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TSP1-producing B cells show immune regulatory property and suppress allergy-related mucosal inflammation

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Specific immunotherapy (SIT) is the only specific remedy for the treatment of allergic diseases currently. B cells are important immune cells in the immunity. The role of B cells in immune regulatory activities has not been fully understood yet. This study aims to elucidate the role of the thrombospondin (TSP)1-producing B cells in the immune regulatory role of SIT. The results showed that after SIT, the frequency of CD35$^+$ B cells was increased in the intestine of mice with food allergy. The CD35$^+$ B cells expressed TSP1 after exposure to specific antigens. Co-culture with the TSP1-producing CD35$^+$ B cells decreased the levels of CD80/CD86 in dendritic cells; the cells convert naïve CD4$^+$ T cells to regulatory T cells to inhibit allergic inflammation in the intestine.

The major functions of B cells include the production of antibodies against specific antigens and the presentation of antigens to T cells[1]. Recent studies have revealed that B cells also have immune regulatory functions[2]. Antigen specific B cells can directly recognize specific antigens. The findings of the immune suppressor functions of B cells have greatly expanded the interest scope of immunologists[3,4]. Similar to the immune suppressor feature of regulatory T cells (Treg), the production of interleukin (IL)-10 or transforming growth factor (TGF)-β has been noted in regulatory B cells[5]. Still, much remains to be understood in the immune regulatory functions of B cells, such as to elucidate the immune regulatory molecules by which tolerogenic B cells modulate immune activities of other immune cells.

Antigen specific immunotherapy (SIT) is the only specific method for the treatment of allergic diseases currently. SIT can suppress ongoing allergic symptoms, activities of antigen specific effector immune cells and up-regulate Tregs and regulatory B cells[6]. Yet, how SIT regulates the tolerogenic properties of B cells is not fully understood yet.

Thrombospondin (TSP), which consists of five extracellular calcium-binding multifunctional proteins: TSP1, TSP2, TSP3, TSP4, and TSP5, was first reported as a component of the α-granule in platelets[7,8]. TSP1 is the best-studied member of the TSP family. A number of normal cells, including endothelial cells, adipocytes, fibroblasts, smooth muscle cells, macrophages, monocytes, and transformed cells such as malignant glioma cells, dendritic cells (DC) and B cell lymphomas, secrete TSP1[9,10]. TSP1 is expressed upon activation of the cells, such as in response to tissue damage or stress[11]. TSP1 can bind latent TGF-β to generate biologically active TGF-β. TSP1 is also involved in the regulation of apoptosis[10]. TSP1-deficient mice are prone to suffering from immune inflammation[8]. The administration of recombinant TSP1 can inhibit allergic disorders[12]. These studies imply that TSP1 may be involved in the immune regulation of the body. However, whether the disturbance of TSP1 expression plays any role in the pathogenesis of allergic disorders is unclear.

CD35 is a monomeric single-pass type I membrane glycoprotein found in a number of cells including erythrocytes, leukocytes, glomerular podocytes, splenic follicular dendritic cells, B cells, thymocytes, monocytes, macrophages, neutrophil, eosinophils and Kupffer cells[13]. The ligands of CD35 include complement C3, C4, C3b, iC3b and C4b. The functions of CD35 include acting as a regulator of complement activation, a cofactor for the Factor I–mediated cleavage of C3b and C4b and as an inhibitor of convertases[14]. CD35 is required in the

development of memory B lymphocytes[15]. The murine $CD35^+$ $CD80^+$ B memory cells were described recently[16]; their role in immune regulation has not yet been fully understood.

The costimulation plays an important role in the T cell activation. CD80 and CD86 expressed on the surface of DCs are the major costimulatory molecules for T cell activation. The amount of costimulatory molecules on the surface of DCs is crucial to determine the subsequent immune activities to switch to immune tolerance or immune activation[17]. If DCs are in the semi-mature state, they may induce immune tolerance[5,18]. The term of the semi-mature of DCs is demonstrated by the low levels of costimulatory molecules on surface of DCs[5,18]. However, factors regulating the amount of costimulatory molecules on the surface of DCs are not fully demonstrated. Based on the above information, we hypothesize that TSP1-producing B cells may play a role in the immune regulation during SIT. In the present study, we observed that the $CD35^+$ B cells expressed TSP1 that was upregualted by SIT; the levels of the costimulatory molecules on the surface of DCs could be regulated by TSP1 derived from antigen specific $CD35^+$ B cells, which could be induced by SIT.

Results

SIT modulates phenotypes of antigen-specific B cells. Published data indicate that CD80 and CD35 can be expressed by the antigen specific B cells[16,19]. In this study, we characterized the $CD19^+$ (a B cell marker) $CD80^+$ B cells and $CD35^+$ B cells in the intestine of a food allergy mouse model. The mice were sensitized to ovalbumin (OVA) and then treated with or without SIT. The mice were sacrificed one week after the last treatment. The lamina propria mononuclear cells (LPMC) were isolated and analyzed by flow cytometry. The frequency of $CD19^+$ B cells was markedly increased in sensitized mice than naïve control mice (Fig. 1A). We then isolated the $CD19^+$ B cells from the intestine by magnetic cell sorting (MACS), labeled with carboxyfluorescein succinimidyl ester (CFSE) and cultured in the presence of the specific Ag (OVA) for 3 days. The $CD19^+$ B cells from the sensitized mice, treated with either saline or SIT, proliferated markedly while those from naïve mice did not (Fig. 1B). Further analysis showed that the phenotypes of the proliferating $CD19^+$ B cells were $CD80^+$ $CD35^-$ (41.2%), $CD80^+$ $CD35^+$ (19.7%) and $CD80^-$ $CD35^+$ (21.0%) in sensitized mice. After treating with SIT, the phenotypes of B cells were altered dramatically to be $CD80^+$ $CD35^-$ (10.8%), $CD80^+$ $CD35^+$ (16.4%) and $CD80^-$ $CD35^+$ (49.2%) (Fig. 1C–D). The results indicate that the Ag-specific B cells have developed in the intestine after the sensitization, which can be activated upon re-exposure to specific Ag. Treating with SIT can alter the phenotypes of antigen specific B cells.

Antigen-specific B cells produce TSP1 upon activation. TSP1 is an immune regulatory molecule[7], which may contribute to the development of immune tolerance. Since one of the mechanisms by which SIT-induced immune tolerance contributes to suppression of the ongoing allergic inflammation[20], we hypothesize that SIT increases the production of TSP1 in the antigen specific B cells. We then assessed the expression of TSP1 in the B cells by flow cytometry. The results showed that TSP1 was detected in 4.27% $CD35^-$ $CD80^+$ B cells, 26.1% $CD80^+$ $CD35^+$ B cells and 89.7% $CD80^-$ $CD35^+$ B cells (Fig. 2A), which was not only observed in the intestine, but in the mesentery lymph nodes and the spleen as well (Fig. 2B). The expression of TSP1 by the B cell subtypes was further confirmed by the assessment of qRT-PCR (Fig. 2C). To elucidate if

