TCCCGACCTAC-3′; R: 5′-CTCTTGGCCAAATGCGTAGC-3′; IL-β1 F: 5′-CCAAACCTCTTCGAGGCACA-3′; R: 5′- AACACGCAGGACAGGTA CAG-3′; IFN-γ F: 5′-GTCGCCAGCAGCTAAAACAG-3′; R: 5′-CCACAGCT AAGAAGACTCCCC-3′; GAPDH F: 5′-GCATCTTCTTGTGCAGTGCC-3′; GAPDH R: 5′-TACGGCCAAATCCGTTCACA-3′.

### 2.6. Western blotting analysis

Total protein was extracted from PBMCs as previously described (Chen et al., 2007). Briefly, samples containing 30–40 μg of protein were separated on 7.5% or 10% SDS-PAGE followed by transfer to a nitrocellulose membrane Bio-Rad Laboratories (Hercules, USA). Incubation with primary antibodies (1:500 dilution) against TIM-3, CXCR5, IL-β1, and IFN-γ was followed by incubation for 2 h with peroxidase-COnjugated secondary antibodies (1:5000 dilution) Santa Cruz biotech (Dallas, USA). Immunoreactive proteins were detected using the Luminata Forte Western HRP substrate (Millipore, Billerica, USA) and quantified relative to β-actin. Images were taken on a C-Digit chemiluminescent Western blot scanner (LI-COR, Lincoln).

