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Forkhead box protein-3 (Foxp3)-producing dendritic cells suppress allergic response

Running title: Foxp3-producing DCs induces Tregs

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Abstract:

Background: The generation of the tolerogenic dendritic cells (DC) is not fully
understood yet. Forkhead box protein-3 (Foxp3) is an important molecule in the
immune tolerance. This study tests a hypothesis that DCs express Foxp3, which can
be up regulated by *Staphylococcal* enterotoxin B (SEB).

Methods: The expression of Foxp3 by DCs was evaluated by real time RT-PCR,

Western blotting, flow cytometry and chromatin immunoprecipitation assay.

Results: We observed that mice treated with SEB at 0.25-0.5 µg/mouse showed high

frequencies of transforming growth factor (TGF)-β-producing CD4⁺ T cells and

TGF-β-producing DCs in the intestine, while the IL-4⁺ CD4⁺ T cells and TIM4⁺ DCs were

dominated in the intestine in mice treated with SEB at 1-10 µg/mouse. Treating DCs

with SEB in the culture induced high levels of Foxp3 at the TGF-β promoter locus. The

function of Foxp3 was blocked by STAT6 (Signal transducer and activator

transcription-6); the latter was induced by exposing DCs to SEB in the culture at

doses of 100-400 ng/ml. Treating allergic mice with specific immunotherapy (SIT)

together with SEB significantly promoted the therapeutic effects on the allergic

responses than treating with SIT alone.

Conclusion: DCs have the capacity to express Foxp3, which can be up regulated by

exposure to SEB.

Keywords: Dendritic cell; Staphylococcal enterotoxin B; T lymphocyte; Intestine; T

cell immunoglobulin mucin domain molecule-4; Forkhead box protein-3.

Introduction

Dendritic cells (DC) are antigen presenting cells in the immune system. The essential functions of DCs are to present the antigen information to T cells to initiate immune responses in the body. The induced types of immune response are dependent on the activated DC phenotypes (1). Yet, the mechanisms governing the DC phenotyping have not been fully understood.

Several DC phenotypes have been defined. Interleukin (IL)-12-producing DCs mainly induce T helper (Th) 1 responses (2); the T cell immunoglobulin mucin domain-4 (TIM4)-producing DCs induce Th2 responses (3); interleukin (IL)-10- producing DCs induce regulatory T cells (Treg) or regulatory B cells (4, 5). It is well documented that exposure to transforming growth factor (TGF)- β can induce forkhead box protein 3 (Foxp3) positive Treg (6). DCs are the critical cell type to determine the immune response types. Our previous work indicated that DC-derived TGF- β could generate Tregs (7). However, the regulatory mechanism of the expression of TGF- β in DCs has not been investigated.

SEB is an enterotoxin secreted by the Gram positive bacteria, *staphylococcus aureus* (*S. aureus*). Ingestion of SEB-contaminated foods can induce severe responses in the digestive system, such as diarrhea and intestinal cramping or even sepsis (8). SEB functions as a superantigen through activating T-cells by cross-linking MHC class II

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molecules to T-cell receptors (9). It is reported that exposure to SEB at 100 ng/ml induces DC maturation to facilitate the initiation of Th2 polarization (10), while relatively low doses of SEB induce immune tolerance, such as via peritoneal injection of SEB at 75 µg/kg promotes the allograft cornea survival (11).

Signal transducer and activator of transcription 6 (STAT6) is a member of the STAT family of transcription factors. This molecule plays a critical role in facilitating IL-4 mediated biological responses and relates the initiation of T helper (Th) 2 polarization. Our recent studies reveal that STAT6 mediates microbial product-induced TIM4 expression in DCs (12). Others found that inhibition of STAT6 suppressed TIM4 expression and promoted the generation of Tregs (13) and reduced Foxp3 expression (14). Whether STAT6 involves the DC-induced Treg generation is to be further investigated.

Based on the above information, we hypothesize that there is a dose-dependent effect of SEB on regulating DC's phenotyping. To test this, we carried out this study. The results showed that SEB induced Foxp3 expression in DCs at lower doses, while higher doses (>100 ng/ml) of SEB induced signal transducer and activator transcription-6 (STAT6) expression in DCs, which bound to Foxp3 to inhibit the functions of Foxp3.

