Thrombospondin-1 (TSP1)-producing B Cells Restore Antigen (Ag)-specific Immune Tolerance in an Allergic Environment*

Received for publication, November 4, 2014, and in revised form, March 21, 2015 Published, JBC Papers in Press, April 3, 2015 DOI 10.1074/jbc.M114.623421

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Background: The generation of tolerogenic dendritic cells (TolDCs) 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 requires further investigation. 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 (TolDC) 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 methylation-specific PCR. The results showed that B cells were required in the generation of the TGF-β-producing TolDCs in mice. B cell-derived TSP1 converted the latent TGF-β to the active TGF-β in DCs, which generated TGF-β-producing TolDCs. Exposure to IL-13 inhibited the expression of TSP1 in B cells by enhancing the TSP1 gene DNA methylation. Treating food allergy mice with Ag-specific immunotherapy and IL-13 antagonists restored the generation of TolDCs 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 facilitated the generation of TolDCs and enhanced the therapeutic effect of immunotherapy.

The underlying mechanism of immune tolerance is proposed to be that the exposure to specific antigens (Ags)4 induces the generation of tolerogenic dendritic cells (TolDCs) and regulatory T cells (Tregs) (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 TolDCs and Tregs in the body (2). However, the generation of TolDCs is quite refractory in subjects with allergic disorders; the mechanism remains to be further elucidated.

TolDCs 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, etc. (3). Various subtypes of TolDCs play important roles in the induction and maintenance of immune tolerance in the body (3). One of the mediators by which TolDCs induce Tregs is TGF-β (4). TGF-β regulates multiple cell functions, including differentiation, migration, and 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 TolDCs can produce TGF-β to generate Tregs; however, after synthesis, TGF-β exists as a precursor, the latent TGF-β (LTGFβ), in the cells. LTGFβ has a latency-associated peptide (LAP) attaching to the TGF-β molecular complex. It is necessary to remove the LAP from the complex to convert the LTGFβ to TGF-β; how the conversion is carried out in DCs has not yet been well defined.

Thrombospondin 1 (TSP1) is a protein that in humans 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

* This work was supported by grants from the National Science Foundation of SZU (8000004), the Shenzhen Innovation of Science and Technology Commission (JCYJ20140141097533538 and JCYJ20140115090170019), and the Natural Science Foundation of China (81373176 and 31400856).

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which TSP1 plays a critical role by converting the LTGFβ to
TGF-β (10). Our previous studies show that the activated B
cells express TSP1 (11); whether the B cell-derived TSP1 con-
verts LTGFβ 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 are present. IL-13 is one of the Th2 type cyto-
kines, 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).
However, how B cell dysfunction in the induction of TolDCs
occurs in an allergic environment is unclear. Thus, B cells may
be a critical checkpoint in regulating the tolerogenic properties
of DCs. Although some reports suggest that B cells are not
important in the induction of immune tolerance (16), cumula-
tive 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 envi-
nronment 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, and the latter activated the LTGFβ 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 con-
current administration of specific Ag vaccines and IL-13
antagonists.

MATERIALS AND METHODS

Reagents—Antibodies of CD20 (H-170, polyclonal antibody),
CD11c (M-20), CD19 (R-20), TGF-β (D-12), LAP (T-17),
STAT6 (D-1), pSTAT6 (Tyr-641), and TSP1 (N-20) were pur-
blished by the Animal Ethics Committee at Shenzhen
University.

Isolation of Immune Cells—Single cells were isolated from
the intestine, mesenteric lymph nodes, 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
instructions. 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
μg/ml for 30 min. The cells were then washed with PBS three
times and analyzed by a flow cytometer (FACSCanto II, BD
Biosciences, Beijing, China).

Induction of TGF-β+ TolDCs in the Intestine—Following
published procedures (2), mice were gavage-fed 1 mg/ml
ovalbumin (OVA) daily for 7 days; control groups were
nontreated with saline alone. The frequency of TGF-β+ DCs
was assessed by flow cytometry.

CD19+ B Cell Depletion and CD11c+ TGF-β+ TolDC
Assessment—B cells were depleted in mice with an anti-CD20
polycyclonal antibody of a single 250-μg dose via tail vein injec-
tion (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, spleen cells,
and lamina propria mononuclear cells (LPMCs) 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 μg/ml) for 48 h. The frequency of
TGF-β+ TolDCs was more than 90% that of the CD11c+ DCs as
assessed by flow cytometry. The CD11c+ DCs were then isolated
by MACS and used as TolDCs in the further experiments.