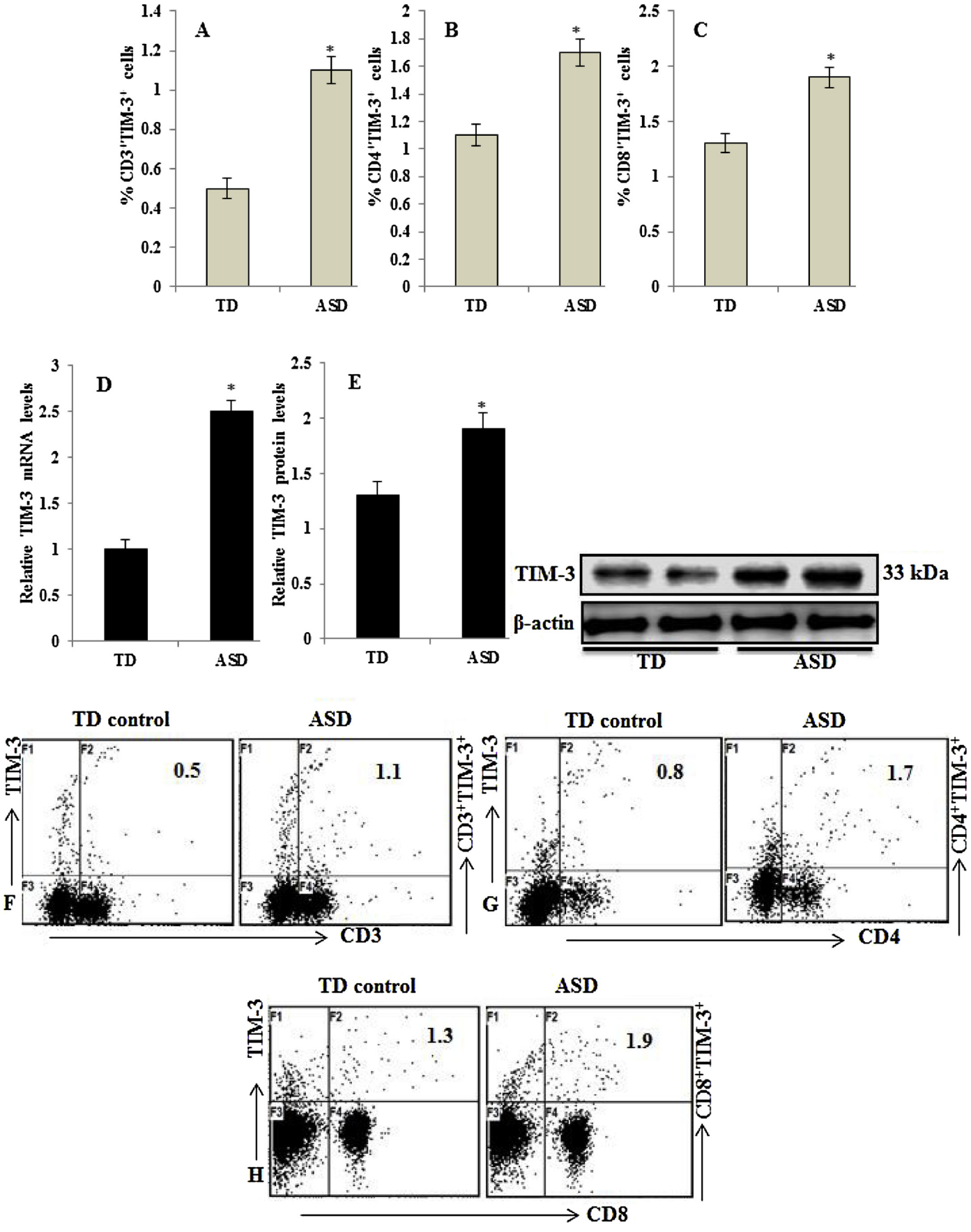


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**Fig. 1. Analysis of TIM-3 production in CD3$^+$, CD4$^+$ and CD8$^+$ cells. A, B and C.** Flow cytometry analysis of TIM-3 production in CD3$^+$, CD4$^+$ and CD8$^+$ cells in children with autism spectrum disorder (ASD) and typically developing (TD) controls in PBMCs. **(D)** RT-PCR analysis of *TIM3* mRNA expression in PBMCs. **(E)** Western blotting analysis of TIM-3 protein expression in PBMCs. **(F)** Data shown in the FACS dot plots are representative from PBMCs taken from each ASD and TD control. The level of significance was set at *p < 0.05 compared with the TD control.

