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Vasoactive intestinal peptide alleviates food allergy via restoring regulatory B cell functions

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ABSTRACT

The immune regulatory cell dysfunction is associated with many immune diseases including food allergy (FA). This study aims to investigate the role of vasoactive intestinal peptide (VIP) in the maintenance of regulatory B cell (Br cell)'s immune suppressive functions by stabilizing thrombospondin (TSP1) expression. In this study, blood samples were collected from patients with food allergy (FA) and healthy control (HC) subjects. Br cells were isolated from the samples through flow cytometry cell sorting and analyzed by immunological approaches to determine the immune regulatory capacity. We found that the immune suppressive functions of Br cells were impaired in FA patients. The serum VIP levels were associated with the production of immune suppressive function-related mediators (interleukin-10, IL-10) of Br cells in FA patients. VIP counteracted IL-10 mRNA decay in Br cells by up regulating the TSP1 expression. TSP1 inhibited tristetraprolin (TTP) to prevent IL-10 mRNA decay in Br cells. Administration of VIP inhibited FA response through restoration of immune suppressive functions in Br cells. In conclusion, administration of VIP can alleviate FA response through up regulating expression of TSP1 to stabilize IL-10 expression in FA Br cells and recover the immune regulatory functions. The results have translational potential for the treatment of FA and other disorders associated with immune regulatory dysfunction of Br cells.

1. Introduction

Food allergy (FA) is an adverse response to food allergens by the immune system in the intestinal mucosa. FA can be categorized into IgE-dependent and non-IgE-dependent subtypes. In the non-IgE-dependent FA, aberrant immune cellular response plays a dominant role. The IgE-dependent FA is featured as the overproduction of antigen-specific IgE. IgE binds to the high affinity IgE receptors on mast cells to make mast cell sensitized. Re-exposure to specific antigens triggers mast cell activation and evokes FA attacks. FA symptoms vary from slightly abdominal discomfort to life-threatening anaphylactic shock (Jones and Burks, 2017). Besides, FA can induce lesions in other organs such as allergic asthma, allergic dermatitis (Jones and Burks, 2017; Yu et al., 2016). Our previous work also found that FA was involved in the pathogenesis of inflammatory bowel disease (Cai et al., 2016). In fact, FA is a great negative impact on human health and social economy (Patel et al., 2017). To date, the efficacy of current therapeutics for FA are not satisfactory (Jones and Burks, 2017). Therefore, to further elucidate the pathogenesis and invent novel and more effective remedies for the treatment of FA are necessary.

It is recognized that the immune regulatory system is dysfunctional in FA subjects (Chinthrajah et al., 2016). The immune regulatory system consists of immune regulatory cells (such as regulatory T cells, Tregs, and regulatory B cells, Brs), and immune regulatory mediators [such as transforming growth factor (TGF)-β and interleukin (IL)-10] (Li and Zheng, 2015; Tedder, 2015). The functions of the immunoregulatory system are suppressing aberrant immune responses of other immune cells (Li and Zheng, 2015; Tedder, 2015). The dominant

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T helper (Th)2 response and overproduction of Th2 cytokines and IgE antibodies mirror dysfunction of the immune regulatory system. The insufficient production of immune regulatory mediators was observed in FA subjects (Li et al., 2016). The underlying mechanism is to be further investigated.

Our previous work revealed that TSP1 played an important role in the immune regulatory activities of Brs by facilitating Tregs' immune regulatory functions (Zhang et al., 2013; Yang et al., 2015). Early works indicate that TSP1 is a factor to activate platelets. Latterly, it was found that, through physical contact with the extracellular matrix, other matricellular proteins, growth factors, cytokines, proteases and cell receptors, TSP1 has multiple functions involving a variety of bioactivities in an environmental context-dependent manner (Resovi et al., 2014). Yet, whether TSP1 is involved in the regulation of immune regulatory mediator activities in Brs remains to be further understood.

Published data indicate that vasoactive intestinal peptide (VIP) has the immune regulatory function and suppression of local inflammation through activating Toll-like receptor (TLR) 2/4/MyD88, nuclear factor-κB and mitogen-activated protein kinase signal transduction pathway (Xu et al., 2014). It was found that VIP was produced by the nerve endings in the intestinal mucosa; latterly, VIP was found being produced by several cell types and tissues, including epithelial cells, endocrine cells and immune cells (Pozo and Delgado, 2004). Although immune cells can produce VIP, whether VIP regulates the immune regulatory mediator activities in Br cells is undetermined. In this study, we analyzed the immune regulatory function of Br cells in FA patients. The role of VIP in the stabilization of Br cells' immune regulatory function by modulating TSP1 expression was investigated. The results showed that VIP could efficiently regulate Br cells' immune regulatory capacity and inhibited allergic inflammation in the intestine.

