Immune function changes of the IDPN-induced Tourette syndrome rat model

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Abstract
There may be immunologic alternations during Tourette syndrome (TS) development. This study aimed to determine the immune function changes in different aspects (spleen or thymus index, plasma cytokines, and T cell) in an 3,3′-iminodipropionitrile (IDPN)-induced rat model of TS. Male Sprague-Dawley rats were assigned to control and TS groups. The control group received intraperitoneal injections of normal saline (5 ml kg−1 day−1), and the TS rats were injected with IDPN (150 mg kg−1 day−1). The spleen and thymus indices were calculated. The expression of anti-inflammatory cytokines and inflammatory cytokines TNF-α, in peripheral blood were measured by ELISA and Western blotting. The proportion of CD3+, CD4+, CD8+, Treg, Th1, and Th2 cells were determined by fluorescence-activated cell sorting analysis. After 1 week of IDPN treatment, TS rats had decreased spleen and thymus weights versus control. The plasma levels of IL-4, IL-10, IL-12, IFN-γ, and TNF-α were significantly increased, while no significant difference in TGF-β was found. Flow cytometry analysis demonstrated that TS rats had significantly reduced CD3+ and CD4+ cells in spleen, without any change in the proportion of CD8+ cells. Furthermore, the ratio of Treg cells (CD4+/CD25+/FoxP3+) was decreased in TS rats; simultaneously, Th1 cells (CD4+/IFN-γ+) and Th2 cells (CD4+/IL4+) were dramatically increased. Together, IDPN can trigger immune dysfunction through impairment of matured Th cells, in particular for the Treg subset.

Keywords
IDPN, immune function, Th cell, Tourette syndrome, Treg

INTRODUCTION

Tourette syndrome (TS) is a neurodevelopmental movement disorder characterized by multiple motor and vocal tics (Brander et al., 2019). Its incidence is increasing every year. Tics usually start at childhood with a peak age between 7 to 15 years and persist to late adolescence or even early adulthood (Zhang & Li, 2015). TS is associated with attention deficit hyperactivity disorder, obsessive-compulsive disorder, and emotional disorder, which may severely influence children's social adaptation ability (Deeb et al., 2019; Ekinci & Erkan, 2020; Essoe et al., 2019; Kuhn & Huys, 2019). So
far, the pathophysiology of TS is still not fully clear, and there are very few safe and effective drugs for treating TS. Different upstream occasions, especially exogenous toxins (e.g., heavy metal) may cause Tourette's disorder (Hsieh et al., 2010). Therefore, theoretically, there may be immune system disorders caused by exogenous toxins at the same time. Actually, there have been reports about immunologic alternations from the serum investigation. For example, TS patients may have reverse CD4/CD8, higher percentages of memory T cells, diminished lymphocyte activation CD69 marker, and impaired NK cytotoxicity (Hsieh et al., 2010). However, there is a lack of direct evidence in following issues. In TS animal models, the immune function changes, especially for T cell changes, are not clear. Also, the expression changes of different immunological cytokines (anti-inflammatory and inflammatory cytokines in peripheral blood) in TS-model animals are still to be revealed. Furthermore, very few studies have directly observed the influence of TS on thymus index and spleen index. Among well-established approaches, 3,3′-iminodipropionitrile (IDPN), a synthetic nitrile, is one of the first-choice manipulations used to develop TS animal models, which quickly induces marked alterations in central nervous system (Xie et al., 2016; Zhang & Li, 2015). After IDPN treatment, the dyskinesia and persistent behavioral syndromes are characterized by repetitive head movements, circling, hyperactivity, and retropulsion. This study applied a 7-day IDPN treatment protocol to establish the TS model and explored the immune function changes in different aspects.

2 | MATERIALS AND METHODS

2.1 | TS modeling by IDPN treatment

A total of 20 male Sprague-Dawley rats (180 g) were randomly assigned to control and TS groups (n = 10 in each group). Rats in the control group received intraperitoneal injections of normal saline (5 ml kg⁻¹ day⁻¹), and the TS rats were injected with IDPN (Sigma Chemical Co., St. Louis, MO, USA, 150 mg kg⁻¹ day⁻¹). After 7 days of treatment, the modeling was validated through the stereotyped behavior assessment. When the rat in the TS group behaved significant head twitching, biting, putting forepaws around mouth, lighting, shaking claws, body raising, or episodic utterance (the total time of stereotypic behaviors in 30 min was longer than 15 min). All TS rats were validated in this batch, as well as in our pilot observation using the identical protocol.