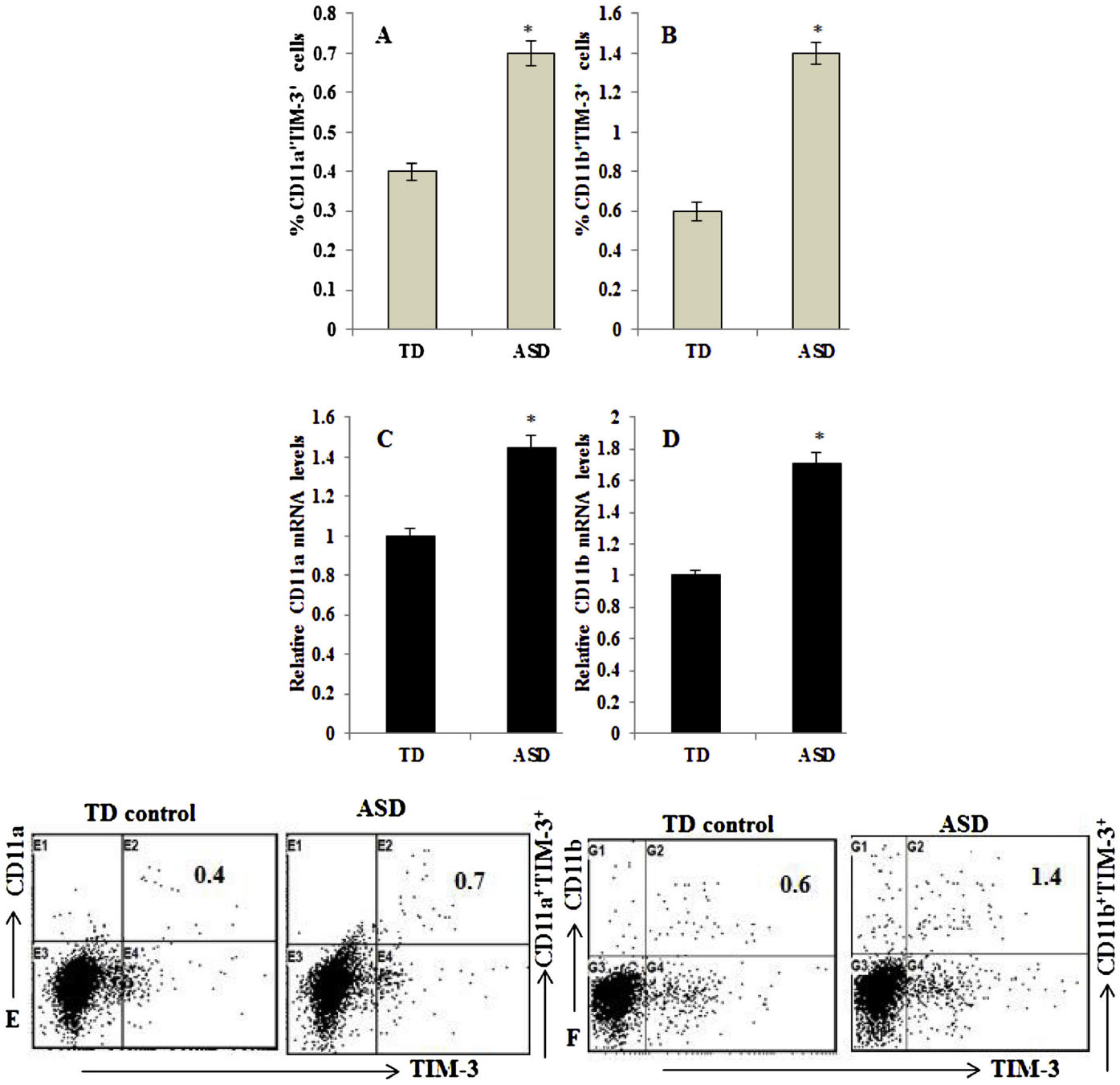


**Fig. 2. Analysis of TIM-3 production in CD11a$^+$ and CD11b$^+$ cells. A and B.** Flow cytometry analysis of TIM-3 production in CD11a$^+$ and CD11b$^+$ cells in children with autism spectrum disorder (ASD) and typically developing (TD) controls in PBMCs. **(C and D)** RT-PCR analysis of CD11a,b mRNA expression levels in PBMCs. **(E)** Data shown in the FACS dot plots are representative from PBMCs taken from each ASD and TD control. The level of significance was set at *p < 0.05 compared with the TD control.

### 2.7. Statistics

Student's *t*-test was used for statistical comparison between two groups. All statistical tests have been indicated in the figure legends. P values of 0.05 or less were considered significant. All statistical analyses were performed using the Graph Pad Prism statistical package. The data were expressed as mean ± SD.

## 3. Results

### 3.1. Altered cell surface receptors response in children with autism

Flow cytometry analysis was performed to measure the TIM-3 production in CD3$^+$ T cells surface receptor cells. The number of CD3$^+$TIM-3$^+$ cells among PBMCs was substantially higher in the ASD