2. Materials and methods

2.1. Human subject recruitment

Patients with FA were recruited into this study at the affiliated hospitals of Shenzhen University (Shenzhen, China). The diagnosis and management of FA were carried out by our physicians. Healthy control (HC) subjects were also recruited. The demographic data of human subjects are presented in Table 1. Patients with any of the following conditions were excluded: Autoimmune diseases, severe organ diseases, malignant tumors, under treatment with immune suppressors or corticosteroids for any reasons. The experimental procedures were approved by the Human Ethics Committee at Shenzhen University. A written informed consent was obtained from each human subject.

Table 1
Demographic data of FA patients.

Items	FA	HC
Number of Subjects	26	26
Age (yr) (median)	29.5 ± 3.6	28.2 ± 5.5
Male	13 (50)	13 (50)
Female	13 (50)	13 (50)
SPT (diameter)*		
< 3 mm	0	
10-15 mm	16 (61.5)	
> 15 mm	10 (38.5)	
Serum sIgE#		
15.3-50 KU/L	8(31)	
50-100 KU/L	12 (46)	
> 100 KU/L	6 (23)	

*Positive criteria: SPT positive was considered when a wheal diameter greater than 3 mm of the negative saline control.
#The serum sIgE to specific antigens was measured by the ImmunoCap test and a value of more than 0.35 kUA/L was considered a positive response.

2.2. Development of an FA mouse model

The animal experimental procedures were approved by the Animal Ethics Committee at Shenzhen University (SZUM180003). BALB/c mice were purchased from Guangdong Experimental Animal Center. Mice were maintained in a specific pathogen-free facility at Shenzhen University with accessing food and water freely. an FA mouse model was developed following our established procedures. Briefly, mice were subcutaneously injected with ovalbumin (OVA, 0.2 mg/mouse, a model antigen) mixed with 0.1 ml alum into the back skin. The mice were boosted on day 3 and day 6, respectively. Challenge with OVA (1 mg/mouse, 0.3 ml saline) by gavage-feeding was carried out on day 11, 13 and 15, respectively. Mice were sacrificed on day 15 at 2 h after the last OVA challenge under isoflurane anesthesia, and followed by cervical dislocation. Assessment of FA response and FA pathological changes in the intestine was carried out following our previous reports (Zhang, et al., 2013).

2.3. Statistical analysis

The difference between two groups was determined with Student *t* test. ANOVA followed by Dunnett's *t*-test or Student-Newman-Keuls test was performed for multiple comparisons. $P < 0.05$ was considered as statistical significance. Correlation between two group data was analyzed by Pearson correlation assay.

Some experimental procedures are presented in supplemental materials.

3. Results

3.1. Immune suppressive function of Br cells is impaired in patients with FA

We firstly assessed the number of Br cells in PBMCs by flow cytometry. The results showed that the frequency of $CD73^-$ $CD19^+$ $CD25^+$ $CD71^+$ Br cells (van de Veen et al., 2013) was not significantly different between the FA group and the HC group (Fig. 1A-C). The results indicate that the FA environment does not significantly affect the lineage development of Br cells. We then assessed the immune suppressive functions of Br cells. FA Br cells (Br cells collected from FA patients) and HC Br cells were prepared by flow cytometry cell sorting. The Br cells were cocultured with effector T cells (Teffs, isolated from HC PBMCs by flow cytometry cell sorting) in the presence of T cell activators (anti-CD3/CD28 Abs) for 3 days. The cells were analyzed by flow cytometry. The results showed that HC Br cells efficiently suppressed Teff proliferation while much weaker suppressive effect on Teff proliferation were observed in FA Br cells (Fig. 1D-E). The results indicate that the immune suppressive capacity of FA Br cells is impaired. As IL-10 is the critical immune regulatory mediator of Br cells (van de Veen et al., 2013), an inhibitor of IL-10, AS101, was added to the culture. The suppressive effects of HC Br cells on Teff proliferation were considerably attenuated by the addition of AS101, indicating the suppressive effects of Br cells were mediated by IL-10 (Fig. 1D-E). As a control, naïve B cells did not show suppressive functions on Teff proliferation. The results emphasize the importance of IL-10 in the immune suppressive functions of Br cells.

3.2. Serum VIP levels are associated with production of immune suppressive function-related mediators of Br cells in FA patients

IL-10 is the canonical mediator in the immune suppressive functions of Br cells (van de Veen et al., 2013; Xiao et al., 2015). Our previous work indicates that TSP1 is involved in Br cell's immune regulatory functions (Zhang, et al., 2013). Therefore, we assessed the production of IL-10 and TSP1 in Br cells. The results showed that the levels of IL-10 and TSP1 in FA Br cells were significantly lower than that in HC Br cells (Fig. 2A-D). Since VIP has distinctive immune regulatory functions and

