

TSP1-producing B cells restore Ag specific immune tolerance in an allergic environment

Running title: B cells facilitate immune tolerance restoration

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Capsule

Background: The generation of tolerogenic dendritic cells (ToIDC) in an allergic environment is refractory.

Results: Administration of IL-13 antagonists increases ToIDCs in an allergic environment.

Conclusions: Blocking IL-13 in an allergic environment facilitates the generation of ToIDCs.

Significance: Using IL-13 antagonists has the potential to enhance the therapeutic effect of the allergic diseases.

Abstract

To restore the antigen (Ag) specific immune tolerance in an allergic environment is refractory. B cells are involved in immune regulation. Whether B cells facilitate the generation of Ag specific immune tolerance in an allergic environment is to be further investigated. This paper aims to elucidate the mechanism by which B cells restore the Ag specific immune tolerance in an allergic environment. In this study, a B cell-deficient mouse model was created by injecting an anti-CD20 antibody. The frequency of tolerogenic dendritic cell (ToIDC) was assessed by flow cytometry. The levels of cytokines were determined by enzyme-linked immunosorbent assay. The expression of thrombospondin-1 (TSP1) was assessed by quantitative real time RT-PCR, Western blotting and the methylation specific PCR. The results

showed that B cells were required in the generation of the transforming growth factor (TGF)- β -producing ToIDCs in mice. B cell-derived TSP1 converted the latent TGF- β to the active TGF- β in DCs, which generated TGF- β -producing ToIDCs. Exposure to interleukin (IL)-13 inhibited the expression of TSP1 in B cells by enhancing the TSP1 gene DNA methylation. Treating food allergy mice with specific Ag immunotherapy and IL-13 antagonists restored the generation of ToIDCs and enhanced the effect of specific immunotherapy. In conclusion, B cells play a critical role in the restoration of specific immune tolerance in an allergic environment. Blocking IL-13 in an allergic environment facilitates the generation of ToIDCs and enhanced the therapeutic effect of immunotherapy.

Introduction

The underlying mechanism of the immune tolerance is proposed as that the exposure to the specific antigens (Ag) induces the generation of tolerogenic dendritic cells (ToIDC) and regulatory T cells (Treg) (1). This has been supported by a large number of animal model studies, which indicate that exposure to Ags at small doses for several times, or exposure to one large dose of Ags can induce ToIDCs and Tregs in the body (2). However, the generation of ToIDCs is quite refractory in subjects with allergic disorders; the mechanism remains to be further elucidated.

ToIDCs are categorized into several subtypes based on their expression of high levels of interleukin (IL)-10, transforming growth factor (TGF)- β , or indolamine-2, 3-dioxygenase (IDO), etc. (3). Various sub-types of ToIDCs play important roles in the induction and maintenance of immune tolerance in the body (3). One of the mediators by which ToIDCs induce Tregs is TGF- β (4). TGF- β regulates multiple cell functions including differentiation, migration, proliferation, inhibiting the functions of inflammatory cells and promoting the function of Tregs (5). TGF- β induces expression of Foxp3 in T cells to facilitate the generation of Tregs (6,7). It is accepted that a subtype of ToIDCs can produce TGF- β to generate Tregs, however, after synthesis, TGF- β exists as a precursor, the latent TGF- β (LTGF β), in the cells. LTGF β has a latency associated peptide (LAP) attaching to the TGF- β molecular complex. It is required to remove the LAP from the complex to convert the LTGF β to TGF- β ; how the conversion is carried out in DCs has not been well defined yet.

Thrombospondin 1 (TSP1) is a protein that in human is encoded by the THBS1 gene (8). TSP1 has multiple functions, such as in platelet aggregation, angiogenesis, and tumorigenesis (9). Previous reports suggest a pathway to generate Tregs from human CD4⁺ CD25⁻ T cells in response to inflammation, in which TSP1 plays a critical role by converting the LTGF β to TGF- β (10). Our previous studies show that the activated B cells express TSP1 (11); whether the B cell-derived TSP1 converts LTGF β to TGF- β in DCs is to be further investigated.

B cells can be activated by receiving the Ag stimulation and/or other stimuli from T cells (12), including various T cell-produced cytokines. In the allergic organs or tissues, high levels of Th2 cytokines present. IL-13 is one of the Th2 type cytokines, which is involved in the regulation of gene transcription of a large number of cytokines via the gene DNA methylation (13). Whether IL-13 also regulates TSP1 expression in B cells to alter the behavior of B cells is unclear. Published data indicate that B cells can regulate dendritic cell functions (14). Recent reports indicate that coculture of CD27⁺ CD35⁺ B cells with naive DCs generated TGF- β -expressing tolerogenic DCs (15). Yet, how B cell dysfunction in the induction of ToIDCs occurs in an allergic environment is unclear. Thus, B cells may be a critical checkpoint in regulating DC's tolerogenic properties. Although some reports suggest that B cells are not important in the induction of immune tolerance (16), cumulative evidence has emphasized the importance of B cells in the immune tolerance (17-19). Specifically, the role of B cells in the restoration of Ag

specific immune tolerance in an allergic environment has not been investigated. In the present studies, we observed that B cells were required in the generation of the TGF- β^+ TolDCs in an allergic environment. Upon activation, B cells produced TSP1, the latter activated the

LTGF β in DCs to convert the DCs to TGF- β^+ TolDCs. The expression of TSP1 in B cells was suppressed in the allergic environment by the T helper (Th)-2 cytokine IL-13, which could be reversed by concurrent administration of specific Ag vaccines and IL-13 antagonists.

Materials and methods

Reagents

Antibodies of CD20 (H-170, pAb), CD11c (M-20), CD19 (R-20), TGF- β (D-12), LAP (T-17), STAT6 (D-1), pSTAT6 (Tyr 641) and TSP1 (N-20) were purchased from Santa Cruz Biotech (Guangzhou, China). The neutralizing anti-mouse-TGF- β antibody was purchased from Abcam (Guangzhou, China). Fluorescence labeled antibodies for flow cytometry were purchased from BD Bioscience (Guangzhou, China). The anti-mouse TGF- β antibody for flow cytometry was purchased from Biol-equip (Beijing, China). Reagents for qRT-PCR and DNA extraction were purchased from Bio-Rad (Guangzhou, China). Reagents for immune cell isolation were purchased from Miltenyi Biotech (Shanghai, China). Peptides of LSKL and SLLK were synthesized by Shuguang Biotech (Shanghai, China). The ELISA kit of TSP1 was purchased from Shanghai Qayee Biological Technology Co., Ltd. (Shanghai, China). Recombinant IL-13 protein and neutralizing anti-IL-13 mAb were purchased from R&D Systems (Shanghai, China). The methylation inhibitor 5-aza-2'-deoxycytidine (5-aza) and ChIP kit were purchased from Sigma Aldrich (Shanghai, China). The recombinant TSP1 was purchased from Shanghai Feixian Biotech (Shanghai, China). The endotoxin levels in all reagents were detected using the Limulus assay (Limulus amoebocyte lysate QCL 1000, Bio Whittaker, Walkersville, MD, USA). The reagents used in this study contained <0.2U endotoxin/10 μ g reagents.

Mice

Male C57BL/6J (B6) mice (6-8 week old) were purchased from the National Rodent Laboratory Animal Resources, Shanghai

Branch (Shanghai, China). TSP1-null mice and IL-13-null mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Mice were maintained in a pathogen-free animal facility. The experimental procedures were approved by the Animal Ethic Committee at Shenzhen University.