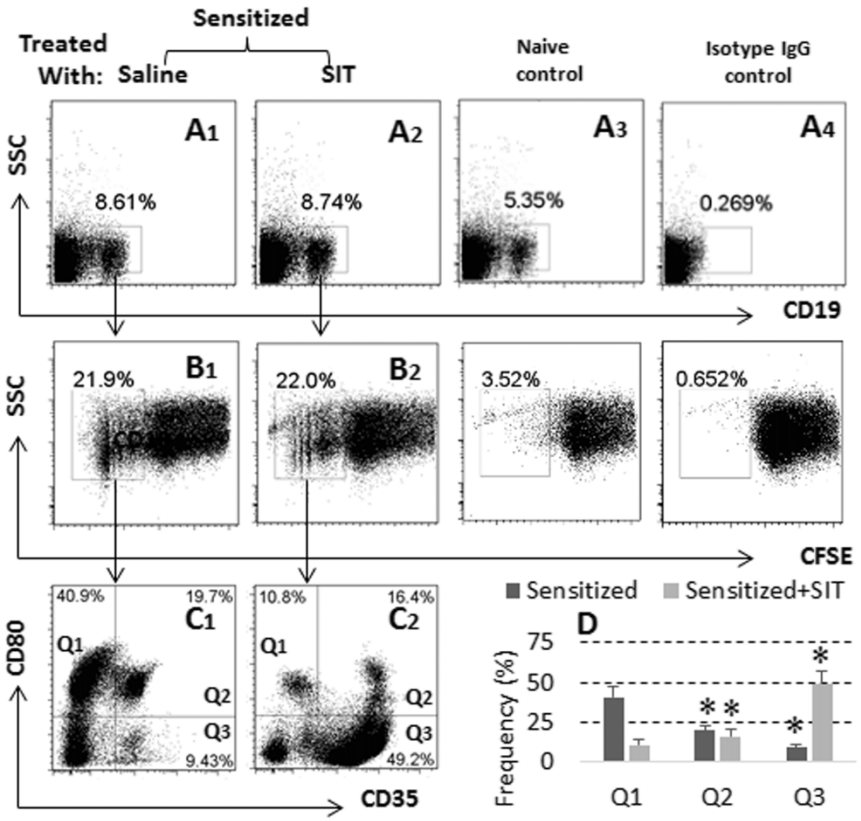

Figure 1 | Specific Ags modulate Bmcs' phenotypes. BALB/c mice were sensitized to OVA and treated with SIT or saline. LPMCs were isolated. (A), the dot plots indicate the frequency of $CD19^+$ B cells (the gated cells). (B), $CD19^+$ B cells were isolated from the LPMCs by magnetic cell sorting (MACS), labeled with CFSE and cultured in the presence of the specific Ag (OVA) at 1 μg/ml for 3 days. The dot plots show the frequency of the proliferating $CD19^+$ B cells. (C), the dot plots show the phenotypes of the activated $CD19^+$ B cells (gated $CD80^+$ or/and $CD35^+$ cells) in (B). (D), the bars indicate the frequency of TSP1 in the B cell subtypes (the labels on the X axis are the same as that in panel C). The data represent 3 separate experiments.

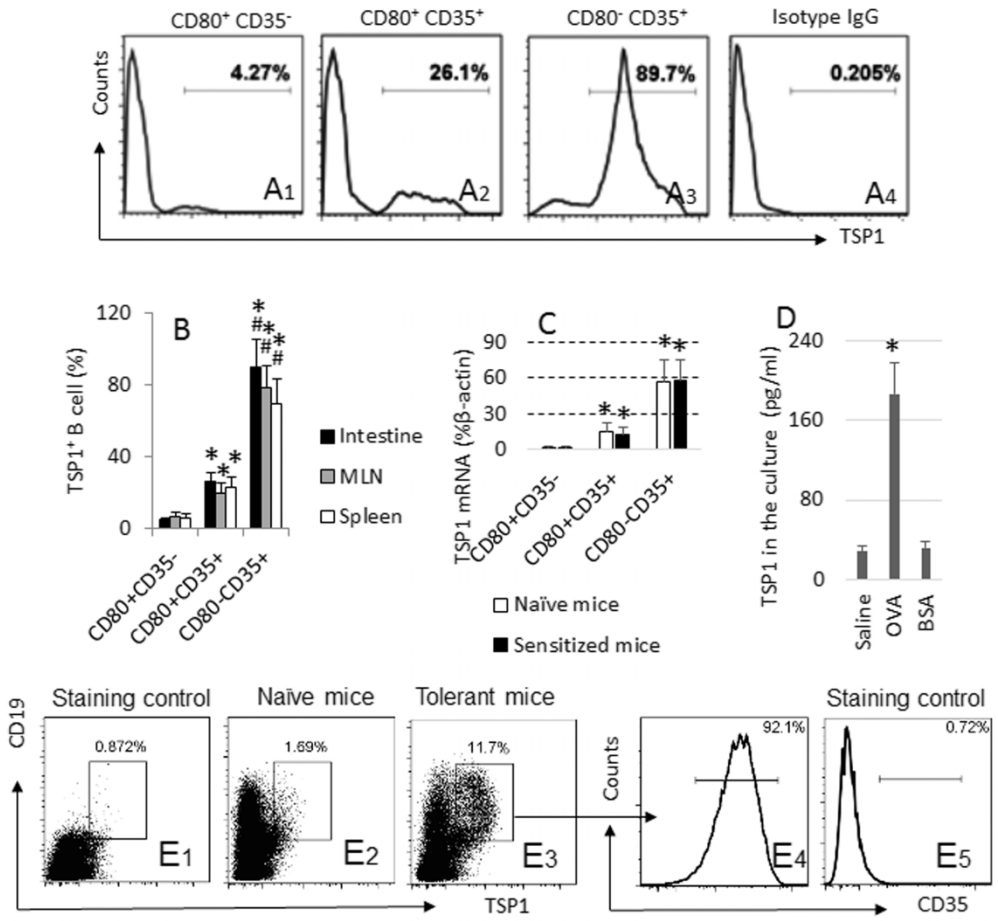

Figure 2 | CD19$^+$ CD35$^+$ B cells produce TSP1. (A), cells in Fig. 1C were further analyzed with gating technique. The histograms indicate the frequency of TSP1 cells. (B), cells were isolated from the spleen and mesentery lymph nodes; phenotypes of B cells (denoted on the X axis) were analyzed in the same procedures of Fig. 1C. The bars indicate the frequency of TSP1$^+$ B cells (the item of ''intestine'' was the summarized data of panel A). (C), phenotypes of B cells (denoted on the X axis) were isolated by MACS and analyzed by qRT-PCR. The bars indicate the mRNA levels of TSP1. (D), the bars indicate the levels of TSP1 in culture supernatant (measured by ELISA), in which the CD19$^+$ CD35$^+$ B cells were isolated from the OVA-tolerant mouse intestine and cultured in the presence of OVA or BSA (control) for 3 days. E, the LPMCs were isolated from tolerant mice and analyzed by flow cytometry. (E1–E3), the dot plots indicate the frequency of CD19$^+$ TSP1$^+$ B cells. (E4–E5), the histograms indicate the frequency of CD35$^+$ B cells in the gated cells (E4 is from E3; E5 is from E1). The data represent 3 separate experiments.

the TSP1 can be released to the microenvironment, we measured the levels of TSP1 in the culture medium, in which CD19$^+$ CD35$^+$ B cells (isolated from the OVA-tolerant mouse intestine; the mice were fed with OVA at 5 mg/mouse daily for 7 days) were cultured in the presence of OVA or BSA for 3 days. As shown by ELISA data, the levels of TSP1 in the supernatant were detectable in the saline group, which were markedly increased when the cells stimulated by OVA, but not in those stimulated by BSA (Fig. 2D). The results indicate that exposure to specific antigen can induce TSP1 release from CD35$^+$ B cells. In addition, treating mice with a specific antigen, OVA, daily for 7 days also increased the CD35$^+$ TSP1$^+$ B cells in the intestine (Fig. 2E). Since TSP1 has the immune regulatory property, the CD35$^+$ TSP1$^+$ B cells may be a subset of tolerogenic B cells, which was further explored as presented below.