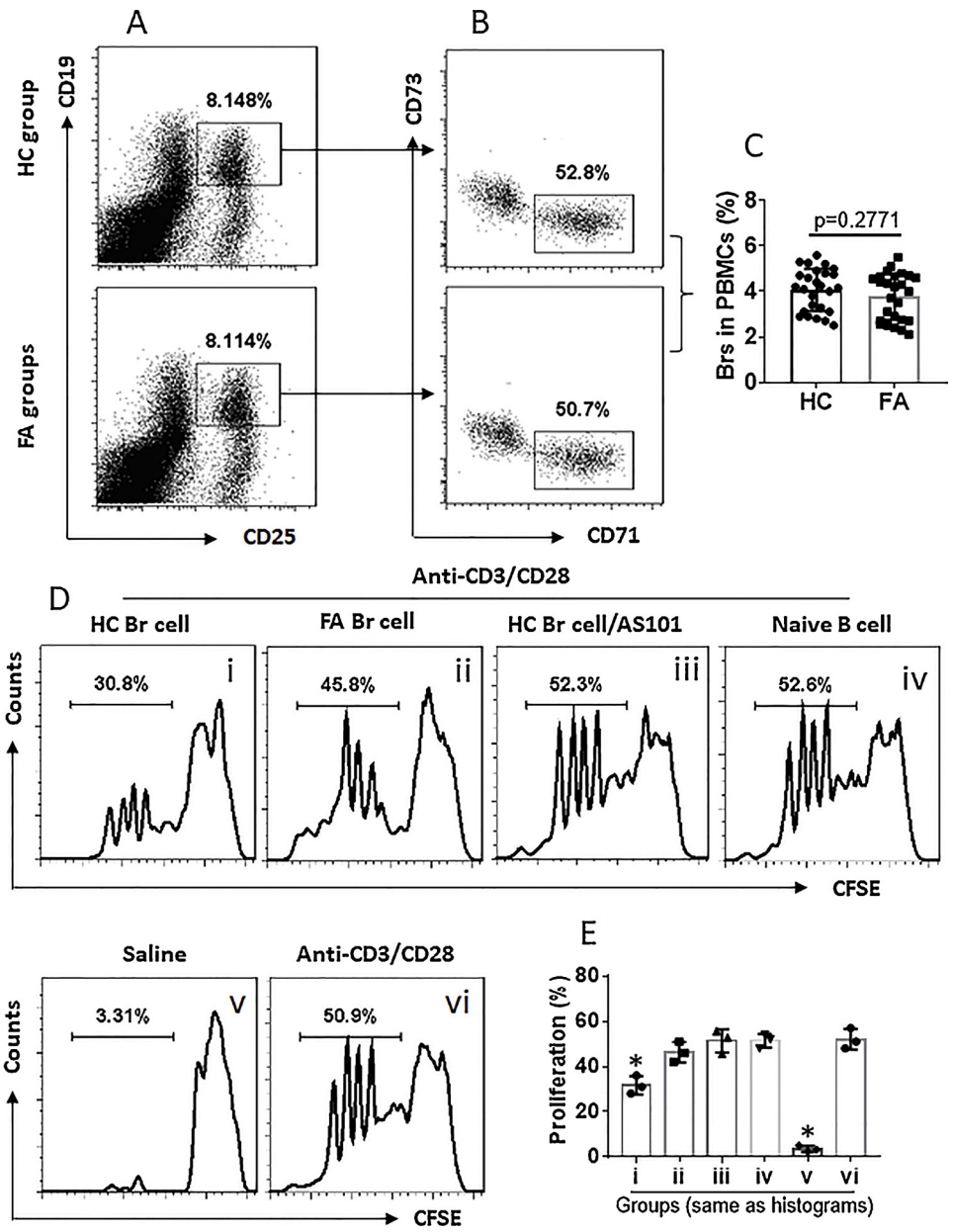

Fig. 1. FA Br cells show incompetent immune suppressive function. A–C, blood samples were collected from HC subjects (n = 26) and FA patients (n = 26). PBMCs were isolated from the blood samples and analyzed by flow cytometry. A, gated dot plots show frequency of $CD19^+$ $CD25^+$ B cells. B, gated dot plots show frequency of $CD73^-$ $CD71^+$ cells in the gated $CD19^+$ $CD25^+$ B cells of panel A. C, bars indicate summarized frequency of Br cells. D, Br cells were isolated from PBMCs and cocultured with $CD4^+$ $CD25^-$ T cells (labeled with CFSE) under the conditions denoted above the histogram panels. The gated histograms indicate frequency of proliferating $CD4^+$ $CD25^-$ T cells. E, bars show summarized data of panel D. AS101: An IL-10 inhibitor (10 μg/ml). Data of bars are presented as mean ± SEM. Each dot inside bars present data obtained from an independent experiment. *p < 0.01, compared with group vi.

associated with immune cell functions (Delgado et al., 2004), we assessed the serum levels of VIP by ELISA. The results showed that the serum levels of VIP were lower in FA patients than that in HC subjects (Fig. 2E). A positive correlation was identified between serum VIP levels and IL-10 in Br cells (Fig. 2F-G) or serum VIP levels and TSP1 (Fig. 2H-I) in Br cells. The results demonstrate the production of IL-10 and TSP1 in FA Br cells is impaired, which may be attributed to the lower serum levels of VIP.