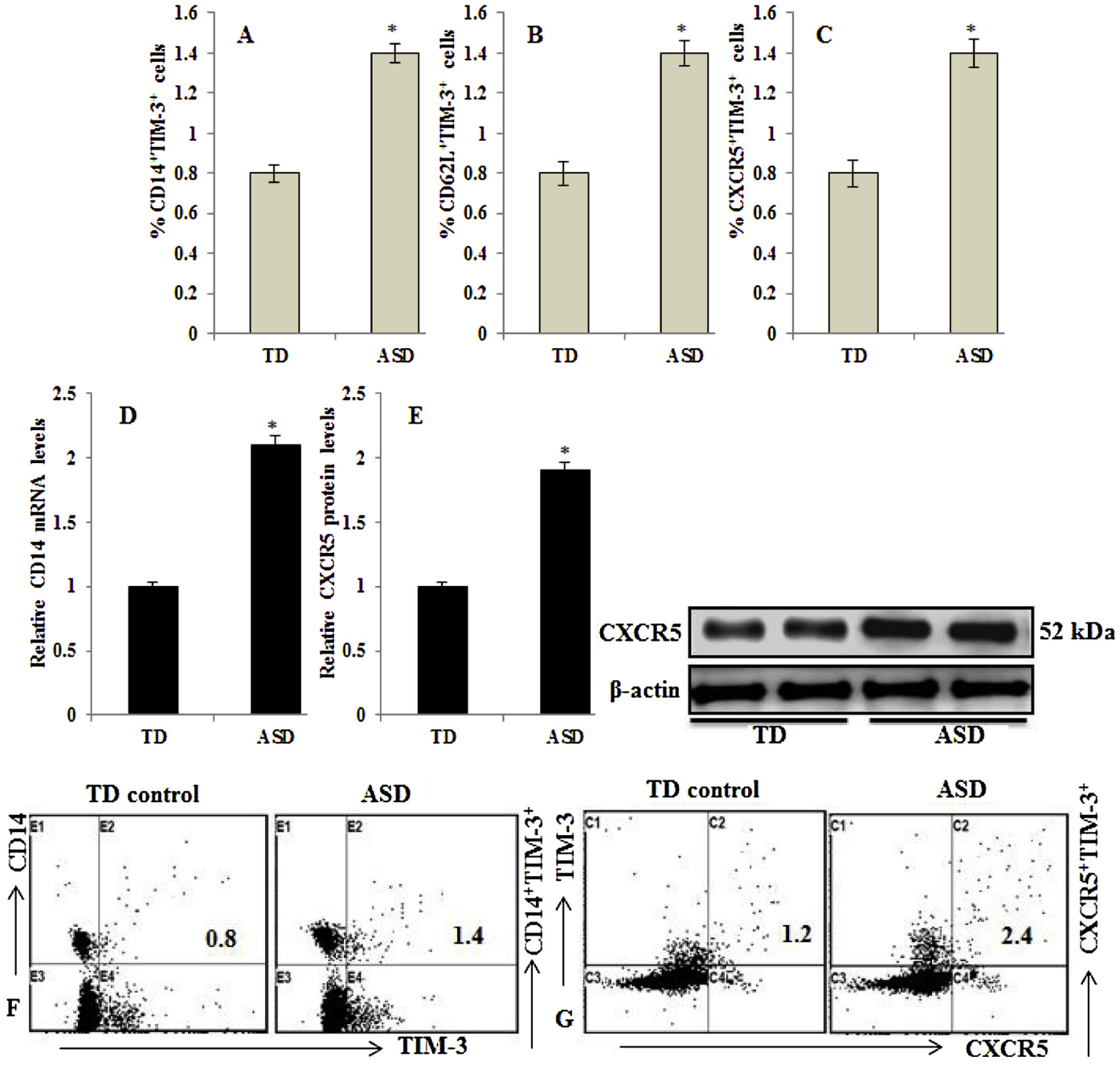


**Fig. 3. Analysis of TIM-3 production in CD14$^+$, CD62P$^+$, and CXCR5$^+$ cells. A, B and C.** Flow cytometry analysis of TIM-3 production in CD14$^+$, CD62P$^+$, and CXCR5$^+$ cells in children with autism spectrum disorder (ASD) and typically developing (TD) controls in PBMCs. **(D)** RT-PCR analysis of *CD14* mRNA expression levels in PBMCs. **(E)** Western blotting analysis of *CXCR5* protein expression in PBMCs. **(F)** Data shown in the FACS dot plots are representative from PBMCs taken from each ASD and TD control. The level of significance was set at *p $<$ 0.05 compared with the TD control.

group compared with that in the TD controls (Fig. 1A). Children with ASD had a significantly increased number of CD4$^+$TIM-3$^+$ PBMCs compared with that in the TD controls (Fig. 1B). Children with ASD also showed a significant increase in CD8$^+$TIM-3$^+$ cells compared with that in the TD controls (Fig. 1C). *TIM3* mRNA expression was significantly increased in PBMCs from children with ASD as compared with that in the TD controls (Fig. 1D). Protein expression of TIM-3 was observed in PBMCs from children with ASD and TD controls. Children with ASD showed a significant increase in the TIM-3 level compared with that in TD controls (Fig. 1E). These results suggested that TIM-3$^+$ cells could be responsible for the altered immune dysfunction in children with ASD.

Children with ASD had increased numbers of CD11a$^+$TIM-3$^+$ PBMCs compared with those in the TD controls (Fig. 2A). Fig. 2B shows that the children with ASD had significantly increased numbers of CD11b$^+$TIM-3$^+$ PBMCs compared with those in the TD controls (Fig. 2C). The expression of the mRNAs encoding *CD11a* and *CD11b* were significantly higher in PBMCs from children with ASD compared with that in the TD controls (Fig. 2C,D), confirming the strong induction of CD11a,b in children with ASD. These results suggested that CD11a,b could play an important role in the development of ASD.

We further found that children with ASD had significantly increased numbers of CD14$^+$TIM-3$^+$, CD62L$^+$TIM-3$^+$, and CXCR5$^+$TIM-3$^+$ PBMCs compared with those in the TD controls (Fig. 3A-C). We further used RT-PCR to determine the *CD14* mRNA expression level in PBMCs. The children with ASD showed significantly higher *CD14* mRNA