Materials and methods:

Mice

Male BALB/c mice and C57B6 mice (6-8 week old) were purchased from the Guangzhou Experimental Animal Center (Guangzhou, China). GFP-tagged Foxp3 mice, STAT6-deficient mice and MHC II-deficient mice were purchased from Jackson Laboratory. The mice were maintained in a pathogen free environment with freely accessing water and food. The animal experimental procedures were approved by the Animal Ethic Committee at Shenzhen University. The experiments were performed in accordance with the approved guidelines.

Immunizing mice with SEB and OVA

Balb/c mice were subcutaneously injected with 100 μ g OVA/mouse and an indicated dose of SEB (μ g/ml: 0.1, 0.2, 0.5, 1, 2, 5 and 10 respectively) on day 0 and were boosted with the same dose of OVA and SEB on day 3 and 7. The mice were challenged by gavage-feeding with OVA (1 mg/mouse in 0.3 ml saline) on day 9, 11 and 13, respectively. The sensitized mice were sacrificed on day 14 (Fig. S1 in the supplemental materials).

Development of allergic inflammation in the intestine

Balb/c mice were gavage-fed with OVA (1 mg/mouse) mixing with cholera toxin (20 μ g/mouse) in 0.3 ml saline weekly for 5 consecutive weeks (Fig. S2). The mice were

used for further experiments of specific immunotherapy. The parameters of the allergic inflammation in the intestine and the clinical allergic reaction symptoms (core temperature change and diarrhea) were evaluated following our established procedures (15).

Modified antigen specific immunotherapy (SIT)

Referring to the procedures of Dr. Berin's laboratory (16) with modification (17), the sensitized mice were treated with OVA (0.2 mg/mouse) or/and SEB (0.2 µg/mouse) in 0.3 ml saline, gavage-feeding daily for 6 days (Fig. S3).

***S. aureus* gavage**

Following our established procedures (18), grouped sensitized mice were treated on the first day of SIT with oral *S. aureus* (American Type Culture Collection no. 10390; American Type Culture Collection, Manassas, VA) or heat-killed *S. aureus* (prepared by heating a suspension of the *S. aureus* in saline at 60°C for 1 hour) at a dose of 10^5 colony-forming units (cfu)/mouse in 0.3 ml of saline, or mixing in the OVA-vaccine; the oral *S. aureus* was repeated once on day 3 after the first treatment. The dosage of the oral *S. aureus* gavage was based on a *S. aureus* culture study (Fig. S4 in supplemental materials).

Preparation of TGF- β -producing DCs (SEB-primed DCs)

Bone marrow-derived DCs (wild or STAT6-deficient) were cultured in the presence of SEB (50 ng/ml) for 96 h. The viability of the DCs was greater than 99% as checked by Trypan blue exclusion assay. The frequency of TGF- β^+ DCs was greater than 90% as checked by flow cytometry.

Statistics

All the data are normally distributed as tested by Norm.Dist in MS Excel. The data are presented as mean $\pm$ SD. The differences between two groups were determined by Student t test or two-way analysis of variance (ANOVA) along with Bonferroni correction was used in for continuous variable among treatment groups. A $p < 0.05$ was set as a significant criterion.

The following methods are presented in supplemental materials:

Reagents, immune cell isolation from the mouse small intestine, cell culture, CD4⁺ T cell proliferation assay, flow cytometry, enzyme-linked immunosorbent assay (ELISA), real time RT-PCR (RT-qPCR), generation of bone marrow-derived DCs, preparation of cytosolic and nuclear extracts, RNA interference (RNAi), Luciferase-tagged TGF- β expression assay, Western blotting, assessment of intestinal allergic reactions, Chromatin immunoprecipitation (ChIP) and coimmunoprecipitation.

Results

Effects of SEB on CD4⁺ T cell phenotypes in the intestine

To understand the effects of SEB on modulating the immune response in the intestine, we immunized mice with ovalbumin (OVA) together with SEB at gradient doses (used as an adjuvant). Lamina propria mononuclear cells (LPMC) were isolated from the small intestine; the CD4⁺ T cells were further isolated from the LPMCs and analyzed by CFSE-dilution assay. As shown by Fig. 1A, exposure to the specific antigen (OVA), but not to BSA, in the culture markedly induced CD4⁺ T cell proliferation, indicating that OVA-specific CD4⁺ T cells were induced in the intestine by immunization with OVA/SEB.