3.3. VIP counteracts IL-10 mRNA decay in Br cells by up regulating TSP1 expression

To take an insight into the mechanism of the impaired IL-10 production in FA Br cells, we isolated Br cells from FA patients and HC subjects. Adopting an established IL-10 inducing experimental model (Lee et al., 2017), Br cells were exposed to LPS in the culture for 100 min to up regulate the expression of IL-10, washed with fresh medium and cultured with RPMI1640 medium. The cells were harvested from the culture and analyzed the production of IL-10. IL-10 mRNA of HC Br cells and FA Br cells was detected at 0 min time point. However, the levels of IL-10 mRNA in Br cells were declined gradually after 30 min time point in both FA Br cells and HC Br cells. The results indicate that IL-10 mRNA decays spontaneously in Br cells. Since serum VIP levels in Br cells positively correlate with IL-10 levels in Br cells as shown by Fig. 2, we inferred that VIP might counteract with IL-10 mRNA decay in Br cells. To test the inference, VIP was added to the culture. Indeed, the IL-10 mRNA was stabilized in HC Br cells by the presence of VIP in the culture, while in FA Br cells, however, the levels of IL-10 mRNA dropped in the first 40 min, but stabilized for the next 60 min throughout the observation period (Fig. 3A). Since VIP can up regulate the expression of TSP1 (Paparini et al., 2015), we inferred that exposure to VIP in the culture increased the expression of TSP1; the latter stabilized IL-10 mRNA in FA Br cells. To test this, we treated FA Br cells with VIP in the culture for 48 h. It increased the expression of TSP1 in an FA Br cell in a concentration-dependent manner (Fig. 3B-C). We then depleted the TSP1 expression in HC Br cells by Crispr (Fig. 3D). The TSP1-deficient HC Br cells were primed with LPS, then cultured in the presence of VIP for 48 h. Indeed, the presence of VIP could not stabilize the expression of IL-10 in TSP1-deficient HC Br cells (Fig. 3E). The data indicate that IL-10 mRNA decays spontaneously in Br cells.

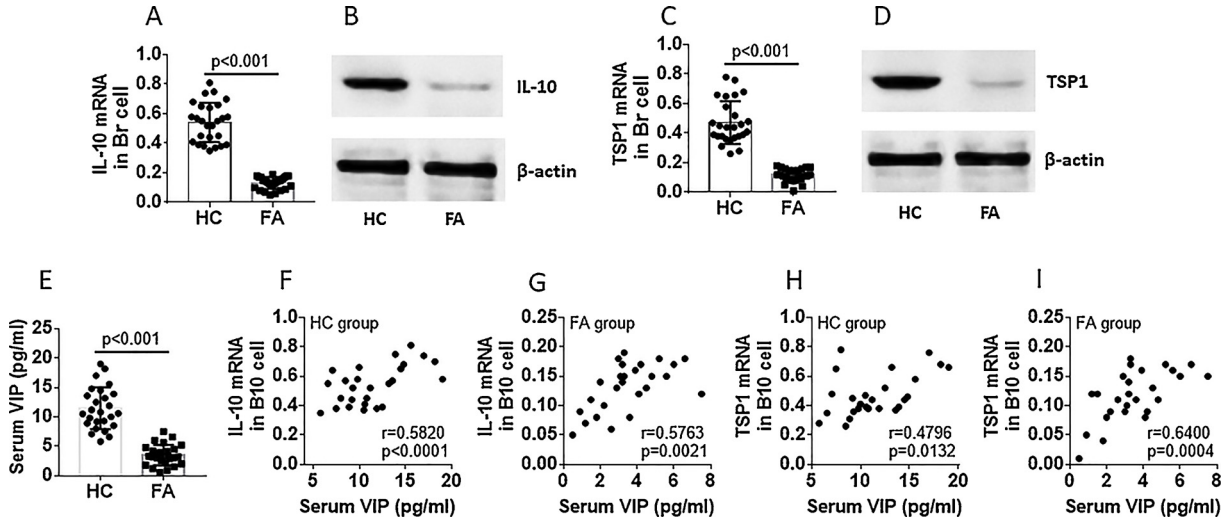

Fig. 2. Serum VIP levels are associated with expression of IL-10 and TSP1 in Br cells. Blood samples were collected from HC subjects (n = 26) and FA patients (n = 26). PBMCs were isolated from the blood samples and cultured overnight in the presence of CD40 L. Br cells were isolated from PBMCs and analyzed by RT-qPCR, Western blotting and ELISA to determine the expression of IL-10 and TSP1. A–B, IL-10 expression in Br cells. C–D, TSP1 expression in Br cells. E, serum levels of VIP. F–I, correlation between serum VIP and IL-10 (F–G) or serum VIP and TSP1 (H–I) in Br cells. Data of bars are presented as mean ± SEM. Each dot inside bars present data obtained from an independent experiment. Protein extracts of Br cells were pooled per group. Immunoblots of B and D are from one experiment representing 3 independent experiments.