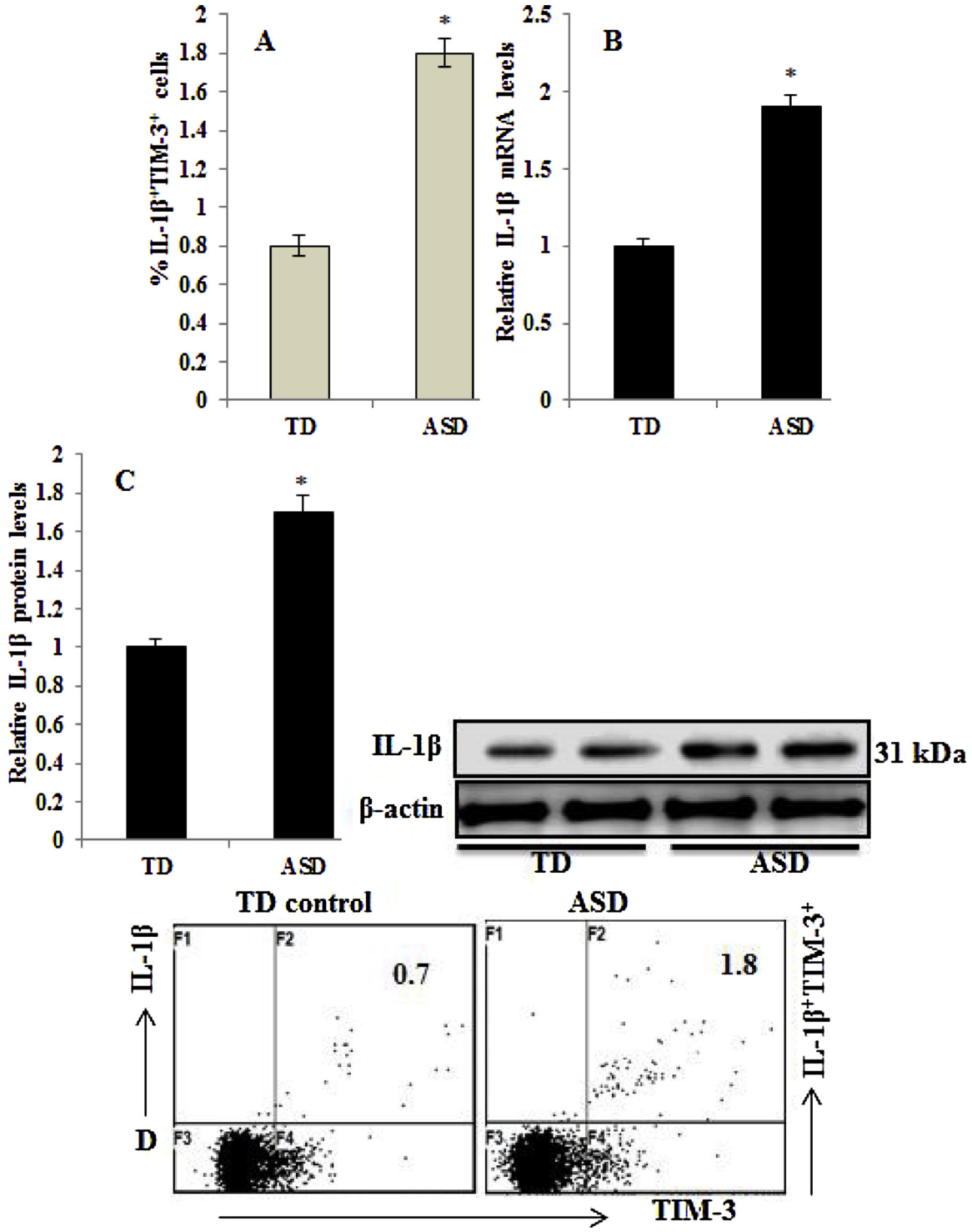


**Fig. 4. Analysis of IL-1β production in TIM-3$^+$ cells. A.** Flow cytometry analysis of IL-1β production in TIM-3$^+$ cells in children with autism spectrum disorder (ASD) and typically developing (TD) controls in PBMCs. **(B)** RT-PCR analysis of *IL1B* (IL-1β) mRNA expression levels in PBMCs. **(C)** Western blotting analysis of IL-1β protein expression in PBMCs. **(D)** Data shown in the FACS dot plots are representative from PBMCs taken from each ASD and TD control. The level of significance was set at *$p < 0.05$ compared with the TD control.

expression compared with that in the TD controls (Fig. 3D). The protein level of CXCR5 in children with ASD was significantly increased compared with that in the TD control (Fig. 3E). Our results indicated that TIM-3 is expressed in several cell types that could be critically involved in the effect of neuroimmune dysfunction in children with ASD.

### 3.2. Cytokines and transcription factors regulated in children with autism

Flow cytometry analysis was further performed to determine the IL-1β production in TIM-3$^+$ cells. We showed that the number of IL-1β$^+$TIM-3$^+$ PBMCs in children with ASD was significantly increased compared with that in the TD controls (Fig. 4A). Moreover, *IL-1β* mRNA expression was also significantly higher in PBMCs from children with