SIT-induced CD35$^+$ B cells to modulate costimulatory molecule levels on the surface of DCs. The amount of costimulatory molecules on the DC surface is one of the indicators of the tolerogenicity of DCs[21]. The fact that TSP1 can cleave cell surface proteins[7] implies that the B cell-derived TSP1 may be a regulator of the amount of costimulatory molecules on the surface of DCs. To test the hypothesis, CD19$^+$ B cells were isolated from the intestine of OVA-sensitized mice with or without treating with SIT and isolated to be CD35$^+$ and CD35$^-$ B cells respectively (the TSP1$^+$ cells were 89.7% of the CD35$^+$ B cells as checked by flow cytometry). DCs were isolated from the naïve mouse spleen and pulsed by lipopolysaccharide (LPS; 100 ng/ml, overnight) in the culture to boost the expression of the costimulatory molecules, CD80 and CD86. CD35$^+$ B cells (or CD35$^-$ B cells) and DCs were co-cultured overnight in the presence or absence of the specific antigen (OVA). The results showed that the exposure to the activated CD35$^+$ B cells significantly reduced the frequency of CD80$^+$ DCs (Fig. 3A1–A4). The exposure to BSA, an irrelevant antigen, did not alter the amount of costimulatory molecules from DCs (Fig. 3A5). To elucidate the role of TSP1 in the reduction of CD80 from DCs, the TSP1 inhibitor, peptide LSKL, was added to the culture, which blocked the reduction of CD80 in the DCs (Fig. A6–A7). The suppressor effect of CD35$^+$ B cells was also observed on CD86 in DCs (Fig. S1A). In contrast to CD35$^+$ B cells, the CD35$^-$ B cells did not have any effect on the levels of CD80/CD86 in DCs (Fig. S1B). The results were confirmed by Western blotting data (Fig. 3B) and by exposing the LPS-pulsed DCs to recombinant TSP1 in the culture (Fig. S1C–D). To clarify if the effect of TSP1 on the suppression of CD80/CD86 in DCs was to suppress their gene expression, DCs were isolated by MACS and analyzed by qRT-PCR. However, the treatment did not disturb the gene expression of CD80/CD86 in DCs (Fig. 3C). The data indicate that the CD35$^+$ B cell-derived TSP1 can reduce the amount of costimulatory molecules in DCs; but does not alter the gene transcription in the cells. The results implicate that

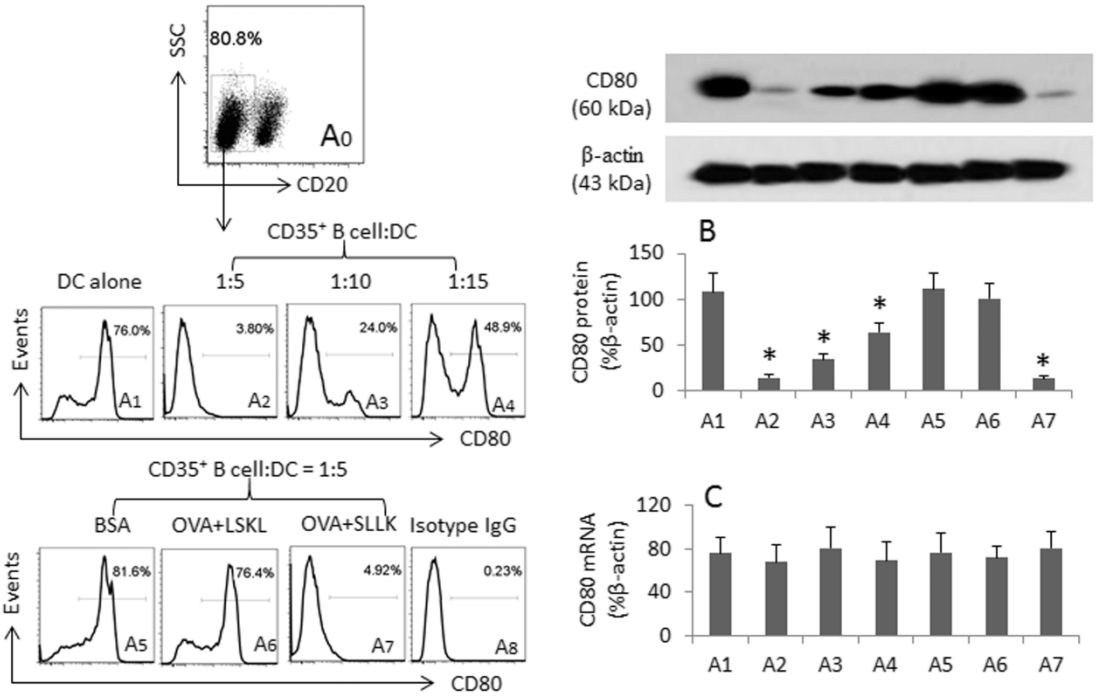

Figure 3 | TSP1 cleaves costimulator molecules. The $CD20^+$ $CD19^+$ $CD35^+$ B cells were isolated from the OVA-sensitized mouse intestine by MACS. The $CD11c^+$ DCs were isolated from the naïve mouse spleen by MACS (the $CD35^+$ DCs were selected out) and cultured with LPS (500 ng/ml) for 48 h. The $CD35^+$ B cells were co-cultured with LPS-pulsed DCs (10^5 cells/ml) at the indicated ratios in the presence of OVA (1 μg/ml) for 24 h. The cells were analyzed by flow cytometry. (A0), the $CD20^+$ B cells were gated out first; the remained DCs were further analyzed. The histograms indicate the frequency of $CD80^+$ DCs. The treatment of (A1–A7) was annotated above each histogram. (B–C), the CD35− DCs were selected by MACS (the purity was >98%); the cell extracts were analyzed by Western blotting and qRT-PCR. B, the immune blots indicate the levels of CD80; the bars below the blots show the summarized integrated density of the blots. (C), the bars indicate the levels of CD80 mRNA. The labels on the X axis of (B) and (C) are the same as those in panel A. LSKL: TSP1 inhibitor (10 μg/ml). SLLK: A control peptide of LSKL. BSA: The antigen specific $CD35^+$ B cells were treated with BSA (using as an irrelevant antigen) instead of the specific antigen (OVA). The data represent 3 separate experiments.