VIP counteracts IL-10 mRNA decay in Br cells by up regulating the expression of TSP1.

3.4. TSP1 inhibits tristetraprolin (TTP) to prevent IL-10 mRNA decay in Br cells

TTP is an RNA-binding protein. By physically contacting with RNA, TTP causes RNA degradation (Brooks and Blackshear, 2013). As expected, we found a complex of TTP and IL-10 mRNA in Br cells (Fig. 4A-B). The finding prompted us to assess the expression of TTP in Br cells. More TTP proteins were found in FA Br cells while the TTP mRNA levels were not significantly different from each other (Fig. 4C-D). The results suggest that the degradation of TTP protein is impaired in FA Br cells. Since ubiquitination is involved in proteolysis (Ji and Kwon, 2017), we looked at the TTP ubiquitination in Br cells. The results showed that the ubiquitinated TTP was much less in FA Br cells as compared to that in HC Br cells (Fig. 4E). The results suggest that TSP1 binds TTP to prevent TTP from binding IL-10 mRNA and thus to prevent IL-10 mRNA from decay. Since ubiquitin E3 ligase A20 (A20, in short) plays a role in IL-10 expression in B cells (Li et al., 2016), we measured A20 expression in Br cells. The results showed that A20 expression was higher in the HC group than that in the FA groups (Fig. S1 in the Supplemental

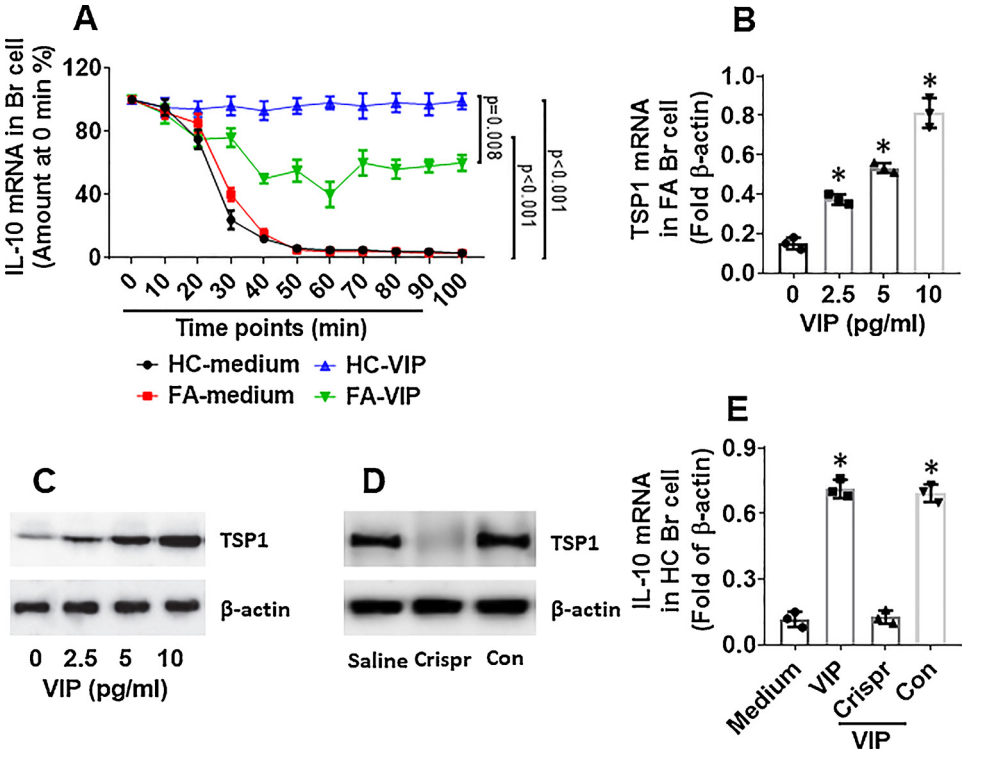

Fig. 3. VIP counteracts IL-10 mRNA decay in Br cells through up regulating TSP1. A, HC and FA Br cells were isolated from blood samples of human subjects and cultured in the presence of LPS (100 ng/ml) for 100 min. The cells were washed with fresh medium and cultured in RPMI1640 medium. Cells were harvested at the indicated timepoints (on the x axis) and analyzed by RT-qPCR. The curves indicate IL-10 mRNA levels in Br cells. B–C, RT-qPCR and Western blotting data show expression of TSP1 in FA Br cells after exposing to VIP in the culture for 48 h. D, TSP1 Crispr results show depletion of TSP1 expression in HC Br cells. E, RT-qPCR results show IL-10 mRNA levels in HC Br cells after treating with the procedures denoted on the x axis. Crispr: TSP1 Crispr. Con: Control RNAi. Data of bars are presented as mean ± SEM. Each dot inside bars present data obtained from an independent experiment. The data represent 3 independent experiments.