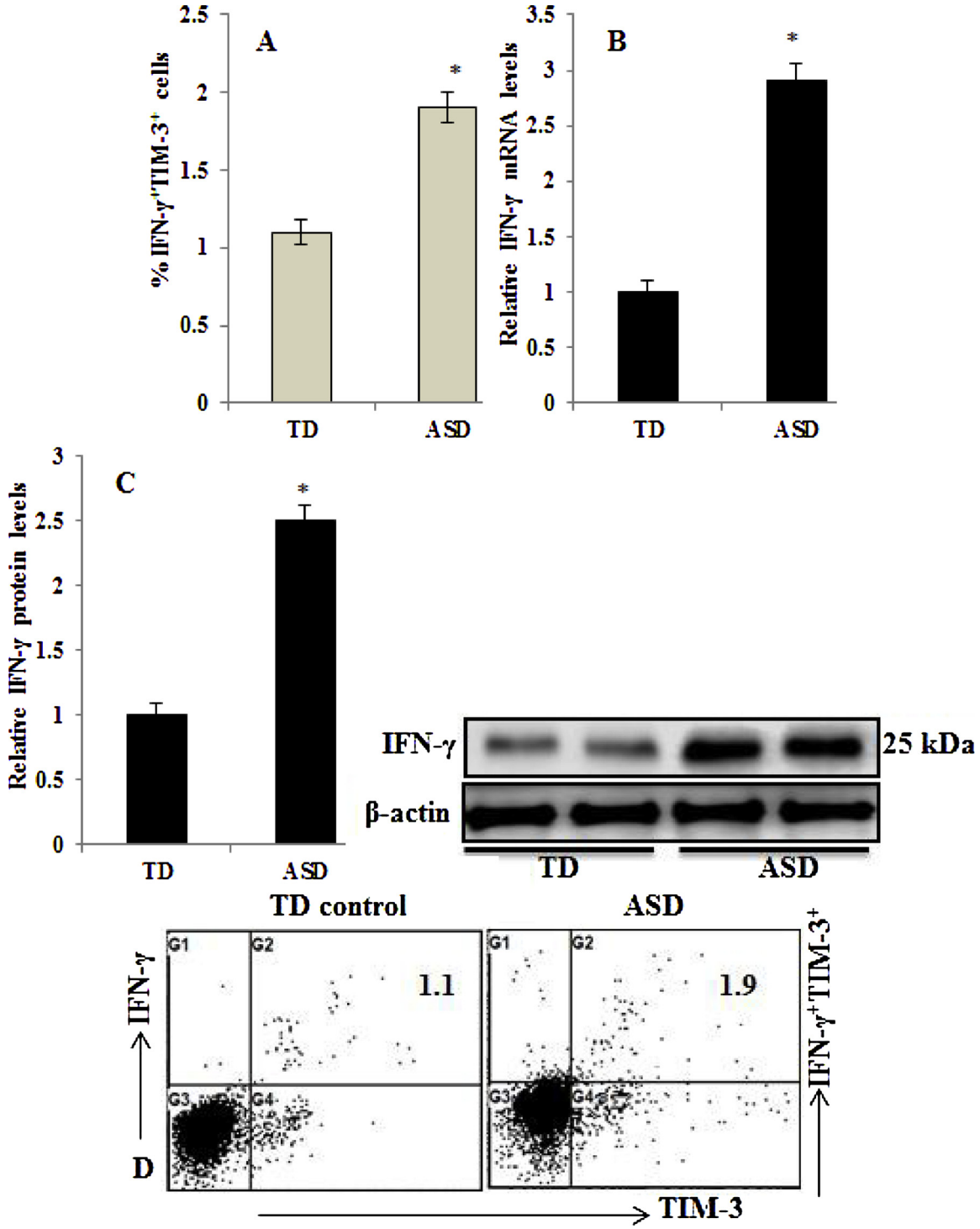


**Fig. 5. Analysis of IFN-γ production in TIM-3$^+$ cells. A.** Flow cytometry analysis of IFN-γ production in TIM-3$^+$ cells in children with autism spectrum disorder (ASD) and typically developing (TD) controls in PBMCs. **(B)** RT-PCR analysis of *IFNG* (IFN-γ) mRNA expression levels in PBMCs. **(C)** Western blot analysis of IFN-γ protein expression in PBMCs. **(E)** Data shown in the FACS dot plots are representative from PBMCs taken from each ASD and TD control. The level of significance was set at *p $<$ 0.05 compared with the TD control.

ASD compared with that in the TD controls (Fig. 4B). We further showed that the protein level of IL-1β in PBMCs from children with ASD was significantly increased compared with that in the TD controls (Fig. 4C). Our findings demonstrated, for the first time, that IL-1β production in TIM-3$^+$ PBMCs is greatly increased in children with ASD. Thus, cytokine IL-1β might also be involved to neuroimmune progression of ASD.

We next investigated IFN-γ proinflammatory cytokine signaling in children with ASD. The number of IFN-γ producing TIM-3$^+$ PBMCs was significantly increased in children with ASD compared with that in with TD controls (Fig. 5A). We further revealed that higher *IFN*-γ mRNA expression was observed in PBMCs from children with ASD compared with that in the TD controls (Fig. 5B). Similarly, the IFN-γ protein level was significantly higher in PBMCs from children with ASD compared with that in the TD controls (Fig. 5C). These results showed that IFN-γ could be associated with upregulation of the neuroimmune response in