Isolation of Peripheral Mononuclear Blood Cells—Mice were
decapitated, and the blood was collected immediately in a tube
precoated with heparin. The peripheral blood mononuclear
cells were isolated by gradient density centrifugation.

Cell Culture—The isolated immune cells were cultured in
RPMI1640 medium supplemented with 10% fetal bovine
serum, 100 units/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 cells were used for fur-
ther experiments.
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Western Blotting—Total proteins were extracted from isolated cells and separated by SDS-PAGE. 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) three 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 with a UVP BioSpectrum Imaging System (Upland, CA). The integrated density of the immunoblots was analyzed by Photoshop software version CS5. The results were expressed as a percentage of the immunoblots of the internal control β-actin.

Quantitative Real-time RT-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. Quantitative PCR was carried out using the MiniOpticon real-time PCR detection system with SYBR® Green Master Mixes (Invitrogen). The relative expression of mRNA was calculated as 2−ΔΔCT, the results were normalized to a percentage of the internal control β-actin. The primers of used in this study were as follows: TSP1, forward (ccaaagcctgcaagaaagac) and reverse (atgcgatgctgttccatgac); integrin 6, forward (tacggaagcatctacg-gcc) and reverse (tttctgcagacacacttgcc); integrin β6, forward (gcactgtgtcaactccaagg) and reverse (gtgacc) and reverse (tttctgcagacacacttgcc); integrin β6, forward (gcactgtgtcaactCCAagg) and 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 units/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 days 6–8; at this stage, >95% cells expressed CD11c, and less than 20% cells expressed CD80 and CD86, indicating that these cells were in the immature state.

Food Allergy (FA) Mouse Model—Following published procedures (21) with minor modification, mice were gavage-fed with OVA (1 mg/mouse) mixed with cholera toxin (20 μg/mouse) in 0.3 ml of saline once per 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 Antibody Ab—Some FA mice were treated with anti-IL-13 mAb (100 μg/mouse, intraperitoneal) or an isotype IgG (control Ab) every other day four 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 of 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, naive B cells, or TSP1+ BCs at 10^6 cells/mouse via tail vein injection at week 0, 2, and 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 μg of DNA were denatured with 2 m NaOH at 37 °C for 10 min and incubated with 3 m sodium bisulfite (pH 5.0), overlaid with mineral oil to cover the surface of the aqueous phase, and incubated at 50 °C overnight. The samples were incubated with 3 m NaOH for 5 min, concentrated by ammonium precipitation and 100% ethanol, washed with 70% ethanol, and resuspended in 20 μl of distilled water.

The methylation status of the TSP1 promoter was then determined by methylation-specific PCR. The methylated and unmethylated DNA sequences were distinguished by specific PCR. The primer sequences for the methylated form were TATTTTATAGTTGTTGGGGAGAATAAC (sense) and TAA-CCTTCTAATACGAAAGCAAC (antisense) (134 bp), and the primer sequences for the unmethylated form were GTATTTTAGAGTTGTTGGGGAGATAAAT (sense) and TAA-CCTTCTAATACGAAAGCAAC (antisense) (135 bp). Genomic DNA, methylated in vitro by CpG methyltransferase (SssI) following the manufacturer’s directions (New England Biolabs, Inc., Beverly, MA), was used as a positive control. A water blank was used as a negative control.

Activation of Naive B Cells—The CD19+ IL-7R+ CD45R+B cells were isolated from the mouse bone marrow by magnetic cell sorting 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 a protein A/G-agarose/salmon sperm DNA slurry, and the immunoprecipitation was carried out using an anti-STAT6 antibody or normal mouse IgG (used as a negative control) overnight at 4 °C with mild agitation. The complexes of antibody-chromatin were collected by incubation with protein A/G-agarose and analyzed by quantitative PCR. For the STAT6 binding sites in the TSP1 promoter, the primers were as follows: forward, ggttcagggaggctctgct; reverse, atcccctttgcaactggg. 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 three times. The data are presented as mean ± S.D. The data were analyzed using analysis of variance, and the means were compared by the Student-Newman-Keuls test. p < 0.05 was set as the criterion for significance.
RESULTS

B Cells Are Required in the Generation of TGF-β+/H9252/H11001 TolDCs—To determine the role of B cells in the generation of TGF-β+/H9252/H11001 TolDCs, we treated mice with anti-CD20 Abs 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+/H9252/H11001 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. 1, B–D). The injection of a control Ab did not apparently alter the frequency (p > 0.05) of either B cells or TolDCs (Fig. 1, A and B). The results imply that B cells are required in the generation and/or maintenance of TGF-β+/H9252/H11001 TolDCs. To strengthen the results, we fed B6 mice with OVA daily for 7 days. As analyzed by flow cytometry, the frequency of TGF-β+/H9252/H11001 TolDCs was increased in the intestine of the B cell-sufficient mice, but not in the B cell-deficient mice (Fig. 1, E and F). The results suggest that depletion of B cells reduces the frequency of TGF-β+/H9252/H11001 TolDCs.