Isolation of immune cells

Single cells were isolated from the intestine, MLN and spleen with our established procedures (20). The immune cells were further isolated from the single cells by magnetic cell sorting (MACS) with commercial reagent kits following the manufacturer's instruction. In the isolation of DCs, T cells and B cells were positively selected out; CD11c⁺ DCs were then isolated from the remaining cells. As checked by flow cytometry, the purity of the isolated cells was more than 98%.

Flow cytometry

Single cells were prepared and fixed with 2% paraformaldehyde for 60 min. The cells were then treated with 0.1% saponin (Sigma Aldrich) for 30 min in the case of intracellular staining. After washing with phosphate buffered saline (PBS), the cells were stained with fluorescence labeled antibodies (isotype IgG was used as a negative control) at 0.5-1 μ g/ml for 30 min. The cells were then washed with PBS for 3 times and analyzed by a flow cytometer (FACSCanto II, BD Bioscience, Beijing, China).

Induction of TGF- β ⁺ TolDCs in the intestine

Following published procedures (2), mice were gavage-fed 1 mg/ml of OVA daily for 7 days; control groups were treated with

saline instead. The frequency of TGF- β ⁺ DCs were assessed by flow cytometry.

CD19⁺ B cell depletion and CD11c⁺ TGF- β ⁺ TolDC assessment

B cells were depleted in mice with an anti-CD20 polyclonal antibody of a single 250 μ g dose via the tail vein injection (control mice were injected with saline or isotype IgG). The mice were sacrificed on day 0, 2, 4, 6 and 14 respectively after the injection. Peripheral blood mononuclear cells (PBMC), spleen cells and lamina propria mononuclear cells (LPMC) were prepared from the mice, stained with fluorochrome-labeled antibodies of CD19, CD11c and TGF- β , and analyzed by flow cytometry.

Generation of TolDCs

The naive DCs were cocultured with the activated B cells at a ratio of 1:1 in the presence of LPS (5 ng/ml) and/or OVA (10 μ g/ml) for 48 h. The frequency of TGF- β ⁺ TolDCs were more than 90% of the CD11c⁺ DCs as assessed by flow cytometry. The CD11c⁺ DCs were then isolated by MACS using as TolDCs in the further experiments.

Isolation of peripheral mononuclear blood cells (PBMC)

Mice were decapitated; the blood was collected immediately in a tube pre-coated with heparin. The PBMCs were isolated by the gradient density centrifugation.

Cell culture

The isolated immune cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM l-glutamine. As checked with the Trypan blue exclusion, the cell viability was more

than 98% before using for further experiments.

Western blotting

Total proteins were extracted from isolated cells and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred onto a nitrocellulose membrane. After blocking with 5% skim milk for 30 min, the membrane was incubated with the primary antibodies at 50-100 ng/ml (or the corresponding isotype IgG) for 1 h at room temperature. After washing with Tris-buffered saline with Tween 20 (TBST) for 3 times, the membrane was incubated with the secondary antibodies conjugated with horseradish peroxidase at 10-20 ng/ml for 1 h. The immune complex was developed with the enhanced chemiluminescence reagents. The results were photographed by the UVP BioSpectrum Imaging System (Upland, CA, USA). The integrated density of the immune blots was analyzed by PhotoShop software version CS5. The results were expressed as the percentage of the immune blots of the internal control β -actin.

Quantitative real time RT-PCR (qRT-PCR)

The total RNA was extracted from the isolated single cells with a reagent kit. The complement DNA was synthesized with a cDNA synthesis kit. qPCR was carried out using the MiniOpticon real-time PCR detection system with the SYBR[®] Green Master Mixes (Invitrogen). The relative expression of mRNA was calculated as $2^{-\Delta\Delta CT}$; the results were normalized to a percentage of the internal control β -actin. The primers of TSP1 using in this study were: forward, ccaagcctgcaagaaagac; reverse, cctgctgttgcaaaactga. Integrin β 6:

forward, tacggaagcatctacggacc; reverse, tttctgcagacacacttgcc. Integrin β 8: forward, gcactgtgtcaactccaagg; reverse, atgcgatgctgttccatgac.

ELISA

The levels of TSP1 were determined by ELISA with a commercial reagent kit following the manufacturer's instruction.

Generation of BMDC

Freshly isolated bone marrow cells were cultured in 24-well tissue culture plates at 1×10^6 cells/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). Cells were stimulated with 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 100 ng/ml IL-4. Cells were used for further experiments on day 6-8, at this stage, >95% cells expressed CD11c; less than 20% cells expressed CD80 and CD86 indicating these cells were at the immature state.

Food allergy (FA) mouse model

Following published procedures (21) with minor modification, mice were gavage-fed with ovalbumin (OVA; 1 mg/mouse) mixing with cholera toxin (20 μ g/mouse) in 0.3 ml saline; once a week for 5 consecutive weeks.

Assessment of the role of TolDCs in generation of Tregs

CD4⁺ CD25⁻ T cells were isolated from the mouse spleen and cultured with TolDCs at a ratio of 1:1 in the presence of IL-2 (20 ng/ml) for 6 days. The medium was changed in every other day.

Treating FA mice with anti-IL-13 Ab

Some FA mice were treated with anti-IL-13 mAb (100 μ g/mouse, i.p.) or an isotype IgG (control Ab) every other day for 4 times.

Modified Ag specific immunotherapy (MSIT)

Following published procedures of MSIT (22) with minor modification, the FA mice were treated with OVA (1 mg/mouse, in 0.3 ml saline, gavage) daily for 6 days. MSIT was started 48 h after the first injection of anti-IL-13 mAb.

Adoptive transfer with TolDCs or TSP1⁺ B cells

TolDCs and TSP1⁺ BCs were prepared as described above. The mice were adoptively transferred with either naive DCs, or TolDCs, or naive B cell, or TSP1⁺ B cells at 10^6 cells/mouse via tail vein injection at week 0, week 2 and week 4 respectively.

Methylation analysis of TSP1 in B cells

DNA was extracted from B cells with a reagent kit following the manufacturer's instruction. The genomic DNA was treated with sodium bisulfite (Sigma Aldrich) as described by Herman et al (23). Briefly, 2 μ g of DNA were denatured with 2M NaOH at 37°C for 10 minutes, and incubated with 3M sodium bisulphite (pH5.0), overlaid with mineral oil to cover the surface of the aqueous phase, and incubated at 50°C overnight. The samples were incubated with 3M NaOH for 5 minutes, concentrated by ammonium precipitation and 100% ethanol, washed with 70% ethanol, and resuspended in 20 μ l of distilled water.

The methylation status of the TSP1 promoter was then determined by methylation-specific PCR (MSP). The methylated and unmethylated DNA sequences were distinguished by the specific PCR. The primer sequences for the methylated form were TATTTAGAGTTTGGGGGAGATAAC (sense) and TAACATTCTCAATACGAAAAACGAA (antisense) (134bp), and the primer sequences for the unmethylated form were GTATTTAGAGTTTGGGGGAGATAAT (sense) and TAACATTCTCAATACAAAAACAAA (antisense) (135bp). Genomic DNA, methylated in vitro by CpG methyltransferase (Sss I) following the manufacturer's directions (New England BioLabs, Inc., Beverly, MA), was used as a positive control. Water blank was used as a negative control.