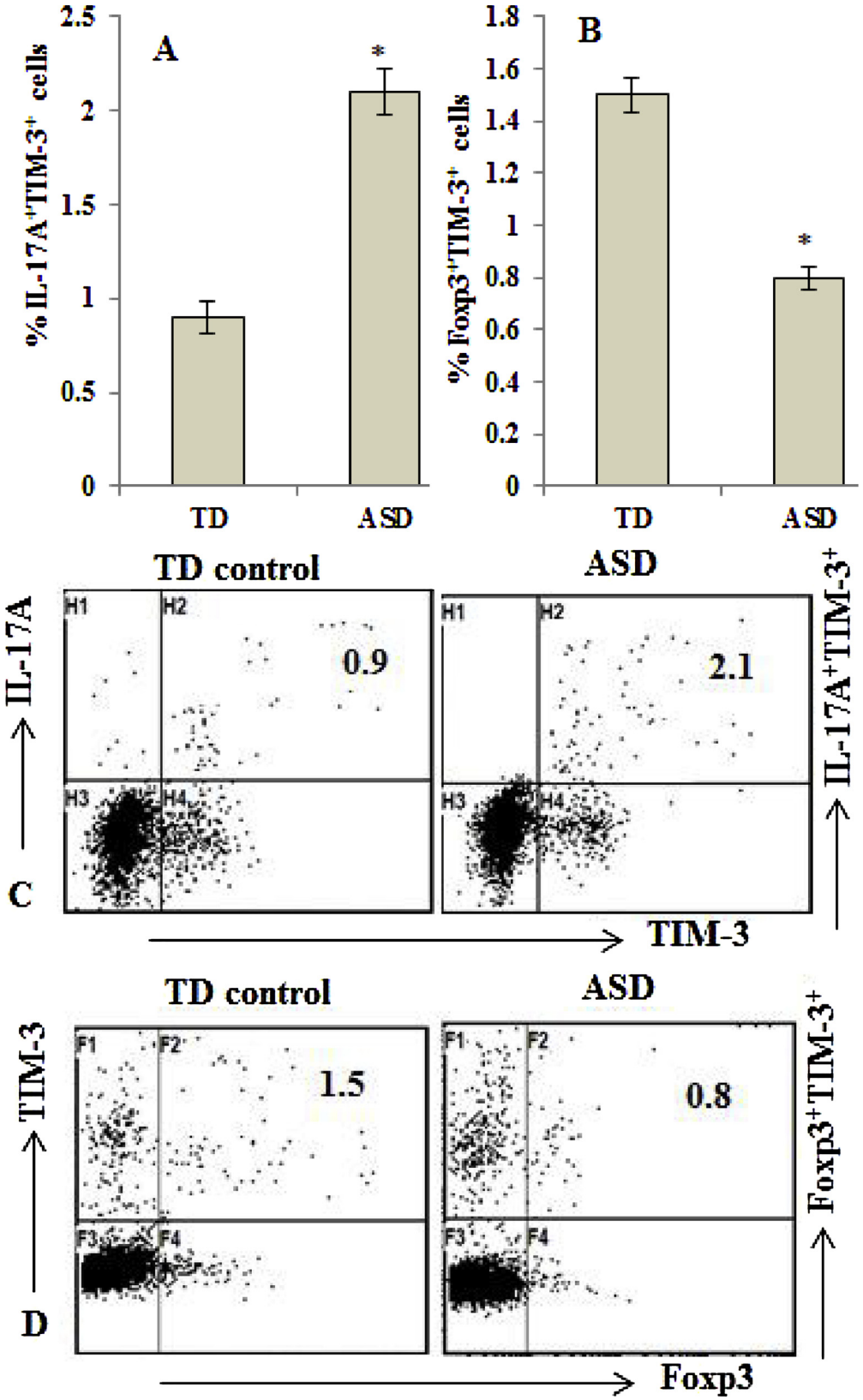


**Fig. 6. Analysis of IL-17 A and Foxp3 production in TIM-3$^+$ cells. A and B.** Flow cytometry analysis of IL-17 A and Foxp3 production in TIM-3$^+$ cells in children with autism spectrum disorder (ASD) and typically developing (TD) controls in PBMCs. **(C)** Data shown in the FACS dot plots are representative from PBMCs taken from each ASD and TD control. The level of significance was set at *$p < 0.05$ compared with the TD control.

ASD.

It has been demonstrated that IL-17 A has a significant role in several neuroinflammatory disorders, including ASD in children. In the present study, we showed, for the first time, that IL- 17 A is produced by TIM-3$^+$ PBMCs. The number of IL-17 A cytokine producing TIM-3$^+$ PBMCs was significantly higher in children with ASD compared with that in the TD controls (Fig. 6A). Furthermore, Foxp3 production in TIM-3$^+$ PBMCs from children with ASD was significantly decrease compared with that in the TD controls (Fig. 6B). Our results suggested that TIM-3 cells express several inflammatory cytokines and transcription factors that might have critical roles in neuroimmune dysfunction in ASD.

## 4. Discussion

Previous studies indicated that neuroinflammatory responses involve increased pro-inflammatory cytokine expression and excess microglial activation (Kirkley et al., 2017; Magnus et al., 2002). Immune abnormalities have been demonstrated in children with ASD (Ashwood et al., 2006). Previous studies have shown that inflammatory cytokines are significantly higher in the peripheral blood of patients with ASD (Molloy et al., 2006; Zimmerman et al., 2005). We previously revealed an imbalance in cytokine production and altered transcription factor pathways in children with ASD (Ahmad et al., 2017a, b). Children with ASD also show higher pro inflammatory cytokine levels (Jyonouchi et al., 2002), which might be one explanation for their immune dysfunction.