B Cell-derived TSP1 Maintains the Tolerogenic Properties of DCs—The data of Fig. 1 show that a population of DCs produces TGF-β in response to proper stimulation. Because TGF-β exists as a precursor, LTGFβ, after synthesis, the LTGFβ must have been converted to TGF-β somehow in the present experimental system. Thus, we next measured the levels of LTGFβ in DCs after activation. Naive BMDCs were prepared and conditioned in no-serum medium overnight; the conditioned BMDCs were exposed to LPS (used as a DC activator) in the culture for 48 h. The data showed that LTGFβ was induced in the BMDCs (Fig. 2, A and C), but no TGF-β was
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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. 3, A (a), B, and C (a)); the activated B cells increased the levels of TGF-β in BMDCs in a B cell number-dependent manner (Fig. 3, A (b–d), B, and C (b–d)). Based on the fact 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. 3, A (e and f), B, and C (e and f)). We then added recombinant TSP1 to the DC culture, which markedly increased the levels of TGF-β in the DCs (Fig. 3, A (g), B, and C (g)), which did not occur when DCs were cultured alone (Fig. 3, A (h), B, and C (h)). Culture in conditioned medium (B cells were activated by anti-IgM in the culture) also induced TolDCs (Fig. 3, A (i), B, and C (i)). More than 90% of naïve 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 activated B cells release TSP1 to facilitate the generation of TolDCs. In addition, we also observed that the TolDCs had immune suppressive functions on CD4+ T cell proliferation (Fig. 3F).

IL-13 Compromises the Tolerogenic Properties of DCs via Suppressing TSP1 in B Cells—Whether allergic disorders disturb the generation of TolDCs has not been well defined. In an FA mouse model, we observed a much lower (p < 0.01) frequency of TGF-β+ TolDCs in the intestine of FA mice than in saline control mice (Fig. 4A). To elucidate whether the DCs in the allergic mouse intestine still produce LTGFβ, we extracted proteins from isolated DCs to be analyzed by Western blotting. The results showed that the levels of LTGFβ in DC extracts of FA mice were not significantly different from those of control mice (Fig. 4B). The results imply that the LTGFβ-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, fewer TSP1+ CD19+ B cells were detected in the intestine of FA mice as compared with 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. Because there are two subtypes of IL-13 receptor (IL-13R), we first 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-13Rα2 at both mRNA and protein levels, whereas only negligible expression of IL-13Rα1 was detected in the B cells (Fig. 5A).

We then observed the effect of IL-13 on regulation of 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-13Rα2 antibody (Fig. 5, B and C).

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 the 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 in naive mice (Fig. 5F). To strengthen the data, we stimulated naive B cells with anti-IgM and LPS to induce IL-13 production (Fig. 5F). The results showed that the induction of IL-13 increased the frequency of TGF-β+ TolDCs (Fig. 5F). The TolDCs showed the ability to generate Tregs (Fig. 5E). The results suggest that under an allergic environment, B cells produce less TSP1 in the intestine.

FIGURE 2. Induction of LTGFβ in DCs. A and 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 in the blots. The immunoblots 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 naïve BMDCs at the 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 three independent experiments.
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-13Rα2 antibody or the presence of a methylation inhibitor (Fig. 5, B, C, and F).

**IL-13 Antagonists Promote the Effect of Specific Immunotherapy via Generation of TGF-β⁺ TolDCs**—The results of Fig. 5 imply that IL-13 may be an obstacle in the generation of TolDCs and that overcoming the effect of IL-13 may facilitate

**FIGURE 3. Induction of TolDCs.** IgM-activated B cells were cultured with naive BMDCs at the indicated ratios (DCs were at a fixed density of 10⁶ cells/ml) for 48 h. A, the cells was analyzed by flow cytometry. The frequency of TGF-β⁺ DCs is shown by the histograms. B, the bars indicate the summarized data of A (a–i). Error bars, S.D. C, the proteins were extracted from remaining DCs (purity was 99%) and analyzed by Western blotting. The immunoblots indicate the contents of TGF-β in DC extracts. The treatment is denoted below the Western blots. nBC, naive BC. aBC, activated BC (stimulated by anti-IgM 10 μg/ml for 6 h). TSP1, the cells were treated with recombinant TSP1 (10 μg/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, naive DCs and TolDCs were analyzed by flow cytometry. The histograms indicate the frequency of CD36⁺ DCs. F, TolDCs were cultured with CD4⁺ CD25⁺ T cells (labeled by carboxyfluorescein succinimidyl ester (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 three independent experiments.