$CD35^+$ B cells may contribute to maintaining the tolerogenic properties of DCs, in which TSP1 plays an important role. In addition, to understand if the cell-cell contact is required in the suppression of costimulatory molecules on DCs by the $CD35^+$ B cell-derived TSP1, in separate experiments, LPS-primed DCs and $CD35^+$ B cells were physically separated in Transwells. The results showed that the amount of costimulatory molecules was still suppressed (Fig. S2A–D). The results indicate that the cell-cell contact is not required in the $CD35^+$ B cell-reducing CD80 amounts on DCs. On the other hand, the supernatant of $CD35^+$ B cells also showed a suppressive effect on the CD80 amounts on DCs (Fig. S2E–H).

SIT-induced $CD35^+$ B cells up-regulate immune tolerance in the intestine. Published data indicate that SIT can induce Tregs in sensitized subjects[22]. In line with the reports, we also detected the expansion of Foxp3+ Tregs in the intestine of the sensitized mice treated with SIT but not in those treated with saline (Fig. 4A, 4C). To clarify if TSP1 plays a role in the generation of Tregs, a group of mice was treated with TSP1 inhibitor during SIT, which abrogated the increase in Tregs in the intestine (Fig. 4A, 4C). Furthermore, we treated mice with a neutralizing anti-CD35 antibody right before the commence of SIT to remove the $CD35^+$ cells from the mice (Fig. S4). Indeed, the increase in Tregs was abolished. To confirm the effect of TSP1 on the induction of Tregs, a group of sensitized mice was treated with recombinant TSP1 daily for 5 days. The frequency of Tregs was increased about 4.7 folds as compared to the controls (Fig. S3B).

We then isolated $CD4^+$ T cells from the intestine of mice sensitized to OVA. Abundant $CD4^+$ T cell proliferation (Fig. 4B, 4D) was observed in response to a specific antigen stimulation in the culture (Fig. 4B1), which was not apparent in the $CD4^+$ T cells isolated from mice treated with SIT (Fig. 4B2). The $CD4^+$ T cells from mice treated with TSP1 inhibitors (Fig. 4B4) also showed marked proliferation in the culture after treated with the specific antigen, OVA. The removal of $CD35^+$ cells resulted in high proliferation of $CD4^+$ T cells. The results indicate that $CD35^+$ B cells play a critical role in the regulation of the properties of $CD4^+$ T cells in the allergic intestine by SIT.

We also measured the levels of IL-4, IL-5 and IL-13 in the culture supernatants of Fig. 4B. The results showed that, compared to the cells isolated from the sensitized mice (not treated by SIT), the $CD4^+$ T cells from SIT-treated mice produced much less Th2 cytokines ($p < 0.01$) in the culture in response to the stimulation of the specific antigen, OVA. The suppressor effect of SIT on Th2 cytokine production was abolished in the cells isolated from mice with $CD35^+$ cell depleted, or treated with TSP1 inhibitors (Fig. 4E). Collectively, the results indicate that the TSP1-producing $CD35^+$ B cells can suppress the skewed Th2 response in the intestine of sensitized mice.

$CD35^+$ B cells show tolerogenic properties. We next observed if TSP1-producing $CD35^+$ B cells could induce Tregs directly. We isolated $CD35^+$ B cells from the OVA-tolerant mice, and Th0 cells from DO11.10 mice. The two cell populations were co-cultured for one week in the presence of the specific antigens. About 25.4% T cells were converted into the $Foxp3^+$ Tregs (Fig. 5B1; Fig. 5C). Because IL-10 or TGF-β from tolerogenic DCs plays the key role in the conversion of naïve T cells to Tregs[23], we added the neutralizing anti-IL-10 antibody or anti-TGF-β antibody to the above co-culture system. The results showed that the addition of anti-TGF-β, but not the anti-IL-10, abolished the generation of Tregs (Fig. 5B2–B3; Fig. 5C). To elucidate if the B cell-derived TSP1

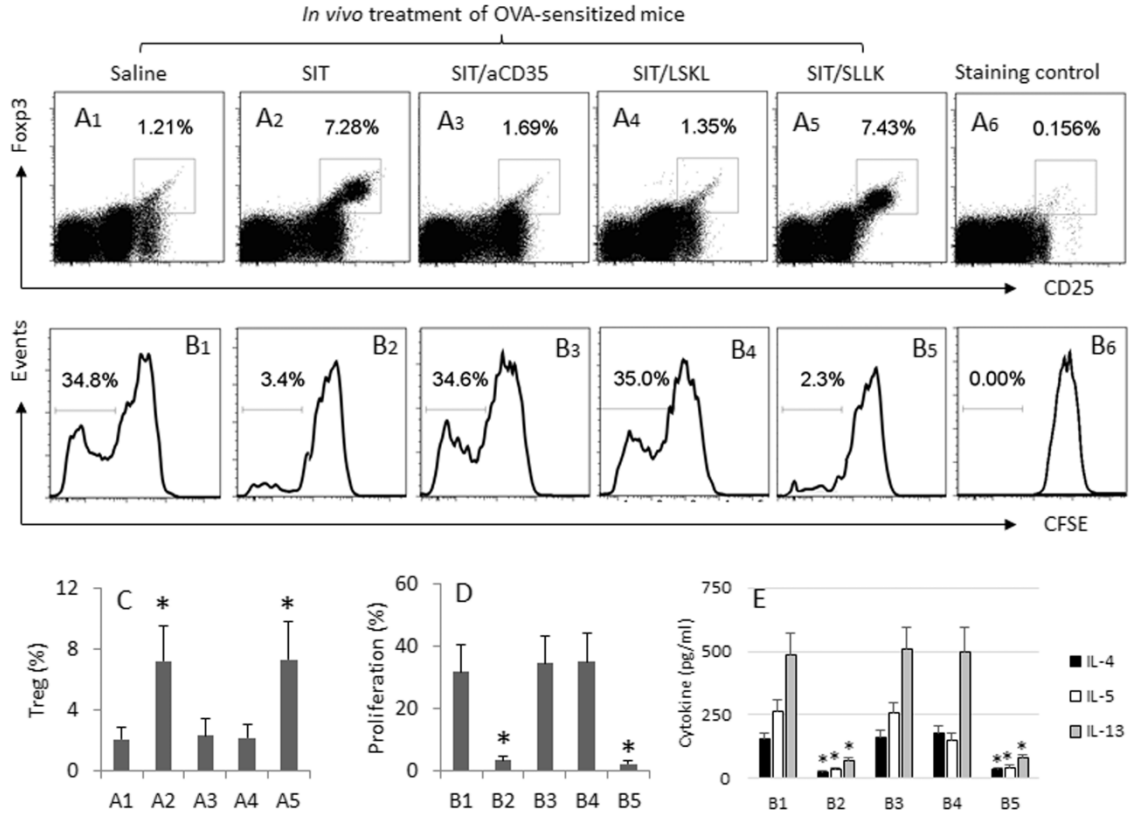

Figure 4 | SIT induces CD35⁺ B cells to modulate immune tolerance. The OVA-sensitized mice were treated with SIT for one week. (A), LPMCs were isolated and analyzed by flow cytometry. The gated cells were Foxp3$^+$ CD25$^+$ T cells. (B), the histograms indicate the proliferation of antigen specific CD4$^+$ T cells isolated from the mouse intestine. (C), the bars indicate the summarized data of panel A. (D), the bars indicate the summarized data of panel B. (E), the bars indicate the levels of IL-4 in culture supernatants of panel B. The data of bar graphs were presented as mean ± SD. *, $p < 0.01$, compared with group A1 (C) or B1 (D, E). The data represent 3 separate experiments. Saline: Mice or cells were treated with saline. SIT: Mice were treated with SIT. OVA: Cells were exposed to OVA (1 μg/ml) in the culture. aCD35: Mice were treated anti-CD35 antibody (0.1 mg/mouse) or anti-CD35 antibody was added to the culture (1 μg/ml). LSKL (SLLK): TSP1 inhibitor (control peptide) (10 mg/kg body weight).