Activation of naïve B cells

The CD19⁺ IL-7R⁺ CD45R⁺ B cells were isolated from the mouse bone marrow by magnetic cell sorting (MACS) with commercial reagent kits following the manufacturer's instructions. The cells were cultured in the presence of IL-7 (10 ng/ml), anti-CD40 (1 µg/ml) and anti-IgM (10 µg/ml) for 7 days. The cells were used in further experiments.

Chromatin immunoprecipitation (ChIP).

ChIP assays were performed using a ChIP kit following the manufacturer's instructions (Sigma Aldrich). Briefly, B cells were cross-linked with 1% formaldehyde for 10 min at room temperature; the cells were lysed in a lysis buffer (containing protease inhibitor). The samples were collected and precleared with protein A/G agarose/salmon sperm DNA slurr; the immunoprecipitation was carried out at 4 °C overnight using an anti-STAT6 antibody or normal mouse IgG (using as a negative control) overnight at 4°C with mild agitation. The complexes of the antibody/chromatin were collected by incubation with protein A/G agarose and analyzed by qPCR. For the STAT6 binding sites in the TSP1 promoter, the primers were: Forward, gtgtcagtggaggaagctct; reverse, atctcccttgcaactcgga. The DNA purified from the sonicated nuclear lysate was analyzed by real-time PCR using the same primer sets. The resulting data were used as an input control. Expression of a target DNA sequence was normalized to the input DNA.

Statistics

Each experiment was repeated at least 3 times. The data were presented as mean ± SD. The data were analyzed using the ANOVA test, and the means were compared by Student–Newman–Keuls test. P<0.05 was set as the significant criterion.

Results

B cells are required in the generation of TGF- β ⁺ TolDCs

To determine the role of B cells in the generation of TGF- β ⁺ TolDCs, we treated mice with anti-CD20 antibodies (Ab) to deplete B cells in the peripheral blood, spleen and lamina propria in a time-dependent manner (Fig. 1A). Meanwhile, we observed that the frequency of TGF- β ⁺ TolDCs was significantly reduced in the peripheral blood, spleen and lamina propria as well; the reduction of TolDC number occurred 2 days after B cell depletion (Fig. 1B, 1C, 1D). The injection of a control Ab (cAb) did not apparently alter the frequency ($p>0.05$) of either B cells or TolDCs (Fig. 1A-B). The results imply that B cells are required in the generation and/or maintenance of TGF- β ⁺ TolDCs. To strengthen the results, we fed B6 mice with ovalbumin (OVA) daily for 7 days. As analyzed by flow cytometry, the frequency of TGF- β ⁺ TolDCs were increased in the intestine of the B cell-sufficient mice, but not in the B cell-deficient mice (Fig. 1E-F). The results suggest that depletion of B cells reduces the frequency of TGF- β ⁺ TolDCs.

B cell-derived TSP1 maintains DC's tolerogenic properties

The data of Fig. 1 implicate that a population of DCs produces TGF- β in response to proper stimulation. Since TGF- β exists as a precursor, the latent TGF- β (LTGF β), after synthesis, the LTGF β must have been converted to TGF- β somehow in the present experimental system. Thus, we next measured the levels of LTGF β in DCs after activation. Naive BMDCs were prepared and conditioned in no-serum medium overnight; the

conditioned BMDCs were exposed to LPS (using as a DC activator) in the culture for 48 h. The data showed that LTGF β was induced in the BMDCs (Fig. 2A, 2C); but no TGF- β was detected in the BMDCs (Fig. 2B, 2D). The results suggest that activation by LPS induces DCs to produce LTGF β , but no TGF- β was converted at this stage.

We cocultured the activated-B cells and BMDCs (primed by LPS) for 48 h. The results showed that TGF- β was not detected in the non-activated B cells (Fig. 3Aa, 3B, 3Ca); the activated B cells increased the levels of TGF- β in BMDCs in a B cell number-dependent manner (Fig. 3Ab-d, 3B, 3Cb-d). Based on that B cells express TSP1 after activation, we isolated B cells from TSP1-deficient mice to be cultured with BMDCs. The results showed that no TGF- β was induced in the DCs (Fig. 3Ae-f, 3B, 3Ce-f). We then added recombinant TSP1 to the DC culture, which markedly increased the levels of TGF- β in the DCs (Fig. 3Ag, 3B, 3Cg), which did not occur when DCs were cultured alone (Fig. 3Ah, 3B, 3Ch). Culture in conditioned medium (B cells were activated by anti-IgM in the culture) also induced TolDCs (Fig. 3Ai, 3B, 3Ci). More than 90% naive DCs expressed CD36, the receptor of TSP1; the expression of CD36 was not altered in TolDCs (Fig. 3D). The TolDCs showed the ability to generate Tregs (Fig. 3E). The results suggest that the 4activated B cells release TSP1 to facilitate the generation of TolDCs. In addition, we also observed the TolDCs had immune suppressive functions on CD4⁺ T cell proliferation (Fig. 3F).

IL-13 compromises DC's tolerogenic properties via suppressing TSP1 in B cells

Whether allergic disorders disturbs the generation of TolDCs has not been well defined. In a food allergy (FA) mouse model, we observed a much lower ($p < 0.01$) frequency of TGF- β^+ TolDCs in the intestine of FA mice than saline control mice (Fig. 4A). To elucidate if the DCs in the allergic mouse intestine still produce LTGF β , we extracted proteins from isolated DCs to be analyzed by Western blotting. The results showed that the levels of LTGF β in DC extracts of FA mice was not significantly different from control mice (Fig. 4B). The results imply that the LTGF β converting system was dysfunctional in the intestinal DCs of FA mice. To test the inference, we isolated DCs from the FA mouse intestine and cocultured with the activated B cells as described in Fig. 2. The results showed that the coculture with activated B cells markedly increased the frequency of TGF- β^+ TolDCs (Fig. 4C). To strengthen the data, we assessed the frequency of TSP1 $^+$ CD19 $^+$ B cells in the intestine of FA mice. Indeed, much less TSP1 $^+$ CD19 $^+$ B cells were detected in the intestine of FA mice as compared to control mice ($p < 0.01$; Fig. 4D). The results suggest that under an allergic environment, B cells produce less TSP1 in the intestine.

IL-13 suppresses the expression of TSP1 in B cells

We next tested the effect of IL-13 on the expression of TSP1 in B cells. Since there are two subtypes of IL-13 receptor (IL-13R), we firstly assessed the expression of the IL-13R in B cells. The results showed that B cells from the lamina propria showed high levels of IL-13R $\alpha 2$ at both mRNA and protein levels while only trifle expression of IL-13R $\alpha 1$ was detected in the B cells (Fig. 5A).

We then observed the effect of IL-13 on regulating the TSP1 expression in B cells. B cells were isolated from naive mice and treated with anti-IgM in the culture; the expression of TSP1 was observed in the B cells. The presence of IL-13 in the culture inhibited the expression of TSP1 in the B cells in an IL-13 dose-dependent manner, which was abolished by the presence of anti-IL-13R $\alpha 2$ antibody (Fig. 5B, 5C).