Further analysis showed that Foxp3⁺ cells (11.2% and 18.5%) were detected in the proliferating CD4⁺ T cells from mice treated with SEB at doses of 0.1 and 0.2 µg/mouse, which was declined (6.3%) in mice treated with SEB at 0.5 µg/mouse, and was further declined in mice treated with SEB from 1-10 µg/mouse (Fig. 1B). The results implicate that at SEB doses of 0.1~0.2 µg/mouse facilitates the generation of Foxp3⁺ CD4⁺ T cells, which is abolished when SEB doses are increased to ≥1 µg/mouse. Flow cytometry data demonstrated that these cells were CD4⁺ CD25⁺ Foxp3⁺ TGF-β⁺ (Fig. S5 in supplemental materials), indicating these cells are Tregs. On the other hand, the results showed that the frequency of OVA-specific CD4⁺ IL-4⁺ T cells and CD4⁺ IFN-γ⁺ T cells were significantly increased in the intestine of mice treated with SEB at doses of 1-10 µg/mouse in a dose-dependent manner (Fig. 1B).

To corroborate the results of Fig. 1A, total proteins were extracted from the small intestine and analyzed by ELISA. The results showed high levels of TGF- β in the mice treated with SEB at 0.1 and 0.2 $\mu\text{g}/\text{mouse}$ (Fig. 1C); the levels of IL-4 (Fig. 1D) or IFN- γ (Fig. 1E) were higher in the mice treated with SEB at 1-10 $\mu\text{g}/\text{mouse}$.

SEB modulates DC phenotypes

The data of Fig. 1 implicate that administration with OVA/SEB at the dose-range of 0.1 and 0.2 $\mu\text{g}/\text{mouse}$ induces Tregs in the intestine. Since DCs are the cells to initiate further immune responses, we inferred that SEB also modulated DC's phenotypes. Thus, we next evaluated the effects of SEB on regulating DC's phenotypes in two approaches. Firstly, after treating with SEB in the same dosage of Fig. 1, LPMCs were isolated from the mice and analyzed by flow cytometry. The results showed that the frequency of CD11c⁺ TGF- β ⁺ Foxp3⁺ DCs was higher in mice treated with SEB at 0.1 and 0.2 $\mu\text{g}/\text{mouse}$ as compared to the naive control mice, which was declined in mice treated with SEB at 0.5 $\mu\text{g}/\text{mouse}$ and further declined in those treated with 1-10 μg SEB per mouse (Fig. 2A-B). On the other hand, the frequency of T cell immunoglobulin mucin domain molecule (TIM)4⁺ CD11c⁺ DCs increased at 0.5 μg SEB per mouse and further increased in those treated with SEB at 1-10 $\mu\text{g}/\text{mouse}$ in a dose-dependent manner (Fig. 2C).

Secondly, naive DCs were generated from the mouse bone marrow and cultured in the presence of SEB at gradient concentrations for 4 days. The phenotypes of DCs were analyzed. The results showed that exposure to SEB at 25-50 ng/ml induced DCs to express TGF- β that was declined when SEB concentration reached 100 ng/ml. The expression of TIM4 in DCs was observed when SEB concentrations were 100-400 ng/ml (Fig. 2D-E). The data of flow cytometry showed that exposure to SEB in the culture increased the expression of CD80 (Fig. 2F) and CD86 (Fig. 2G) in DCs in a SEB dose-dependent manner. The results indicate that SEB can induce DCs to differentiate to either TGF- β -producing DC or the TIM4-producing DCs depending on SEB concentrations in the micro environment. To further corroborate the results, we treated GFP-tagged Foxp3 transgenic mice with SEB at 0.2 μ g/mouse for one week, which markedly increased the CD11c⁺ Foxp3⁺ DCs in the intestine as well as a small part (16.4%) CD4⁺ Foxp3⁺ T cells (Fig. S5C).