played any roles in the conversion of Tregs from Th0 cells, TSP1 inhibitor, the LSKL peptide, was added to the culture; the Treg generation was abolished (Fig. 5B4–B5; Fig. 5C). The results indicate that the CD35$^+$ B cells produce TSP1 upon the exposure to the specific antigen; the TSP1 converts naïve Th0 cells to Tregs. We further co-cultured the CD35$^+$ B cells and Th0 cells in the presence of recombinant TSP1; the Th0 cells were converted to Tregs in a TSP1 dose-dependent manner (Fig. 5B6–8; Fig. 5C). To corroborate the finding, we isolated the Tregs from the co-cultured cells and analyzed the cell extracts by Western blotting. The results showed that the CD35$^+$ B cells or recombinant TSP1 markedly increased the TGF-β levels in the cells (Fig. 5D).

SIT-induced CD35⁺ B cells attenuate the ongoing allergic reactions in the intestine. To further characterize the SIT-induced CD35$^+$ B cells, we developed an intestinal allergy mouse model. The mice were treated with SIT and with or without depletion of CD35$^+$ B cells are treated with TSP1 inhibitors. The effect of SIT was determined by assessing the response of the intestine to the challenge with the specific antigen, OVA, at a dose of 5 mg/mouse in gavage. The results showed that, after the challenge with OVA, the sensitized, saline-control mice showed an allergic attack manifesting high serum levels of OVA-specific IgE (Fig. 6A), high levels of IL-4 (Fig. 6B) and IL-13 (Fig. 6C) in the tissue extracts of the intestine, a significant drop of the core temperature (Fig. 6D) and 100% mice had diarrhea (Fig. S5). The treatment with SIT suppressed the intestinal allergic responses, which was abolished by depletion of the CD35$^+$ cells, or using the TSP1 inhibitors during the SIT. Furthermore, the suppressor effect of SIT on the intestinal allergy phenomenon was mimicked by administration with recombinant TSP1, or adoptive transfer with the CD35$^+$ B cells (isolated from OVA-tolerant mice; Fig. 6). The results emphasize the importance of CD35$^+$ B cells in the maintenance of the immune homeostasis in the intestine.

On the other hand, we also observed the effect of SIT on the frequency of CD11b$^+$ CD35$^+$ DCs, CD11c$^+$ CD35$^+$ DCs and CD19$^+$ CD35$^+$ B cells in the intestine of mice sensitized to OVA. After treating with OVA or saline, LPMCs were isolated from the mouse small intestine and analyzed by flow cytometry. The results showed that much less CD35$^+$ cells were CD11b$^+$ or CD11c$^+$ as compared with the frequency of CD19$^+$ CD35$^+$ B cells in the mice treated with saline. After treating with SIT, markedly increase in the frequency of CD19$^+$ CD35$^+$ B cells was observed ($p < 0.01$), while the frequency of CD11b$^+$ CD35$^+$ DCs and CD11c$^+$ CD35$^+$ DCs was not changed much ($p > 0.05$) (Fig. 7).

Discussion

The present study revealed a novel subset of B cells, the antigen specific CD35$^+$ B cells, could be induced by SIT. The CD35$^+$ B cells expressed high levels of TSP1, the latter can down regulate surface domain of the costimulatory molecules, CD80 and CD86, of DCs in a cell co-culture model. Such an effect was reproduced by adding the recombinant TSP1 to the culture. The results also showed the tolerogenic function of CD35$^+$ B cells. This subset of B cells was capable of inducing Tregs and suppressing the ongoing allergic inflammation.