2.2 | Spleen index and thymus index calculation

Each animal was sacrificed after the peripheral blood collection, the body weight was recorded; spleen and thymus were resected and freshly weighed. The index, namely weight percentage (divided by the body weight), was calculated for each rat.

2.3 | Plasma cytokine determination

The expression of anti-inflammatory cytokines interleukin-4 (IL-4), interleukin-10 (IL-10), transforming growth factor-β (TGF-β) and inflammatory cytokines TNF-α, interferon-gamma (IFN-γ), interleukin-12 (IL-12) in peripheral blood were measured by ELISA, according to manufacturer’s instructions. In brief, 50 μL of plasma samples were added to each well after 1:2 dilution. The concentration was calculated by the standard curve. Simultaneously, several blood serum samples were conducted Western Blotting to further support the variation trend.

2.4 | Western Blotting

The serum samples (four random samples per group) were balanced of the protein amount after appropriate dilution in phosphate-buffered saline (PBS), and about 20 μg protein samples were separated using 10% SDS–PAGE gel, followed by being transferred onto polyvinylidene fluoride membranes. The blots were first blocked in TBST-milk and then incubated with primary antibodies (rabbit anti rat IL-4, IL-10, TGF-β, TNF-α, IFN-γ, and IL-12) overnight at 4°C. Next, the goat anti-rabbit IgG secondary antibodies conjugated with horseradish peroxidase (1:1,000) were used for 2 hr of incubation. GAPDH was used as an endogenous reference. The enhanced chemiluminescence reagent Kit (Pierce) was used for chemiluminescence in the Bio-rad imaging system.

2.5 | Flow cytometry analysis

First, the mononuclear cells were isolated from whole spleens with a complete growth medium (RPMI—1,640, 10% fetal bovine serum). A homogenous suspension was obtained, and cell pellets were resuspended and gently layered on 2.5 ml of Histopaque-1077 (Sigma Aldrich, USA). After centrifugation for 30 min (400 g) at room temperature), the upper layer of the opaque interface containing mononuclear cells was carefully aspirated. Mononuclear cells were washed twice and resuspended in 1 ml of PBS. Next, 200,000 spleen mononuclear cells were incubated with different primary antibodies for fluorescence-activated cell sorting analysis in darkness for 30 min. Next, the cells were rinsed in PBS twice and centrifuged at 400 g for 10 min. Finally, pellets were suspended in 400 μl of PBS and analyzed with the flow cytometer system (BD Biosciences). Specifically, cell surface markers were stained, Treg, Th1, and Th2 cells were
analyzed. The obtained cells were first stained following markers (with corresponding dye): CD3-PE, CD4-FITC, CD8-APC, and 7aad. Next, the obtained cells were stained toward CD4-FITC, CD25-PE, and 7aad; afterwards, the cells were permeabilized and stained for Foxp3. Finally, cells were added to a 96-well U bottom plate and incubated with activation cocktail (2ul reagent per ml medium, at 37°C, 5% CO₂ incubator) for 6 hr, which was performed to block the release of intracellular factors and facilitate the subsequent staining of intracellular factors (IFN-γ-PE, IL-4-PE, IL-17A-PECY7). The dilution of all antibodies were as follow: CD3-PE (1:200), CD4-FITC (1:100), CD8-APC (1:200), 7aad (1:50), CD25-PE(1:200), IFN-γ-PE (1:100), IL-17A-PECY7 (1:100), and IL4-PE(1:100).

2.6 | Statistical analysis

Results were expressed as means ± standard error, and comparison between groups was performed using t test. A P value < .05 was considered statistically significant.

3 | RESULTS

3.1 | TS rats have decreased spleen and thymus weights

In this study, all the IDPN treated rats were validated as successful TS model rats. First, the spleen and thymus weights were compared between groups. After 1 week of IDPN treatment, TS rats had highly significantly decreased spleen and thymus weights versus controls (p < .01, Figure 1a and b).