To gain further insight into the mechanism by which IL-13 suppresses the expression of TSP1, we assessed the STAT6 phosphorylation in B cells. The levels of pSTAT6 were increased in the B cells after exposure to IL-13 in the culture (Fig. 5D); the binding of pSTAT6 of TSP1 promoter was also detected in the B cells (Fig. 5E). Furthermore, the TSP1 gene DNA methylation in the B cells was much higher in FA mice than naive mice (Fig. 5F). To strengthen the data, we stimulated naive B cells with recombinant IL-13 in the culture, which markedly promoted TSP1 gene methylation in B cells, and was inhibited by the presence of the anti-IL-13R $\alpha 2$ antibody or the presence of a methylation inhibitor (Fig. 5B, C, F).

IL-13 antagonists promote the effect of specific immunotherapy via generating TGF- β^+ TolDCs

The results of Fig. 5 imply that IL-13 may be an obstacle in the generation of TolDCs, and that to overcome the effect of IL-13 may facilitate the restoration of generation of TolDCs to attenuate or inhibit allergic inflammation. To test the hypothesis, we developed an FA mouse model. Much fewer TGF- β^+ DCs were observed in the intestine of FA mice than that in naive control mice ($p < 0.01$; Fig. 6A-B, 6J). The FA mice were treated with a modified Ag

specific immunotherapy (MSIT) in a short period (6 days). However, the frequency of TolDCs in the intestine was not apparently increased (Fig. 6C, 6J). Considering IL-13 might interfere with the generation of TolDCs in the allergic environment, we sensitized IL-13-null mice to OVA. The mice were treated with MSIT for 6 days; the results showed that the frequency of TolDCs was markedly increased ($p < 0.01$) in the intestine as compared with those sensitized mice treated with saline (Fig. 6D-E, 6J). Treating the sensitized B6 mice with MSIT and anti-IL-13 antibodies also generated TolDCs in the intestine (Fig. 6F, 6J). On the other hand, treating the sensitized TSP1-null mice with MSIT did not generate TolDCs in the intestine (Fig. 6G, 6J). The results suggest that antagonizing IL-13 facilitates the generation of TolDCs in the intestine in an allergic environment.

We next assessed the effect of IL-13 antagonism on enhancing the inhibitory effect of MSIT on allergic inflammation in the intestine. FA mice were treated with MSIT with or without anti-IL-13 antibodies. Indeed, as shown by Fig. 7, the allergic

inflammation in the intestine was significantly attenuated as compared with mice treated with MSIT alone. To strengthen the results, we treated sensitized IL-13-null mice with MSIT. In contrast to sensitized wild mice, the allergic inflammation was significantly inhibited in the intestine. To test the role of TSP1 in the process, the TSP1-null mice were treated with the same procedures above. The results showed that MSIT had no appreciable effect on suppressing the allergic inflammation in the intestine of sensitized TSP1-null mice. Since we noted that the TGF- β -producing DCs played a critical role in the suppressing of intestinal allergic inflammation (4), we treated a group of FA mice with anti-TGF- β antibody together with anti-IL-13 and MSIT. Indeed, the allergic inflammation was not affected. We also adoptively transferred with TolDC or TSP1⁺ BC to mice, which abolished the allergic response in the intestine (Fig. 7A-F). The results confirm the important role of antagonism of IL-13 in the induction of TolDC and the suppression of allergic inflammation in an allergic environment.

Discussion

The present data show that the B cell-derived TSP1 is required in the generation of TGF- β^+ TolDCs in an allergic environment. After activation, the LTGF β is detected in the DCs, but the active form TGF- β is not detected. On the other hand, activation with anti-IgM induces the production of TSP1 in B cells, which can be released to the micro environment. Coculture of the TSP1-producing B cells and LTGF β -production DCs generates the TGF- β^+ TolDCs; the latter is reduced in FA mice. The data also show that the Th2 cytokine, IL-13, inhibits the expression of TSP1 in B cells and consequently reduces the frequency of TGF- β^+ TolDCs. Administration with anti-IL-13 Ab together with immunotherapy induces TolDC development in FA mice and inhibits allergic inflammation in the intestine.

After capturing Ags, DCs become maturation to induce Th1 response or Th2 response, or become TolDCs, depending the cytokine environment. TolDCs play an important role in the immune tolerance by generating Tregs (24). Despite all the activities of the 3 types of DC are useful in the maintenance of the homeostasis in the body, either one's dysfunction or polarization is harmful to the body. Thus, to understand the regulatory mechanism of the differentiation of DC phenotypes is of significance. The present data show that the B cell-derived TSP1 is critical for TGF- β -producing TolDC development. The data are in line with previous reports. Tang et al indicate that TSP1 activates TGF- β via its proteolytic activity (25). Hayashi et al report that the deficiency of TSP1 significantly reduces the expression of TGF- β in hepatocytes (26). Our previous

data show that TSP1 cleaves the surface CD80 and CD86 of DCs that also contributes to the tolerogenic properties of TolDC (11). TSP1 is a cytokine of multiple sources (27); our data suggest that the B cell-derived TSP1 is important in the development of TGF- β^+ TolDCs, which is supported by the data that the depletion of B cells markedly suppresses the generation of TGF- β^+ TolDCs.

Tregs are one of the major components of the immune tolerant system in the body and are mainly induced by TolDCs (28). The deficiency of TolDCs is associated with the pathogenesis of a large number of immune diseases. This is also mirrored by the present data. In FA mice, we detected much fewer TGF- β^+ TolDCs than that in control mice. The fact suggests that the generation of TGF- β^+ TolDCs is disturbed in the FA mice. There must have been inhibitors of TolDCs in FA mice. The inference is supported by further experimental data, in which IL-13, but not IL-4 and IL-5, is associated with the suppression of the TGF- β^+ TolDCs. IL-13 is one of the major cytokines of Th2 polarization. Published data indicate that IL-13 induces gene methylation of a large number of molecules (13). Our experiments show that IL-13 promotes the TSP1 gene DNA methylation in B cells, which markedly reduces the production of TSP1 in B cells.

The breakdown of immune tolerance plays a critical role in the pathogenesis of a number of immune disorders. It is proposed that restoration of the immune tolerance has the therapeutic benefit for patients with immune diseases (29,30). The present data suggest that under an

allergic environment, or in the Th2 polarization, the high levels of IL-13 prevents the expression of TSP1 in B cells, which suppresses the expression of TSP1 and disturb the generation of TolDCs in the body. Therefore, blocking IL-13 has the potential to up-regulate the generation of TolDCs in an allergic environment. Indeed, the concurrent administration with IL-13 antagonists and the specific Ag vaccines significantly increased the frequency of TolDCs in the intestine of FA mice as compared with FA mice treated with either the Ags alone or an anti-IL-13 mAb alone.

SIT has been employed in the treatment of allergic diseases; but the therapeutic efficiency is to be improved. The present study modified the procedures of SIT by using full doses of specific Ags throughout the therapy period. Although treating with MSIT alone does not improve much the allergic inflammation in the intestine, treating with anti-IL-13 antibody together with MSIT significantly suppressed the intestinal allergic inflammation. The results have mirrored the importance of the antagonism of IL-13 in the suppression of allergic inflammation in the intestine. The underlying mechanism can be that the antagonism of IL-13 removes the IL-13 polarization environment, restores the production of TSP1 and to restore the induction of TolDC. The inference is supported by further experimental data that 1) treating the sensitized IL-13-null mice with MSIT significantly inhibited the allergic inflammation; 2) treating the sensitized TSP1-null mice with both MSIT and anti-IL-13 did not inhibit the inflammation.