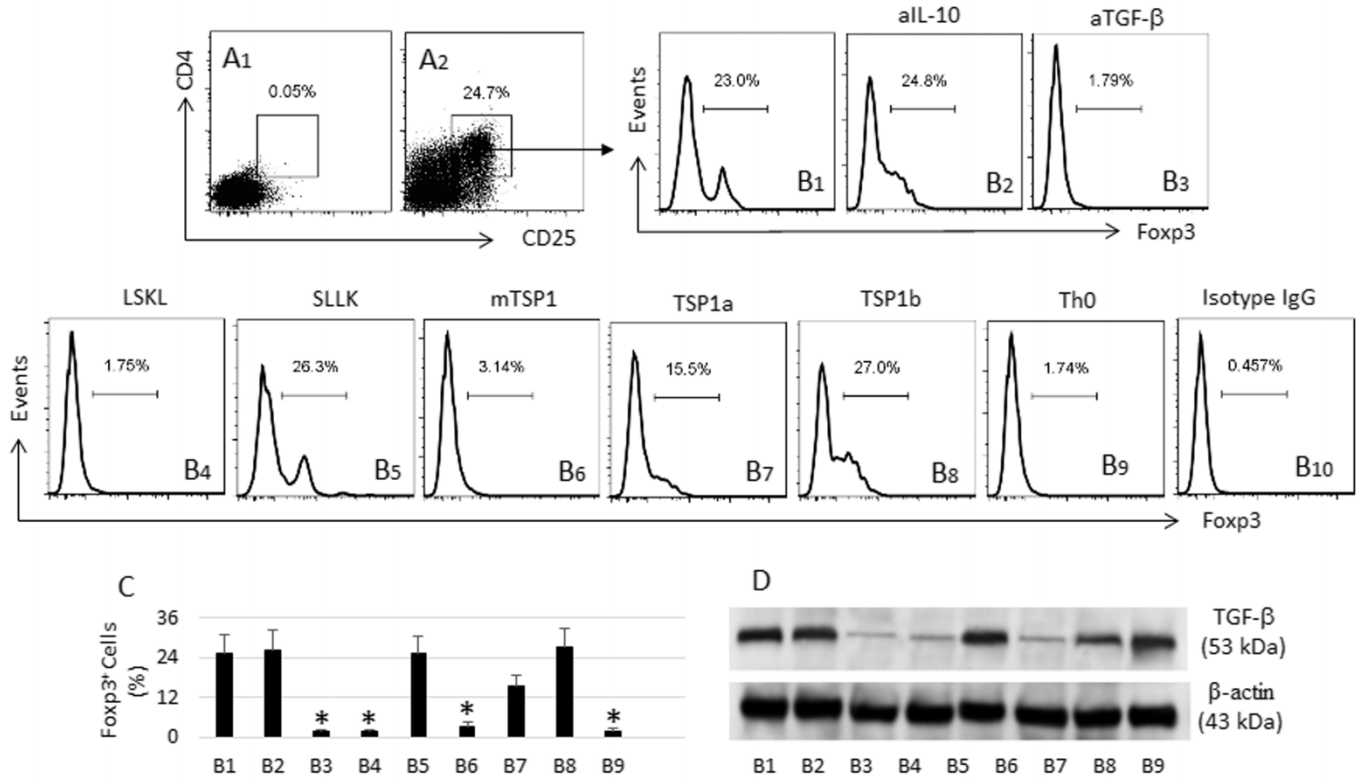

Figure 5 | CD35$^+$ B cells possess tolerogenic properties. CD35$^+$ B cells were isolated from the intestine of OVA-tolerated mice treated with SIT; Th0 cells were isolated from the naïve DO11.10 mouse spleen. The B cells and Th0 cells were cultured at a ratio of 1 : 5 ($1 \times 10^5 : 5 \times 10^5$) in the presence of the specific antigen, OVA, for one week. A0, isotype control. A1, the CD4$^+$ CD25$^+$ T cells were gated out first. B1–B10, the gated cells of A2 were further analyzed; the histograms indicate the frequency of Foxp3$^+$ Tregs. The experimental design was denoted above each histogram. Neutralizing antibodies of anti-IL-10 (aIL-10) or aTGF-β: 100 ng/ml. TSP1 doses: a = 5 μg/ml; b = 10 μg/ml; mTSP1 = 10 μg/ml (m = mutation). B10 is an isotype control. C, the bars indicate the summarized data of B1–B9 (mean ± SD. *, $p < 0.01$, compared with the group A1). D, the CD35$^+$ B cells were isolated by MACS from the culture and analyzed by Western blotting. The immune blots indicate the levels of TGF-β in CD35$^+$ B cells. The data represent 3 separate experiments.

The promotion of immune tolerance by SIT in the subjects with allergic disorders has been noted. Akdis et al.[24] noted that SIT could up-regulate the expression of IL-10 and T cell anergy. The production of TGF-β was also observed after SIT; the resulted inhibitory effect on allergic reactions could be blocked by pretreatment with TGF-β antagonists[23]. Thus, it seems that SIT can promote the expression of both IL-10 and TGF-β by immune cells under an allergic environment. Our data have expanded the knowledge in this area. By treating the sensitized mice with SIT, A novel subset of B cells, the CD35$^+$ B cells, was induced in mice with intestinal allergy. In line with the published data[23,24], such a subset of B cells could induce Treg development in the tissue with allergic inflammation and could suppress the ongoing allergic reactions. Further finding from this study pinpointed that TSP1 mediated the immune suppression of the CD35$^+$ B cells. Blocking TSP1 abolished the production of Foxp3$^+$ Tregs in the intestine of mice with intestinal allergy.

The role of the costimulatory molecules in the T cell activation has been well described. CD80 and CD86 are the mostly investigated costimulatory molecules produced by DCs or B cells. An amplificatory role of CD80/CD86 in T cell activation is suggested based on the finding of blocking CD80/CD86 can dampen the interactions between DCs and T cells[25]. The levels of CD80/CD86 on DCs are one of the major factors to dictate naïve T cells to differentiate into the inducible Tregs or other effector T cells[26]. A number of factors, such as the stimulation of Toll-like receptors, are suggested in up-regulation of the expression of costimulatory molecules in DCs and so as to induce skewed immunity such as autoimmune diseases and other immune disorders[27]. Thus, the control of the expression of costimulatory molecules in DCs may provide an important tool to modulate aberrant immune responses[28]. The present data indicate that the antigen specific CD35$^+$ B cells are capable of modulating the levels of CD80/CD86 on the surface of DCs; the underlying mechanism is that upon the stimulation of the specific antigens, the CD35$^+$ B cells are activated to produce TSP1; the latter down regulate the levels of CD80/CD86 on surface domain of DCs. TSP1 does not affect the gene expression of CD80/CD86 manifesting the mRNA levels of CD80/CD86 were not altered in DCs after the treatment. The data showed that the TSP1 levels were increased in the supernatants of the co-culture of DCs and CD35$^+$ B cells indicating that the CD35$^+$ B cells can release TSP1 to the microenvironment and has the potential to interact with other immune cells.

It is accepted that the major function of Tregs is to suppress other effector T cells' activities and to suppress or prevent immune inflammation. Various approaches[29] have been tried to generate Tregs in *in vivo* experiments; SIT is one of them. The key point in the mechanism of Treg generation is the induction of Foxp3 expression[30]. In line with previous studies, our data also show that SIT induces an increase in the Foxp3$^+$ Tregs in sensitized mice. Such an increase could be blocked by antagonizing the activities of TSP1, which also could be mimicked by the administration with the recombinant TSP1. The results indicate that the antigen specific CD35$^+$ B cells induce Tregs via producing TSP1 upon the exposure to specific antigens.

The administration of the DC vaccines has been used in the studies of immune regulation[31]. The immature DCs produce IL-10 or/and TGF-β, can induce Th0 cells to differentiate into Tregs[32]. In line with

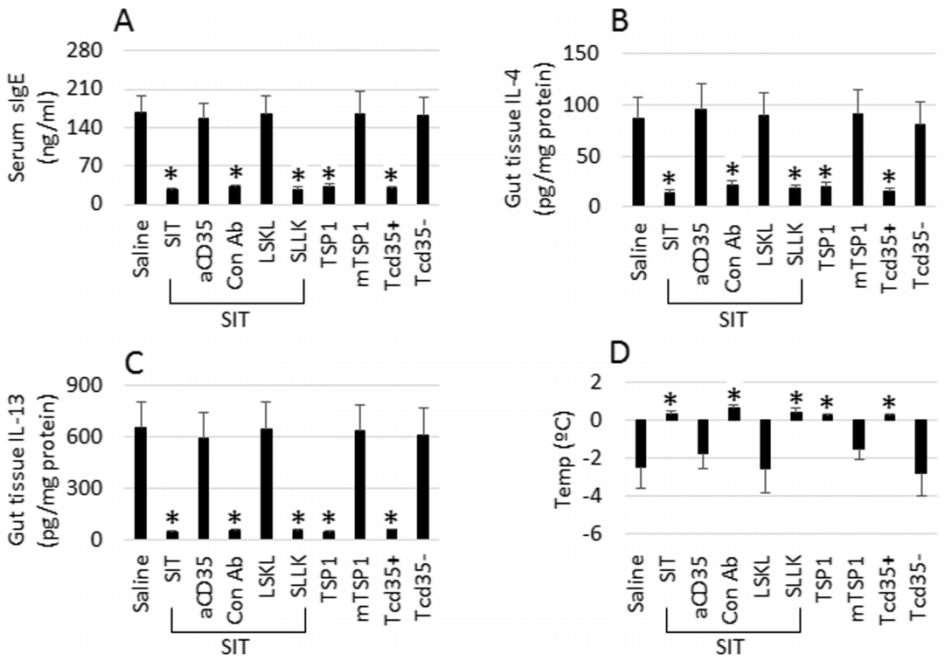

Figure 6 | Parameters of intestinal allergy. (A), the bars indicate the serum levels of specific IgE (sIgE). (B), the bars indicate the IL-4 protein levels in the intestinal tissue extracts. (C), the bars indicate the protein levels of IL-13 in the intestinal tissue extracts. (D), the bars indicate the core temperature of the experimental mice recorded after challenging with specific antigens; the core temperature was taken from the rectum with a digital thermometer before and 30 min after the specific antigen challenge. The data were presented as mean ± SD. *, $p < 0.01$, compared with the saline group. Each group consisted of 6 mice. Samples collected from each mouse were processed separately. Saline: The sensitized mice were treated with saline. SIT: The sensitized mice were treated with SIT. aCD35 (or con Ab): The mice were treated with neutralizing anti-CD35 antibody (or a control Ab). LSKL (or SLLK): The mice were treated with TSP1 inhibitor, LSKL (or SLLK, the control peptide). TSP1 (or mTSP1): The sensitized mice were treated with recombinant TSP1 (or mTSP1; 0.25 mg/mouse) daily for one week. $Tcd35^+$ (or $Tcd35^-$): The mice were adoptively transferred with $CD35^+$ (or $CD35^-$) B cells (10^6 cells/mouse).