3.2 | Immune cytokine changes in TS rats

Next, the immune cytokines in peripheral blood were determined by Western blotting and ELISA. As Western blotting shown, the expression of IL-4, IL-10, IL-12, IFN-γ, and TNF-α significantly increased, and TGF-β just showed an elevation trend (Figure 1c). Consistently, the plasma concentrations of above cytokines were measured, the IL-4, IL-10, IL-12, IFN-γ, and TNF-α levels were increased in the TS model rats (Figure 1d-H), while no difference in TGF-β level was found (Figure 1i).

3.3 | T cell changes in TS rats

Flow cytometry analysis demonstrated that TS rats had highly significantly reduced CD3+ and CD4+ cells in spleen, without any change in the proportion of CD8+ cells (p < .01) (Figure 2a-c). This finding suggests that IDPN may interfere with the maturation of lymphocytes and impair immune function, in particular, Th cells were the major victims under IDPN-induced TS stress. Furthermore, we observed the detailed changes in different Th cell subsets. As Figure 3 shown, the ratio of Treg cells (CD4+/CD25+/FoxP3+) was decreased (Figure 3a); simultaneously, Th1 cells (CD4+/IFN-γ+) and Th2 cells (CD4+/IL4+) were dramatically increased. Together, in our TS model, IDPN can trigger immune dysfunction through impairment of matured Th cells, in particular for the Treg subset.

4 | DISCUSSION

The major findings of this study include: (1) decreased spleen and thymus weights in TS rats, (2) elevated plasma IL-4, IL-10, IL-12, IFN-γ, and TNF-α levels in TS rats, and (3) decreased ratio of Treg cells and increased Th1 and Th2 cells in TS rats. Collectively, IDPN-induced TS is accompanied by immune dysfunction especially impairment in the Treg Cells.

The variation of immune function (in particular T lymphocytes) in TS were mentioned in some previous studies. In TS cases, there are overactive innate and adaptive immune response, abnormal immune response to streptococci and other pathogen, and microglial activation in the central nervous system. Additionally, immune-related and neurotransmitter signaling genes are differently expressed in lymphocytes of TS patients (Martino et al., 2015). In lymphocyte subpopulation analysis, there may be a systemic activation of several T- and B-cell subtypes, especially decreased numbers of T regulatory lymphocytes might predispose to autoimmunity (Elamin et al., 2013). Other relative evidence also supports the influence on T lymphocytes in TS individuals. D8/17 B cell overexpression was regarded as a peripheral blood marker in TS patients (Hoekstra et al., 2001; Murphy et al., 2001). However, there is no report pointing out the significant decrease of spleen and thymus weights in TS individuals. Also, this is the first time when TS onset is found associated with decreased Treg but increased Th1 and Th 2 cells.