The data also show that the frequency of TolDCs of FA mice is decreased in the intestine, which can be up regulated by SIT. The results suggest that IL-13 is not responsible for the decrease in TolDCs in the intestine of FA mice, but plays a critical role in the generation of TolDCs in an allergic environment. There are some other factors involving in the suppression of TolDCs under allergic environment; such as the T cell Ig mucin domain molecule-4 (TIM4)-expressing DCs are a strong promoter for the Th2 polarization; the TIM4⁺ DCs are increased in allergic disorders (31).

In summary, the present study has revealed that TSP1-producing B cells are required in the generation of TGF- β ⁺ TolDCs in the mouse intestine. B cell-derived TSP1 facilitates the generation of TolDCs by converting the LTGF β to TGF- β . The Th2 cytokine IL-13 induces the hypermethylation of TSP1 gene DNA in B cells, which interferes with the production of TSP1 to disturb the generation of TGF- β ⁺ TolDCs. Concurrent administration with MSIT and IL-13 antagonists restores the generation of TGF- β ⁺ TolDCs in an allergic environment and inhibits the allergic inflammation in the intestine.

Declare of the conflict of interest

None to declare.

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Author contribution

GY, XRG, ZQL, JQL, XYL, HPZ and YXS performed experiments, analyzed experimental data and reviewed the

manuscript. ZGL and PCY organized and supervised the experiments. PCY designed the project and wrote the manuscript.

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Figure legends

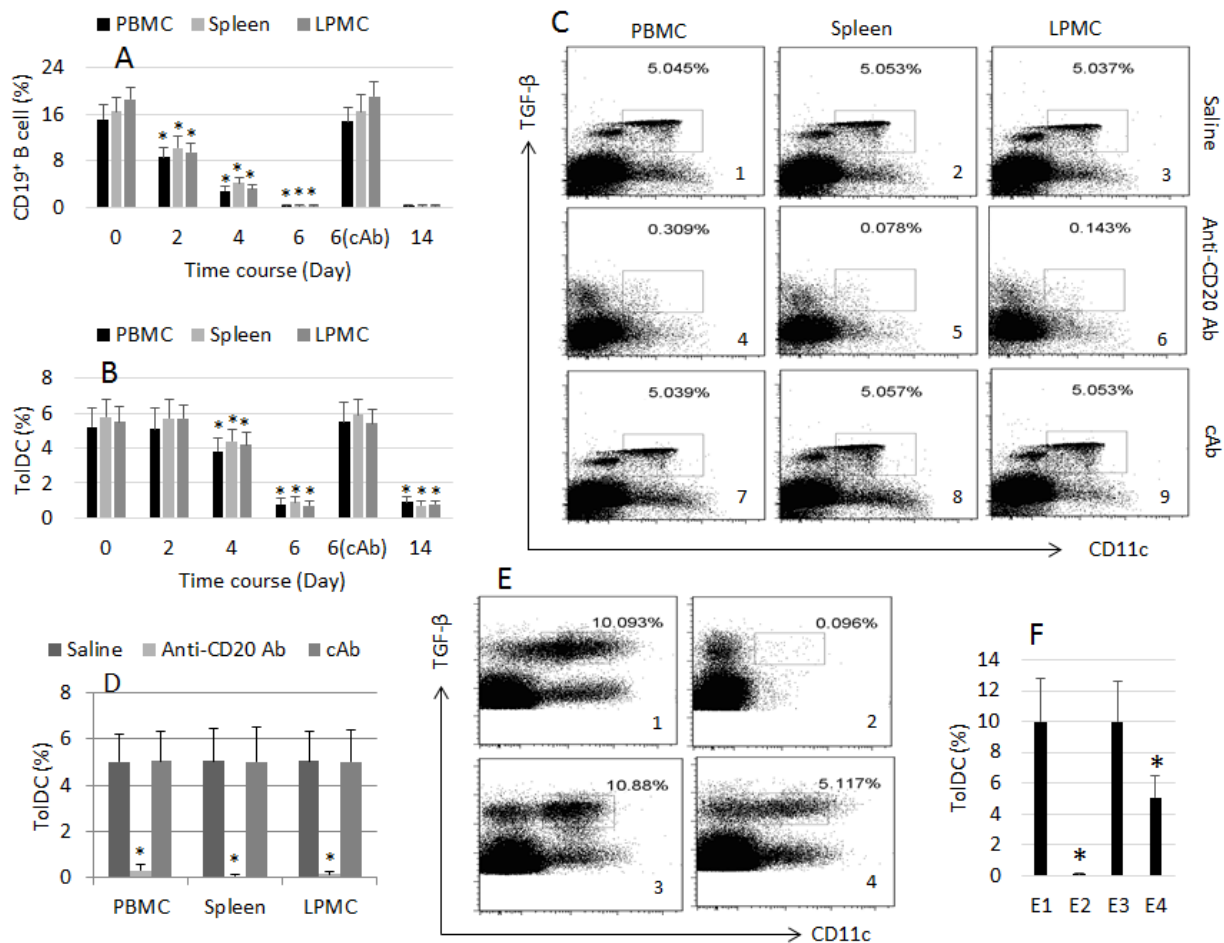


Figure 1. Role of B cells in the generation of TolDCs. A-B, naïve B6 mice were treated with an anti-CD20 pAb (10 mg/Kg body weight), or a control Ab (cAb; isotype IgG; 10 mg/Kg body weight), via tail vein injection. The mice were sacrificed on several time points from day 0 to day 14 as denoted on the X axis of panel A. PBMC (peripheral blood mononuclear cells), spleen cells and LPMC (lamina propria mononuclear cells) were prepared from the mice and analyzed by flow cytometry. The bars indicate the frequency of CD19⁺ B cells (A) or TGF- β ⁺ TolDCs (B). C-D, the mice were treated with anti-CD20 and sacrificed on day 7. The bars of D indicate the frequency of TGF- β ⁺ DCs in panel C. Panel D shows the flow cytometry dot plots of panel C. E, naïve mice (E1), B cell-deficient mice (E2), and cAb-treated mice (E3) were gavage-fed with OVA (5 mg/mouse in 0.3 ml saline) daily for 7 days to create a tolerant condition in the intestine. Another group mice was treated with saline using as controls (E4). The LPMC were prepared and analyzed by flow cytometry. The gated dot plots indicate the frequency of CD11c⁺ TGF- β ⁺ TolDCs. F, the bars indicate the summarized TolDC frequency in E. The data of bars are presented as mean $\pm$ SD. *, $p < 0.01$, compared with Day 0 (A, B), or saline group (E), or group D4 (F). Each group consists of 6 mice. Samples from individual mice were processed separately. The data represent 6 independent experiments.