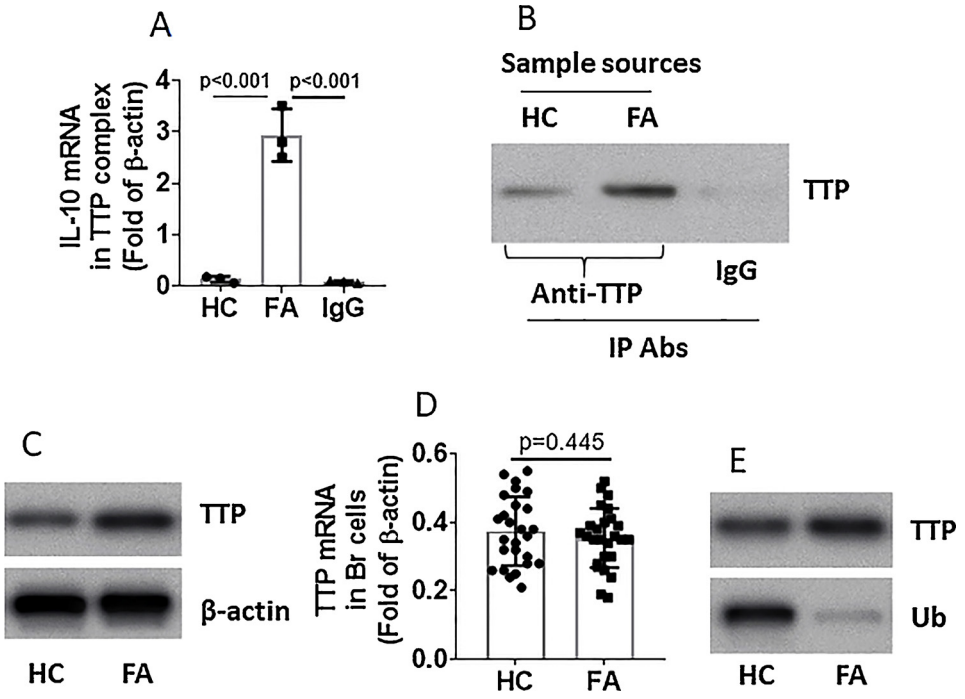

Fig. 4. Assessment of TTP activities in Br cells. A–B, a complex of IL-10 mRNA and TTP in Br cells (By RIP). C–D, expression of TTP in Br cells. E, protein samples were obtained from Br cells collected from HC subjects and FA patients. Upper binds show TTP-positive staining. Lower bands show ubiquitin-positive staining in TTP protein. Data of bars are presented as mean ± SEM. Each dot inside bars present data obtained from one sample. Immunoblots are from one experiment representing 3 independent experiments (samples of C were pooled per group).

Materials).

3.5. Administration of VIP inhibits FA response through restoration of immune suppressive functions of Br cells

Data of Figs. 14 suggest that insufficient VIP may be an important factor in the pathogenesis of FA. To reproduce this *in vivo*, an FA mouse model was developed. In addition to showing FA-like response and Th2-dominant pathological changes (including higher levels of Th2 cytokines and lower levels of IFN-γ in intestinal protein extracts) in the intestine (Fig. 5), FA mice also showed lower serum levels of VIP (Fig. S2 in supplemental materials), lower expression of IL-10 (Fig. S3) and TSP1 (Fig. S4) in intestinal Br cells. The immune suppressive functions of intestinal Br cells were impaired (Fig. S5). Administration of VIP significantly suppressed FA response and Th2-dominant pathological changes in the intestine, which were abolished by blocking TSP1 (Fig. 5). A20 expression in Br cells was also assessed. The results showed there was no significant difference between the FA group and the control group, nor was in Br cells isolated from FA mice treated with VIP (Fig. S6). The results demonstrate that VIP ameliorates FA response and the Th2-dominant pathological changes in the intestine by restoration of Br cell immune suppressive functions.

4. Discussion

The dysfunction of the immune regulatory system is associated with the pathogenesis of many immune diseases. The present data add novel mechanistic information to this area by showing that the deficiency or insufficiency of TSP1 impairs Br's immune suppressive functions. This phenomenon was found in FA patients as well as reproduced in an FA mouse model.