Similar evidence and potential mechanisms are as follow. First, TS can be triggered by pathogenic microorganism infection (e.g., streptococcal infection), and this can be an important reason of immune changes (Eftimiadi et al., 2016; Hornig & Lipkin, 2013). However, in our animal study, the TS model was established by IDPN injection, which excludes the direct reason of microorganisms. Second, T cell involved immune dysfunction can induce autoimmunity and TS development through different mechanisms (Elamin et al., 2013). Clinically, Turkish scholars enrolled the chronic tic disorder (CTD) and TS children and found Tregs may have a role in the pathogenesis of TS/CTD (Yildirim et al., 2020). However, they noticed that Th number and percentage were decreased but activated Treg percentage...
was elevated, which was opposite to our results. Their clinical samples were peripheral blood, and this may be the main reason of our difference. In line with our animal results, an article published in 2007 showed that some TS patients may have an impaired capacity to inhibit autoreactive lymphocytes through a deficit in T reg cells (Kawikova et al., 2007). Taken together, it is reasonable to believe that suppressed Treg cells/function can be an important feature of TS.
The correlation among Th1/Th2/Treg cells and diseases associated with their imbalance have been studied by different teams. Treg is a group of T cells with immunosuppressive effects. They are CD4 and CD25 positive with a high expression of Foxp3. This type of an immunoregulatory T cell is different from Th1 and Th2 cells. It plays an important role in maintaining the own tolerance and suppressing autoimmune responses. In contrast, Th1 cells directly produce cytokines like IFN-γ, IL-2 and TNF. Infection of bacteria, fungi, and viruses in can induce the differentiation of initial T into Th1. Generally, IL-12, IL-18, IFN-γ, and type-1 interferon promote Th1 differentiation, while IL-4, IL-10, and TGF-β inhibit Th1 differentiation. Th2 cells produce IL-4, IL-5, IL-9, IL-10, and IL-13. The Th2 differentiation largely depends on the presence of exogenous IL-4 and the absence of IFN-γ during T cell activation. A disorder of the balance in Th1/Th2/Treg cells and relative cytokines is associated with different autoimmune diseases, such as Guillain-Barre syndrome, allergic asthma, erythematous, and arthritis (Peter et al., 2020; Shi et al., 2011; Talaat et al., 2015; Zhang et al., 2013; Zou et al., 2018). Also, imbalance of Th1/Th2 and Th17/Treg cells were observed in carcinogenesis, asthma, colitis, and encephalomyelitis (Guan et al., 2011; Liang et al., 2017; Lin et al., 2020; Yang et al., 2017). Although Th1 and Th2 differentiation may have a mutual inhibition, they are both important in immunity activation. In contrast, Treg mainly play an immunosuppressive role. Therefore, decreased Treg cells implied excessive immune responses, which are in consistency with the known fact that overactive adaptive immune responses can be observed in TS cases. To date, our finding
is very rare that in the IDPN-induced TS model, Treg and Th1 or 2 have a negative correlation. However, for the limited sample size, the direct negative correlation needs for evidence to support, and the mutual causation of Treg and Th1/2 is still largely unclear in this model.

Disorders in immune cytokines suggest abnormalities in T lymphocytes as well. TNF-α, IFN-γ, and IL-12 are important pro-inflammatory factors. As mentioned above, TS is associated with overactive immune responses in this study, and this result is as expected. However, very few similar reports exist. A study in 2009 suggested that IL-12 and IL-2 may play a role in TS (Gabbay et al., 2009), and this was further confirmed in a pediatric study in 2017 (Yeon et al., 2017). Besides, a traditional Chinese medicine granule can modulate abnormal serum levels of IL-12 and TNF-α in TS children, which might be one of its pharmacodynamic mechanisms for treating TS (Tang et al., 2014). Our study, for the first time supports this trend in an IDPN-induced TS rat model, and jointly highlights a potential pathogenic role of these pro-inflammatory factors. At the same time, we discovered that the levels of anti-inflammatory factors IL-4 and IL-10 were also increased. This may be due to a homeostasis effect under toxic stress. In a balanced system, increased IL-4 may help fight the physical and psychological disorders (Parker-Athill et al., 2015; Pranzatelli et al., 2017). Intuitively, acute IDPN treatment
may cause impairment in the Treg cells and hence immune dysfunction. The pro-inflammatory cytokines were over-expressed, and consequently the anti-inflammatory cytokines were secreted in a homeostasis response. However, the deep mechanisms are still to be investigated.

The major limitation of this study is as follow. IDPN is a synthetic organic nitrile. It is effective in establishing the TS model (Zhang & Li, 2015). However, the toxicity of IDPN can act on systemic organs and tissues (not only neural system) (Boadas-Vaello et al., 2017; Takahashi et al., 2012, 2014). Therefore, the necrotic tissues/cells may activate the immune response and induce the expression of inflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α) as well (Alwelaie et al., 2019). Therefore, we cannot strongly confirm that the immune function changes are derived from TS but not from the toxicity of IDPN. In the further study, more consistent evidence from clinical observation is urgently needed.

5 CONCLUSION

Immune dysfunction, especially impairment in the Treg cells, is associated with development of TS in the IDPN induced rat model.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL

All animal experiments have been approved by the ethics committee of Fujian Provincial Maternity and Children's Hospital.

AUTHOR CONTRIBUTIONS

Xiumei Liu conceived and designed the study. Xiumei Liu and Xueming Wan performed the experiments, analyzed the data, and wrote the paper. Aihua Cao and Xiaoling Zhang performed the experiments, analyzed the data. All authors declared that they read and approve manuscript final version.

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