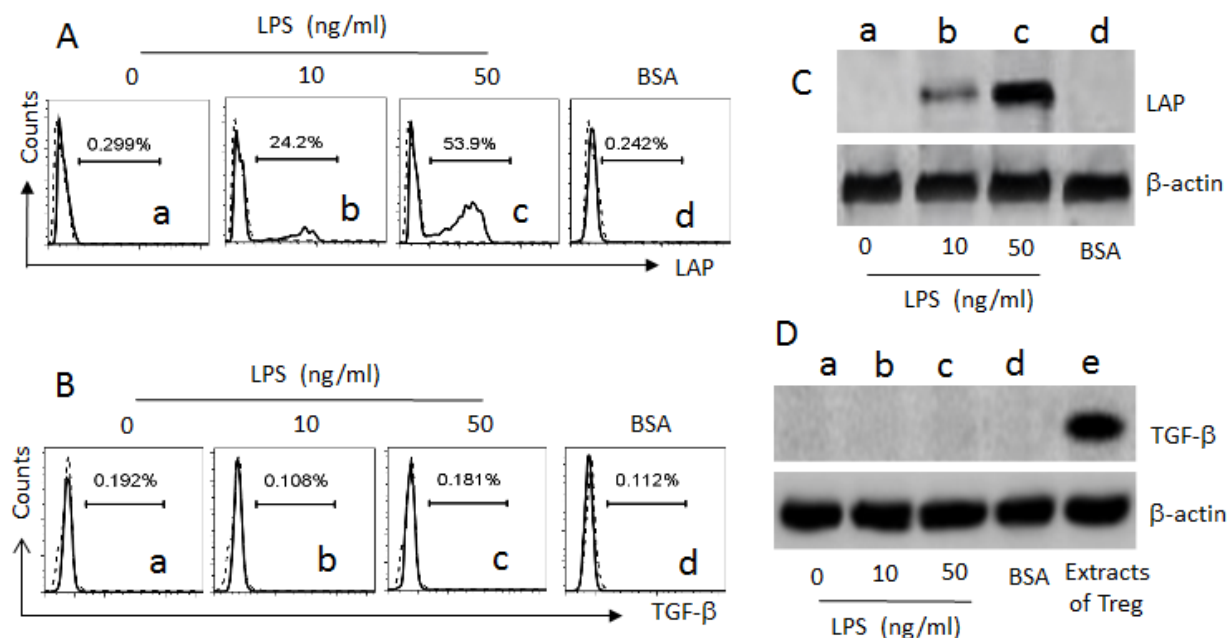


Figure 2. Induction of LTGFβ in DCs. A-B, BMDCs were cultured at 10^6 cells/ml in the presence of LPS (The doses are denoted below the blots) for 48 h; the DCs were designated activated DCs (aDC). The DCs were analyzed by flow cytometry and Western blotting respectively. The histograms indicate the frequency of the latency associated peptide (LAP)⁺ DCs (A) and TGF-β⁺ DCs (B) respectively. The treatment is denoted above the histograms. The immune blots indicate the levels of LAP (C) and TGF-β (D) in the cell extracts of the BMDCs. Extracts from CD4⁺ CD25⁺ Foxp3⁺ Tregs were used as a positive control. C, activated B cells were cultured with naive BMDCs at indicated ratios (DCs were at a fixed density of 10^6 cells/ml) for 48 h. Treatments were denoted below the blots. The cells were collected at the end of culture; B cells were negatively selected out by MACS. The data are representative of 3 independent experiments.

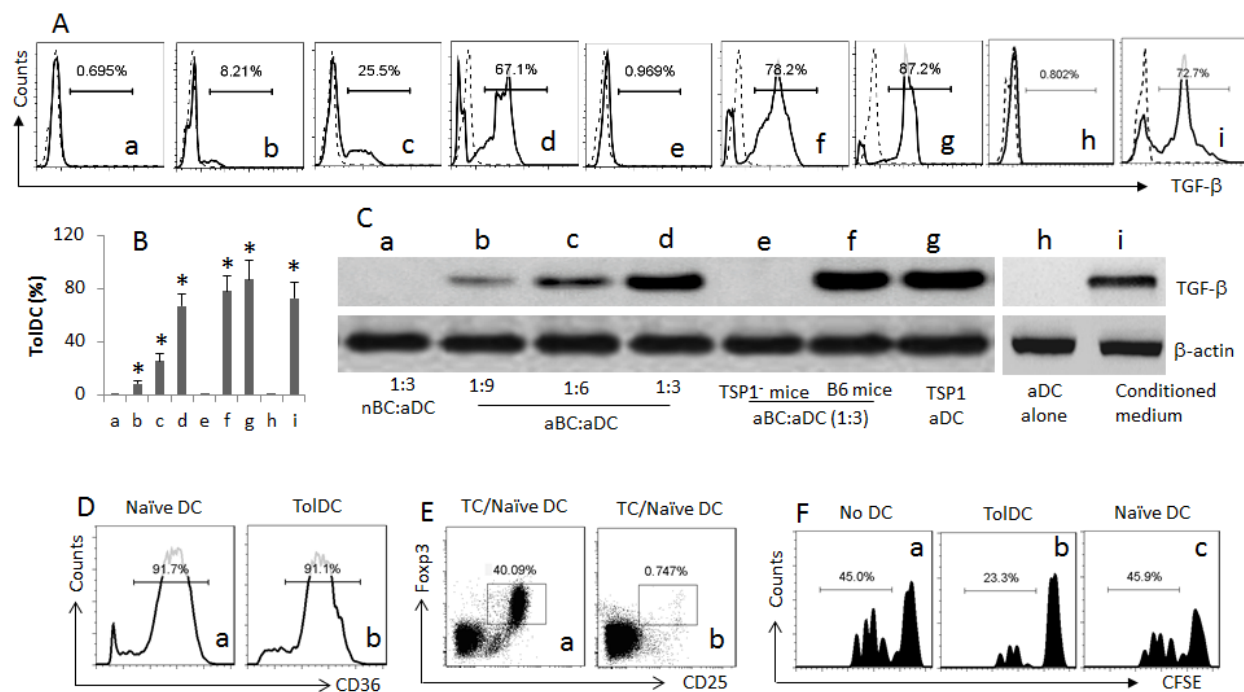


Figure 3. Induction of TolDCs. IgM-activated B cells were cultured with naïve BMDCs at indicated ratios (DCs were at a fixed density of 10^6 cells/ml) for 48 h. **A**, the cells were analyzed by flow cytometry. The frequency of TGF- β^+ DCs is shown by the histograms. **B**, the bars indicate the summarized data of Aa-Ai. **C**, the proteins were extracted from remained DCs (purity was 99%) and analyzed by Western blotting. The immune blots indicate the contents of TGF- β in DC extracts. The treatment is denoted below the Western blots. nBC: Naïve BC. aBC: Activated BC (stimulated by anti-IgM 10 μ g/ml for 6 h). TSP1: The cells were treated with recombinant TSP1 (10 μ g/ml). The histograms with broken lines present the negative control staining (with isotype IgG). **i**, DCs were treated with conditioned medium (B cells were activated by anti-IgM in the culture). **D**, naïve DCs and TolDCs were analysed by flow cytometry. The histograms indicate the frequency of CD36 $^+$ DCs. **E**, the gated dot plots show the frequency of Tregs induced by TolDCs (Ea) or naïve DC (Eb). The lower case letters in the histograms correspond to those above the immune blots. Each experiment was repeated 3 times. **F**, TolDCs were cultured with CD4 $^+$ CD25 $^-$ T cells (labeled by CFSE) at a ratio of 1:1 in the presence of anti-CD3/CD28 mAb. The cells were analyzed by flow cytometry. The histograms indicate the T cell proliferation. The data are representative of 3 independent experiments.