The phenomenon of immune regulatory system dysfunction has been recognized in a large number of immune disorders, such as allergic diseases and autoimmune diseases (Bacchetta et al., 2007; Chatila, 2005). As aforementioned, immune regulatory system consists of cellular components (such as Tregs and Brs) and mediators (such as TGF-beta and IL-10). The reduction of immune regulatory cell number plays an important role in the pathogenesis of immune dysfunction. Patients with FA show significantly a smaller number of Tregs than HC subjects (Prince et al., 2017). Lee et al found that $CD5^+$ Br cells were in a smaller number in FA patients as compared to that of HC subjects (Lee et al., 2010). The present data, however, show that the frequency of Brs is not significantly different between FA patients and HC subjects. The data suggest that the FA environment does not affect the Br lineage development. The difference between our data and Lee's data (Lee et al., 2010) can be the different cell markers used to sort Br cells. Lee et al sorted $CD5^+$ Br cells, while we sorted $CD73^-$ $CD25^+$ $CD71^+$ Br cells.

Although the frequency of Brs was comparable between FA patients and HC subjects, the immune suppressive function was impaired in FA Brs. The expression of IL-10, the canonical immune regulatory mediator, was significantly less in FA Brs as compared to that in HC Brs. The results are in line with previous reports. Li et al reported that the expression of IL-10 was generally lower in B cells. The authors found that IL-13, one of the canonical Th2 cytokines, increased HDAC11 and suppressed ubiquitin E3 ligase A20 and suppressed the expression of IL-10 in B cells (Li et al., 2016). Our data are in line with Li's data by showing lower expression of IL-10 in FA Br cells. However, A20 is not involved in the IL-10 mRNA decay in Br cells as shown by the present data, nor is relevant with the effects of VIP-induced restoring Br immune regulatory capacity. The results suggest that the FA environment does not affect the lineage of Br cell development, but affect the production of IL-10.

The data show that IL-10 mRNA decays spontaneously in both FA Br cells and HC Br cells. This phenomenon suggests that, after gene transcription, IL-10 mRNA needs extra bioactivities to stabilize until the completion of IL-10 protein synthesis. These bioactivities may be provided by a fraction of the protein. A fraction of proteins can speed up RNA decay. Although this is a physiological phenomenon to eliminate infidelity RNAs, over production of these proteins may cause RNAs to be mistakenly eliminated (Fradejas-Villar et al., 2017; Guo et al., 2017). Our data are in line with those pioneer studies by showing that the expression of TTP was unusually higher in FA Br cells. The results implicate that TTP may be attributed to IL-10 mRNA decay in Br cells. The inference was supported by further experimental data that depletion of TTP stabilized IL-10 mRNA in Br cells.

The data also show that the mRNA levels of TTP in FA Br cells were comparable with that in HC Br cells, while the protein levels of TTP are