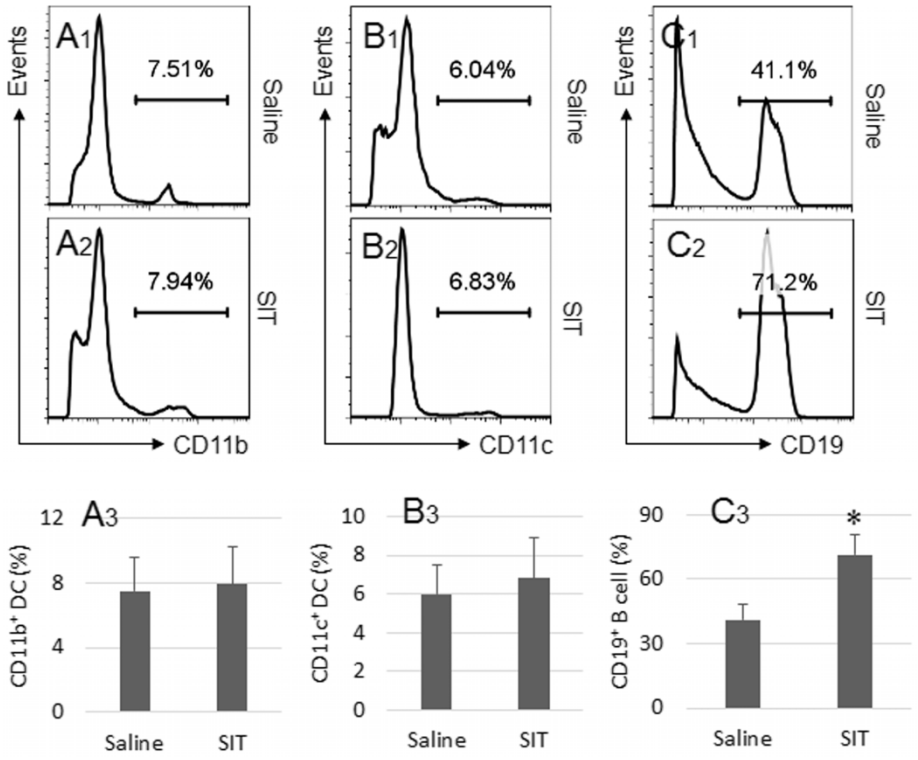

Figure 7 | SIT increases $CD35^+$ B cells in the intestine. In the same procedures of Figure 6, mice were sensitized to OVA and treated with saline or SIT. After sacrifice, LPMC were isolated from the small intestine and analyzed by flow cytometry. The $CD35^+$ cells were gated first, the gated cells were further analyzed for the frequency of $CD11b^+$ $CD35^+$ DCs (A), $CD11c^+$ $CD35^+$ DCs (B) and $CD19^+$ $CD35^+$ B cells (C). The bars of A3, B3 and C3 show the summarized data of the cell frequency in the histograms. The data are presented as mean ± SD. *, $p < 0.01$, compared with the group treated with saline.

the previous studies[33], the present study also revealed that the exposure to the specific antigens induced DCs to express TGF-β. Similar to previous reports[34], which described the critical role of TGF-β in the conversion of Treg, the present data also showed that DC-derived TGF-β is critical in the present experimental setting to convert the Th0 cells to Tregs. The novel aspect of the present study is that the $CD35^+$ B cell-derived TSP1 plays a critical role in the conversion of Tregs. This is supported by others' studies. Crawford found that TSP1 could convert the latent TGF-β to the active TGF-β[8]; Chen et al demonstrated the role of TGF-β in the conversion of Tregs[33]. Together with the results that the recombinant TSP1 also induces the conversion of Tregs from Th0 cells; it suggests a potential of the exogenous TSP1 in the treatment of immune disorders. The inference is supported by the animal model of intestinal allergy of the present study, which shows that the adoptive transfer with the TSP1-producing $CD35^+$ B cells can suppress the intestinal allergic inflammation.

It is reported that several cell types can produce TSP1, such as endothelial cells, adipose cells, fiberocytes, macrophages, dendritic cells and B cells[9,10]. The production of TSP1 by the cells needs proper stimulation. Our data demonstrate that SIT can drive antigen specific $CD35^+$ B cells to produce TSP1 in mice with intestinal allergy. The production of TSP1 by this subset of B cells is associated with the induction of Tregs in the intestine; the supportive evidence includes that blocking TSP1 could abolish the induction of Tregs in the intestine, which was also occurring in the depletion of the $CD35^+$ B cells. In separate experiments, we noted that none-specific activation of B cells by anti-IgM antibody also increased the expression of TSP1 (data not shown). Therefore, although there are a number of sources of TSP1 in the intestine, the proper stimulation is required for its production.

In summary, the present data indicate that the treatment with SIT can induce the development of TSP1-producing $CD35^+$ B cells; this subset of B cells can down-regulate the costimulatory molecule levels in DCs, induce Tregs and inhibit food allergy associated inflammation in the intestine.

Methods

Food allergy mouse model. Following the published procedures[35] with minor modification, BALB/c mice were i.p. injected with OVA (1 mg/mouse) mixing with cholera toxin (2 μg/mouse) on day 0 and boosted on day 3. In addition, mice were gavage-fed with OVA (1 mg/mouse) or/and cholera toxin (CT; 10 μg/mouse; using as an adjuvant) weekly for 6 weeks. The negative control mice were gavage-fed with PBS only. The experimental procedures were approved by the Animal Ethic Committee at Shenzhen University.

SIT. Following the published procedures[35] with minor modification, the sensitized mice were treated with oral SIT. Briefly, ovalbumin (OVA) was gavage-fed with the doses of 1 mg (days 1 and 2), 5 mg (days 3 and 4), 10 mg (days 5–7), 25 mg (days 8 and 9), and 50 mg (days 10–14). Control mice were treated with bovine serum albumin (BSA).

Administration of TSP1 peptides. Peptides of LSKL, SLLK, recombinant TSP1, mutated TSP1 (mTSP1) were dissolved in saline (1.0 mg/ml), and i.p. injected at 30 mg/kg body weight.

Depletion of $CD35^+$ cells. Mice were received neutralizing anti-CD35 antibody (0.25 mg/mouse; i.p.; one dose). The frequency of $CD35^+$ cells was not detected in the intestine after the treatment (Fig. S4).