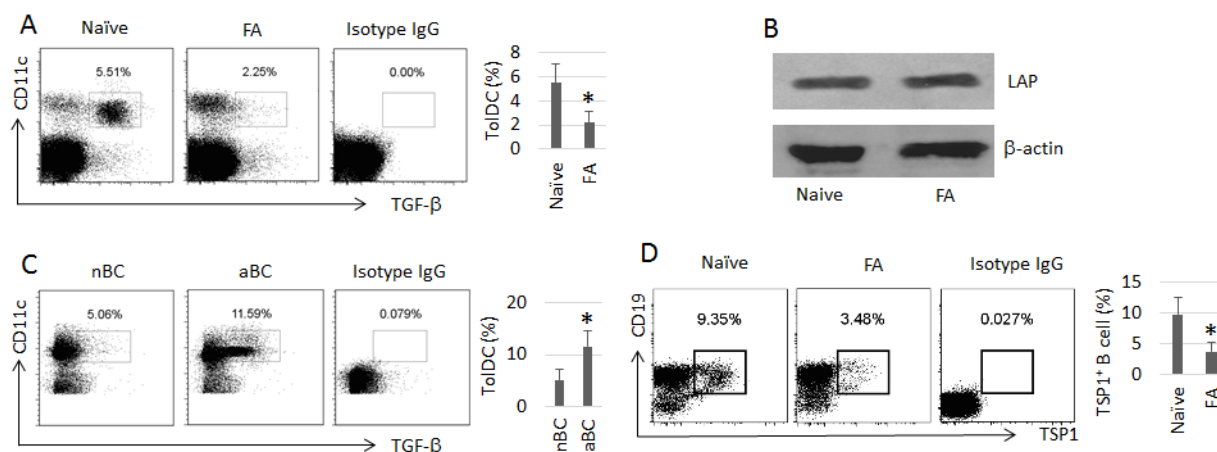


Figure 4. Assessment of TolDCs and TSP1⁺ B cells in FA mice. A, LPMCs were isolated from naïve mice (naïve) and mice with food allergy (FA). The gated dot plots indicate the frequency of TolDCs. B, the CD11c⁺ DCs were isolated from LPMCs of naïve mice and FA mice by MACS. Proteins were extracted from the isolated DCs and analyzed by Western blotting. The Western blots indicate the levels of the latency associated protein (LAP). C, CD11c⁺ DCs were isolated from the FA mouse small intestine and cultured with naïve B cells (nBC) or the anti-IgM-activated B cells (aBC) at a ratio of 1:3 (BC:DC) for 48 h. The gated dot plots indicate the frequency of TolDCs. D, LPMCs were isolated from naïve mice and FA mice, and analyzed by flow cytometry. The gated dot plots indicate the frequency of TSP1⁺ B cells. Isotype IgG: Cells were stained with isotype IgG using as negative staining controls. The bars indicate the summarized data of the dot plots. The data of bars are presented as mean $\pm$ SD. *, $p < 0.01$, compared with the naïve group (A, D) or the nBC group (C). Each experimental group consists of 6 mice. Samples from individual mice were processed separately. The data represent 6 separate experiments.

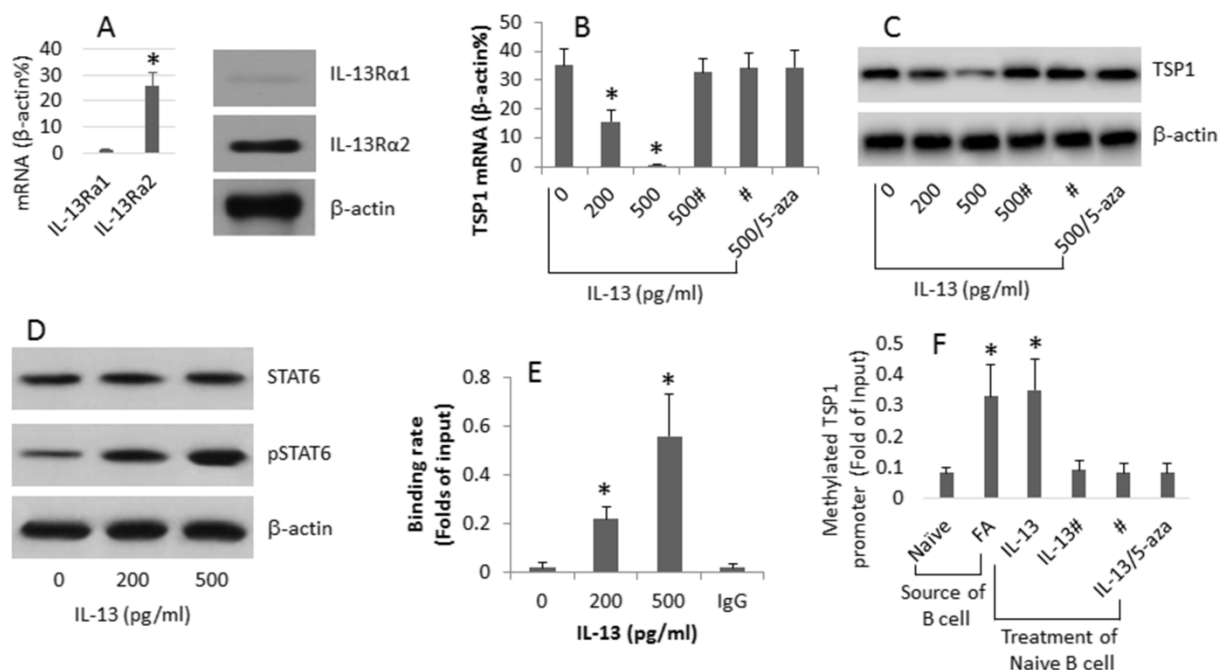


Figure 5. Assessment of TSP1 expression in B cells. A, CD19⁺ B cells were isolated from the mouse lamina propria and analyzed by RT-qPCR and Western blotting. The bars indicate the mRNA levels of IL-13Rα1 and IL-13Rα2. The Western blots indicate the protein levels of IL-13Rα1 and IL-13Rα2. B-E, the naive B cells were cultured for 48 h in the presence of anti-IgM (10 μg/ml) and IL-13 (at gradient concentrations) with or without of the anti-IL-13Rα2 antibody. The bars indicate the mRNA levels of TSP1 (B); the Western blots indicate the protein levels of TSP1 (C). D, the Western blots indicate the levels of STAT6 and pSTAT6 in the B cells. E, the bars indicate the binding rate between pSTAT6 and the TSP1 promoter. F, the bars indicate the levels of methylated DNA of the TSP1 gene. #, anti-IL-13Rα2 antibody (100 ng/ml) in the culture medium. Naive (or FA): CD19⁺ B cells were isolated from the lamina propria of naive mice (or mice sensitized to OVA). IL-13 concentration in the culture = 100 ng/ml. 5-aza: 5 μM. The data are presented as mean ± SD. *, p<0.01, compared with the IL-13a1 group (A), or the “0” group (B, C), or the naive group (D). The data represent 3 independent experiments.