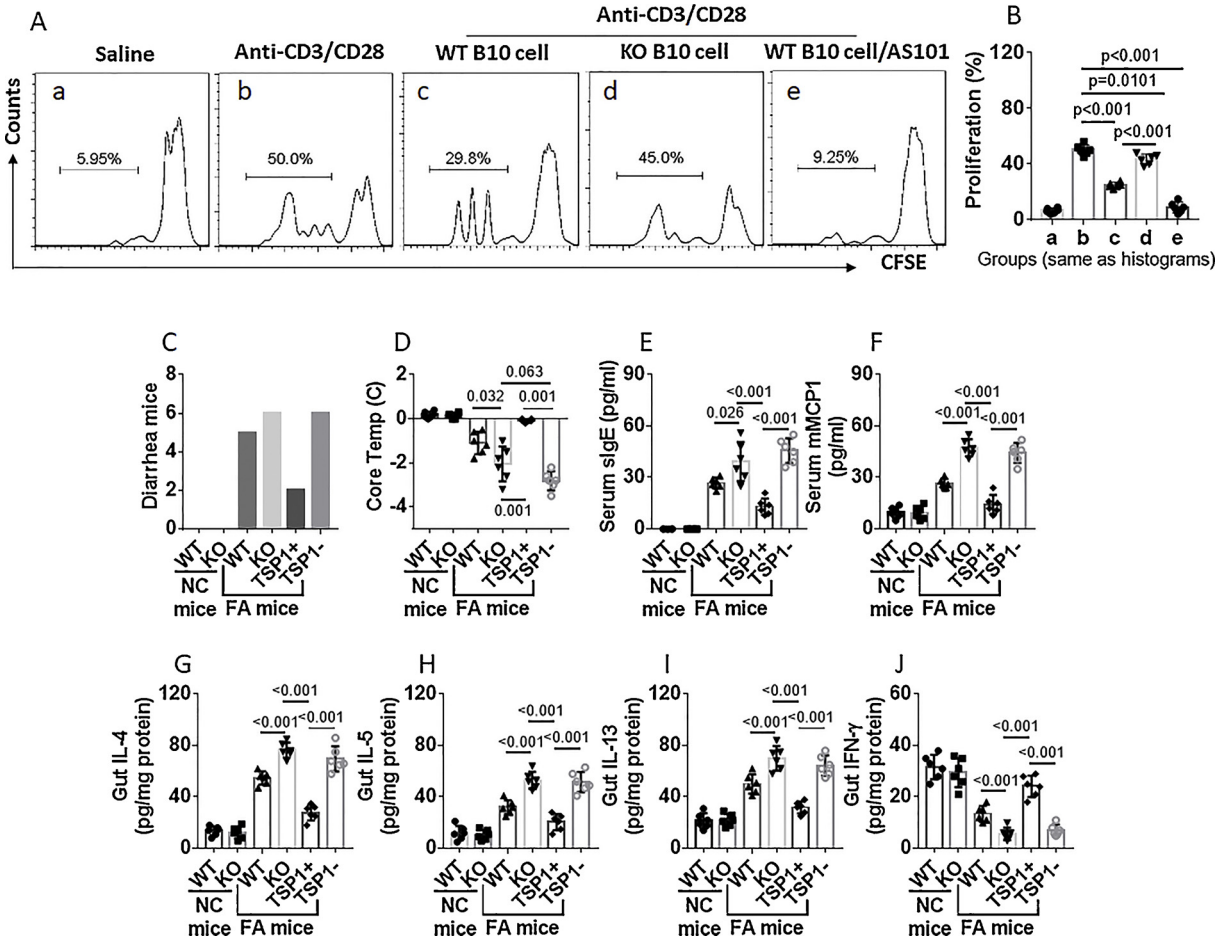

Fig. 5. TSP1 mediates the effects of VIP in the inhibition of FA response. FA mice were developed and treated with VIP and TSP1 inhibitor (LSKL, or control peptide: SLLK; 10 mg/kg body weight, i.p., every other day in the course of sensitization). A–B, diarrhea mice and core temperature recorded in mice during 2 h after oral challenge with specific antigens. Bars show temperature changes. C–D, ELISA results show serum levels of specific IgE and mouse mast cell protease-1 (mMCP1). E–H, ELISA results show cytokine levels in protein extracts of the intestinal tissue. Data of bars are presented as mean ± SEM. Each dot inside bars present data obtained from an independent experiment.

higher in FA Br cells. The fact suggests that the gene transcription of TTP is not altered in FA Br cells, but the degradation of TTP protein is impaired, and thus, TTP is over deposited in FA Br cells as shown by the present data. The data suggest that the over deposit of TTP in Br cells is an important factor for inducing IL-10 mRNA decay. The aberrant lower expression of cytokines is a common phenomenon in FA and other allergic diseases; such as a significantly lower expression of IL-10 in Br patients (Ma and Yin, 2014) and tolerogenic dendritic cells in patients with allergic rhinitis (Luo et al., 2017), lower levels of IFN-γ in $CD4^+$ T cells (Sun et al., 2016). Whether TTP deposit also contributes to these conditions is an interesting topic to be investigated.

We found that expression of TSP1 was less in FA Br cells than that in HC Br cells; this is in line with our previous observation (Zhang et al., 2013). A negative correlation was identified between TSP1 and TTP in Br cells. Besides acting as a platelet activating factor, TSP1 also has the proteolytic property (Kumar et al., 2017). By contacting with target proteins, TSP1 induces proteolytic activities; such as to cleave the latent peptide on the precursor transforming growth factor (TGF)-β to enable its immune activating capacity (Kumar et al., 2017). The present study reveals a novel aspect of TSP1 that TSP1 is required in the stabilization of IL-10 expression in Br cells through a mechanism by which TSP1 restricts the activities of TTP, probably by inducing TTP degradation. TTP is an RNA-binding protein (Guo et al., 2017). By physical contact with target RNAs, TTP induces the RNA degradation (Guo et al., 2017). Such a phenomenon was also found in the present study. Over deposit of TTP in Br cells causes the IL-10 mRNA to decay. Since IL-10 is the canonical mediator in the immune regulatory activities of Br cells, to regulate the production of TTP in Br cells may improve the immune regulatory capacity of Br cells.

We observed the serum VIP levels were lower in FA patients. Similar phenomenon was also observed in patients with arthritis (Seoane et al., 2015; Guan et al., 2019) and patients with inflammatory bowel disease (Sun et al., 2019). The mechanism of low serum levels of VIP is unclear currently; probably because the activities of VIP-autoantibodies are higher that cause VIP hydrolysis (Olopade et al., 2006), this needs to be further investigated.

In summary, the present data show that low VIP levels result in IL-10 mRNA spontaneously decays in FA Br cells to induce Br cell dysfunction. Administration of VIP restores the immune regulatory function in Br cells (Fig. S7), which shows the translational potential for the treatment of FA or other allergic diseases.

Author's contribution

HTZ, HH, SBY, XRG, JQL, GY, DCL and LTY performed experiments, analyzed data and reviewed the manuscript. PCY and PYZ organized the study and supervised experiments. PCY designed the project and wrote the manuscript.

Declaration of Competing Interest

None to declare.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.08.006.

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