Statistics. Each mouse group consisted of 6 mice. Each experiment was repeated at least 3 times. Data were presented as mean ± SD. The differences between two groups were tested with the Student t test or ANOVA if more than two groups. $P < 0.05$ was set as the significant criterion.

More experimental procedures were presented in supplemental materials.

1. Crawford, A., MacLeod, M., Schumacher, T., Corlett, L. & Gray, D. Primary T Cell Expansion and Differentiation In Vivo Requires Antigen Presentation by B Cells. *J Immunol* **176**, 3498–3506 (2006).
2. Lund, F. E. & Randall, T. D. Effector and regulatory B cells: modulators of CD4+ T cell immunity. *Nat Rev Immunol* **10**, 236–247 (2010).
3. Smits, H. H. B cells in allergic diseases: Bad or Better? *Autoimmunity* **45**, 415–426 (2012).
4. Mauri, C. & Bosma, A. Immune Regulatory Function of B Cells. *Annu. Rev. Immunol.* **30**, 221–241 (2012).
5. Noh, G. & Lee, J. H. Regulatory B Cells and Allergic Diseases. *Allergy Asthma Immunol Res* **3**, 168–177 (2011).
6. Akdis, C. A. Therapies for allergic inflammation: refining strategies to induce tolerance. *Nat Med* **18**, 736–749 (2012).
7. Mosher, D. F. Physiology of Thrombospondin. *Annu Rev Med* **41**, 85–97 (1990).
8. Crawford, S. E. *et al.* Thrombospondin-1 Is a Major Activator of TGF-beta1 In Vivo. *Cell* **93**, 1159–1170 (1998).
9. Lopez-Dee, Z. P. K. G. L. S. Thrombospondin-1: multiple paths to inflammation. *Mediators Inflamm.* **2011**, 296069 (2011).
10. Tabib, A. *et al.* Thrombospondin-1-N-Terminal Domain Induces a Phagocytic State and Thrombospondin-1-C-Terminal Domain Induces a Tolerizing Phenotype in Dendritic Cells. *PLoS ONE* **4**, e6840 (2009).
11. Kyriakides, T. R. M. S. The role of thrombospondins in wound healing, ischemia, and the foreign body reaction. *J Cell Commun Signal* **3**, 215–25 (2009).
12. Velasco, P. *et al.* The Angiogenesis Inhibitor Thrombospondin-1 Inhibits Acute Cutaneous Hypersensitivity Reactions. *J Invest Dermatol* **129**, 2022–2030 (2009).
13. Fearon, D. T. Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte, and monocyte. *J Exp Med* **152**, 20–30 (1980).
14. Zipfel, P. F. & Skerka, C. Complement regulators and inhibitory proteins. *Nat Rev Immunol* **9**, 729–740 (2009).
15. Fischer, M. B. *et al.* Dependence of Germinal Center B Cells on Expression of CD21/CD35 for Survival. *Science* **280**, 582–585 (1998).
16. Anderson, S. M., Tomayko, M. M., Ahuja, A., Haberman, A. M. & Shlomchik, M. J. New markers for murine memory B cells that define mutated and unmutated subsets. *J Exp Med* **204**, 2103–2114 (2007).
17. Magee, C. N., Boenisch, O. & Najafian, N. The Role of Costimulatory Molecules in Directing the Functional Differentiation of Alloreactive T Helper Cells. *Am J Transplant* **12**, 2588–2600 (2012).
18. Lutz, M. B. & Schuler, G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol* **23**, 445–449 (2002).
19. Roozendaal, R. & Carroll, M. C. Complement receptors CD21 and CD35 in humoral immunity. *Immunol Rev* **219**, 157–166 (2007).
20. Ozdemir, C., Kucuksezer, U. C., Akdis, M. & Akdis, C. A. Specific immunotherapy and turning off the T cell: how does it work? *Ann Allergy Asthma Immunol* **107**, 381–392 (2011).
21. Bour-Jordan, H. *et al.* Intrinsic and extrinsic control of peripheral T-cell tolerance by costimulatory molecules of the CD28/B7 family. *Immunol Rev* **241**, 180–205 (2011).
22. Kawakita, A. *et al.* Immunotherapy with oligomannose-coated liposomes ameliorates allergic symptoms in a murine food allergy model. *Allergy* **67**, 371–379 (2012).
23. Jutel, M. *et al.* IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol.* **33**, 1205–1214 (2003).
24. Akdis, C. A., Blesken, T., Akdis, M., Wuthrich, B. & Blaser, K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* **102**, 98–106 (1998).
25. Lim, T. S. *et al.* CD80 and CD86 Differentially Regulate Mechanical Interactions of T-Cells with Antigen-Presenting Dendritic Cells and B-Cells. *PLoS ONE* **7**, e45185 (2012).
26. Steinbrink, K., Mahnke, K., Grabbe, S., Enk, A. H. & Jonuleit, H. Myeloid dendritic cell: From sentinel of immunity to key player of peripheral tolerance? *Hum Immunol* **70**, 289–293 (2009).
27. Blanco, P., Palucka, A. K., Gill, M., Pascual, V. & Banchereau, J. Induction of Dendritic Cell Differentiation by IFN-gamma in Systemic Lupus Erythematosus. *Science* **294**, 1540–1543 (2001).
28. Hardin, J. A. Dendritic cells: potential triggers of autoimmunity and targets for therapy. *Ann Rheum Dis* **64**, iv86–iv90 (2005).
29. Lan, Q. *et al.* Induced Foxp3+ regulatory T cells: a potential new weapon to treat autoimmune and inflammatory diseases? *J Mol Cell Biol.* **4**, 22–28 (2012).
30. Baru, A. M. *et al.* Optimal Isolation of Functional Foxp3⁺ Induced Regulatory T Cells Using DEREG Mice. *PLoS ONE* **7**, e44760 (2012).
31. Hillyer, L. *et al.* Adoptively Transferred Dendritic Cells Restore Primary Cell-Mediated Inflammatory Competence to Acutely Malnourished Weanling Mice. *Am J Pathol* **172**, 378–385 (2008).
32. Zahorchak, A. F., Raimondi, G. & Thomson, A. W. Rhesus Monkey Immature Monocyte-Derived Dendritic Cells Generate Alloantigen-Specific Regulatory T Cells From Circulating CD4+CD127−/lo T Cells. *Transplantation* **88**, (2009).
33. Chen, X. *et al.* Intestinal epithelial cell-derived integrin alpha v beta 6 plays an important role in the induction of regulatory T cells and inhibits an antigen-specific Th2 response. *J Leukoc Biol.* **90**, 751–9 (2011).
34. Chen, W. *et al.* Conversion of Peripheral CD4+CD25+ Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-beta Induction of Transcription Factor Foxp3. *J Exp Med* **198**, 1875–1886 (2003).

35. Leonard, S. A., Martos, G., Wang, W., Nowak-W-Ögrzyn, A. & Berin, M. C. Oral immunotherapy induces local protective mechanisms in the gastrointestinal mucosa. *J Allergy Clin Immunol.* **129**, 1579–1587 (2012).

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Author contributions

H.P.Z., Y.W., J.L., J.J., X.R.G., G.Y., L.M., Z.Q.L. and Z.G.L. performed experiments, analyzed data and reviewed the manuscript. P.C.Y. designed the project, supervised the experiments and wrote the manuscript.

Additional information

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