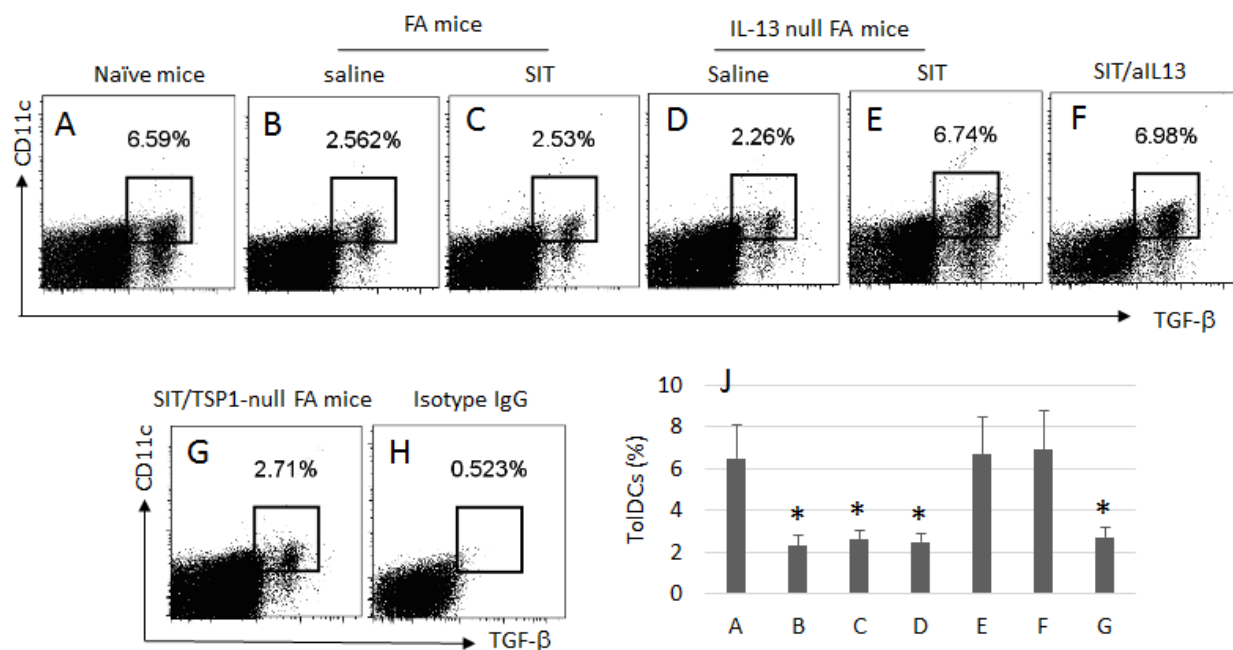


Figure 6. Assessment of TolDC in the intestine. Mice were sensitized to OVA and treated with OVA vaccines (Ag-specific immunotherapy; SIT) or/and anti-IL-13 mAb (aIL13). The treatments were denoted above each dot plot panel. LPMCs were isolated and analyzed by flow cytometry. The gated dot plots indicate the frequency of TGF-β⁺ TolDCs in LPMCs. H, a negative staining control. J, the bars show the summarized data of A-G (mean ± SD. *, p<0.01, compared with group A). Each group consists of 6 mice. Samples from individual mice were processed separately. The data represent 6 independent experiments.

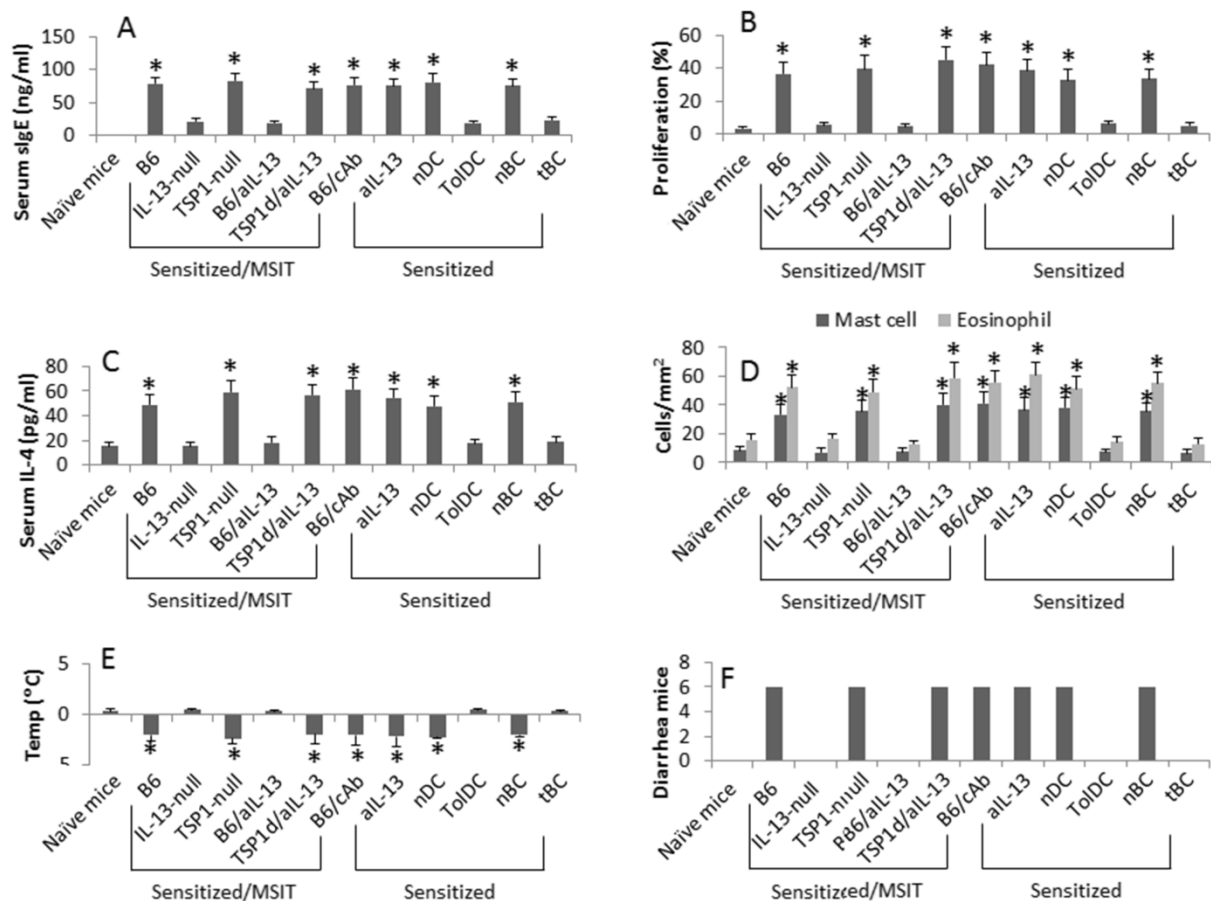


Figure 7. Assessment of allergic inflammation in the intestine. Mice were sensitized to OVA. The treatment was denoted on the X axis. The data are presented as mean $\pm$ SD. *, $p < 0.01$, compared with the naïve group. all-13: Anti-IL-13 antibody. B6d: Sensitized B6 mice were treated with anti-TGF- β antibody (0.25 mg/mouse/injection). cAb: Control antibody (non-specific IgG). nDC [or TolDC, or nBC (naive BC), or tBC (TSP1⁺ BC)]: Adoptive transfer with naive DC [or TolDC, or nBC (naive BC), or tBC (TSP1⁺ BC)]. Each group consists of 6 mice. Specimens from individual mice were processed separately. The data represent 6 separate experiments.

Immunology:

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environment**

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