TIM-3 is involved in modulating immune responses in several autoimmune diseases (Joller et al., 2012). TIM-3 expression has been revealed in astrocytes, hypoxic brain regions, and brain resident immune cells (Koh et al., 2015). Increased TIM-3 expression was demonstrated in brain tissue (Zhao et al., 2011). Increased TIM-3 in CD11b$^+$cells and microglia also plays an important role in brain inflammation (Zhao et al., 2011). Previous studies have demonstrated that chemokines play an important role in CNS trafficking (Biber et al., 2008; Rebenko-Moll et al., 2006). Increased levels of chemokines were linked with deteriorating cognitive and adaptive ability, and behavioral symptoms (Ashwood et al., 2011a) In the present study, we observed that children with ASD exhibited significant increases in the numbers of TIM-3$^+$ cells expressing different cells, such as CD3, CD4, CD8, CD11a,b, CD14, CD62 P, and CXCR5. Our data further showed that increased TIM-3 expression in children with ASD could be associated with development and neuroimmune dysfunction could result in deteriorating behavior in neurodevelopmental disorders. Thus, TIM-3 signaling could represent a therapeutic target in ASD. Our results might have significant implications in the development and monitoring treatments targeted at children with ASD.

Previous studies have reported relations between cytokines and aberrant behavior (Han et al., 2017; Ashwood et al., 2011b). Several lines of evidence support the hypothesis that increased cytokine expression during early brain development is associated with psychiatric disorders (Kim et al., 2016). IL-1β is involved in aberrant behavior associated with inflammation, such loss of appetite, depression, anxiety, impaired ability, and social withdrawal (Harden et al., 2008). Our study showed that IL-1β production in TIM-3$^+$ PBMCs from children with ASD was significantly induced. This significant increase in IL-1β expression could be related with the core features or onset of ASD. Thus, alterations in IL-1β levels could suggest a critical association between neuronal dysregulation and ASD in children.

A previous study highlighted the relation between increased INF-γ levels and autism-related behavior (Jyonouchi et al., 2001), and ASD patients have significantly increased IFN-γ levels (Li et al., 2009). In addition, the higher levels of IFN-γ observed in mid-gestation are associated with increased risk of autistic symptoms in the resulting children (Goines et al., 2011). Earlier studies showed that an elevated IL-17 A level was connected with the severity of ASD (Al-Ayadhi and Mostafa, 2012; Suzuki et al., 2011). The increased level of IL-17 A was also linked with the severity of behavioral symptoms (Akintunde et al., 2015). Our results revealed that children with ASD had significantly increased IFN-γ$^+$TIM-3$^+$ and IL-17 A$^+$TIM-3$^+$ cell populations, which could be related with increased autism-related behaviors. These results suggested that IFN-γ and IL-17 A in TIM-3$^+$ cells could contribute to abnormal behavior and immune dysfunction alterations in ASD. Thus, TIM-3 might play a role in neuroimmune dysfunction disorders, such as ASD and other related behavioral impairments. Further investigation of the role of TIM-3$^+$ cells in neurodevelopment and immune responses in ASD is warranted.

The transcription factor Foxp3 has an important role in self-reactivity and immune activation, and its deficiency contributes to the association between autoimmune disease and ASD (Hsiao et al., 2012). It has been suggested that individuals with reduced Foxp3 in their cells are susceptible to autism (Safari et al., 2017). In our previous study, we showed that children with autism have lower levels of Foxp3, which is associated with higher levels of autism related behaviors (Ahmad et al., 2017b). In the present study, we demonstrate that Foxp3 production in TIM-3$^+$ PBMCs from children with ASD was significantly decreased. The reduction of Foxp3 in TIM-3$^+$ cells could lead to increased susceptibility to ASD. We hypothesized that Foxp3$^+$TIM-3$^+$ cells could contribute to the neurologic symptoms functions in children with ASD. More studies are necessary to determine the role of TIM-3′s association with ASD and the severity of behavioral symptoms.

Our results showed that TIM-3$^+$ cells expressed high levels of several immune factors and this cell population is significantly increased in the children with ASD. In addition, we demonstrated that cytokine and transcription factor production in TIM-3$^+$ cells from children with ASD were significantly increased. Our results suggested that TIM-3 signaling has an important role in the development of ASD and is associated with ASD-related immune dysfunction. These results warrant for further studies, not only in children with ASD, but also in other neurodevelopmental disorders whose initiation and progression remain unknown.

## Conflict of interest

The authors declare no conflict of interest.

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