**FIGURE 4. Assessment of TolDCs and TSP1⁺ B cells in FA mice.** A, LPMCs were isolated from naive mice (naive) and mice with food allergy (FA). The gated dot plots indicate the frequency of TolDCs. B, the CD11c⁺ DCs were isolated from LPMCs of naive 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 LAP. C, CD11c⁺ DCs were isolated from the FA mouse small intestine and cultured with naive 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 TSP1⁺ B cells. Isotype IgG, Cells were stained with isotype IgG used as negative staining controls. The bars indicate the summarized data of the dot plots. The data of bars are presented as mean ± S.D. (error bars). *, p < 0.01, compared with the naive group (A and D) or the nBC group (C). Each experimental group consists of six mice. Samples from individual mice were processed separately. The data represent six separate experiments.
the restoration of generation of TolDCs to attenuate or inhibit allergic inflammation. To test this hypothesis, we developed an FA mouse model. Far fewer TGF-β+ DCs were observed in the intestine of FA mice than in the naive control mice (p < 0.01; Fig. 6, A, B, and F). The FA mice were treated with MSIT in a short period (6 days). However, the frequency of TolDCs in the intestine was not apparently increased (Fig. 6, C and J). 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 compared with mice treated with saline (Fig. 6, D, E, and J). Treating the sensitized B6 mice with MSIT and anti-IL-13 antibodies also generated TolDCs in the intestine (Fig. 6, F and J). On the other hand, treating the sensitized TSP1-null mice with MSIT did not generate TolDCs in the intestine (Fig. 6, G and J). 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. Because 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 TolDC or TSP1+ BC to mice, which abolished the allergic response in the intestine (Fig. 7, A–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 LTGFβ 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 microenvironment. Coculture of the TSP1-producing B cells and LTGFβ-producing DCs generates the TGF-β+ TolDCs; the latter are 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 of 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 mature to induce Th1 response or Th2 response or become TolDCs, depending on the cytokine environment. TolDCs play an important role in immune tolerance by generating Tregs (24). Because all of the activities of the three types of DC are useful in the maintenance of homeostasis in the body, dysfunction or polarization of any one of them is harmful. 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+ TolDC development. The data are in line with previous reports. Tang et al. (25) reported that TSP1 activates TGF-β via its proteolytic activity, and Hayashi et al. (26) reported that a deficiency of TSP1 significantly reduces the expression of TGF-β in hepatocytes. Our previous data show that TSP1 cleaves the surface CD80 and CD86 of DCs, which 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 finding 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). A 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 far fewer TGF-β+ TolDCs than in control mice. This fact suggests that the generation of TGF-β+ TolDCs is disturbed in the FA mice, which must possess inhibitors of TolDCs. This inference is supported by further experimental data, in which 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 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 a therapeutic benefit for patients with immune diseases (29, 30). The present data suggest that under an allergic environment or in Th2 polarization, the high levels of IL-13 prevent the expression of TSP1 in B cells, which suppresses the expression of TSP1 and disturbs 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 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 its therapeutic efficiency must be improved. The present study modified the procedures of Ag-specific immunotherapy (SIT) by using full doses of specific Ags throughout the therapy period. Although treating with MSIT alone does not significantly improve the allergic inflammation in the intestine, treating with anti-IL-13 antibody together with MSIT significantly

FIGURE 6. Assessment of TolDC in the intestine. Mice were sensitized to OVA and treated with OVA vaccines (SIT) and/or anti-IL-13 mAb (aIL13). The treatments are 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 ± S.D. (error bars); *, p < 0.01, compared with group A). Each group consists of six mice. Samples from individual mice were processed separately. The data represent six independent experiments.
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 could be that the antagonism of IL-13 removes the IL-13 polarization environment, restores the production of TSP1, and restores the induction of TolDC. This inference is supported by further experimental data showing that 1) treating the sensitized IL-13-null mice with MSIT significantly inhibited the allergic inflammation, and 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 and 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 involved in the suppression of TolDCs in an allergic environment, such as the TIM4 (T cell Ig mucin domain molecule-4)-expressing DCs, which 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 LTGFβ 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.

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