SEB induces STAT6 to suppress Foxp3 in DCs

The data of Fig. 2 implicate that SEB regulates the expression of TGF- β in DCs. To corroborate the results, we evaluated the levels of Foxp3, the transcriptional factor of TGF- β , at the TGF- β promoter locus in DCs after exposure to SEB in the culture. The results of ChIP assay showed that SEB did increase the levels of Foxp3 at the TGF- β promoter locus in a SEB concentration-dependent manner (Fig. 3A). The results implicate that Foxp3 is dysfunctional in DCs somehow when SEB

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concentrations reaches 100-400 ng/ml. Since the levels of TIM4 were not increased in DCs at doses of 25-50 ng/ml until the doses reached 100-400 ng/ml and published data indicate that STAT6 is the transcriptional factor of TIM4 (12, 19), we inferred that the STAT6 might interfere with the function of Foxp3 in DCs. We then assessed the levels of STAT6 in DCs. The results showed that SEB increased the levels of STAT6 at the TGF- β promoter locus in a dose-dependent manner (Fig. 3B).

Prompted by the data that STAT6 was also increased at the TGF- β promoter locus, we inferred that the STAT6 might interact with Foxp3 to interfere with its function. To test this, we analyzed the DC extracts by coimmunoprecipitation. The results showed that a complex of Foxp3 and STAT6 was detected in DCs after exposure to SEB, which was immunoprecipitated by either anti-Foxp3 antibody or anti-STAT6 antibody (Fig. 3C). The data indicate that Foxp3 and STAT6 physically associate with each other at the TGF- β promoter locus in DCs.

To assess if STAT6 affects the SEB-induced function of Foxp3 in DCs, we transfected wild type and STAT6^{-/-} DCs with the adenovirus encoding luciferase-tagged TGF- β promoter sequences. The DCs were exposed to SEB at 400 ng/ml in the culture for 48 h. The luciferase assay showed that the luciferase activities in the STAT6^{-/-} DCs were 6.5 folds higher than that in wild DCs (Fig. 3D). On the other hand, we assessed the TGF- β levels in the culture supernatant by ELISA. The results showed that about 16.5 pg/ml TGF- β were detected in the supernatant of the STAT6^{-/-} DCs while it was below the detective levels in that of wild type DCs (Fig. 3E). In addition, we cultured wild

type or STAT6^{-/-} DCs and naive CD4⁺ T cells in the presence or absence of SEB (400 ng/ml) for 3 days. The cells were analyzed by flow cytometry. The results showed that the STAT6^{-/-} DCs induced about half of the T cells to express TGF-β while the wild type DCs did not induce appreciable TGF-β expression in the CD4⁺ T cells (Fig. 3F). To stimulate CD4⁺ T cells with SEB alone without the presence of DCs did not induce detectable TGF-β in the T cells (Fig. S6 in supplemental materials).

SEB-primed DCs show tolerogenic properties

TGF-β-producing DCs have tolerogenic properties (20) since TGF-β is an essential factor for the inducible Treg cell induction (21). We next tested the tolerogenic properties of the SEB-primed DCs. Naive DCs and Foxp3-deficient DCs were stimulated with SEB at 50 ng/ml. The DCs were then co-cultured with naive CD4⁺ T cells for 3 days. The cells were analyzed by flow cytometry. The results showed that SEB-primed DCs induced about 30% Foxp3⁺ T cells, while the SEB-primed Foxp3-deficient DCs did not induce detectable Foxp3⁺ T cells (Fig. 4A-B). To corroborate the results, the T cells were isolated from the cocultured cells by MACS and analyzed by RT-qPCR and Western blotting. The results showed that the SEB-primed DCs induced high levels of Foxp3 and TGF-β in the T cells, which were not detected in the T cells co-cultured with the SEB-primed Foxp3-deficient DCs (Fig. 4C-F). In addition, the SEB-primed DCs showed immune suppressor effect on CD4⁺ T cell proliferation (Fig. S7).

SEB enhances the effects of specific immunotherapy

To further test the immune regulatory capacity of SEB, a mouse food allergy model was developed. The sensitized mice showed the food allergy signs, including high serum levels of IL-4, IL-5, IL-13 (Fig. 5A) and OVA-specific IgE (Fig. 5B); abundant infiltration of mast cells and eosinophils in the intestinal mucosa (Fig. 5C); OVA-specific intestinal CD4⁺ T cell proliferation in the culture (D); decrease in the core temperature (Fig. 5E) and had diarrhea (Fig. 5F). Although treatment with SEB alone did not show an apparent effect on suppressing the allergic response, mice received both SEB and SIT significantly inhibited the allergic responses in the intestine (Fig. 5). To corroborate the results, we reconstituted DC-deficient mice with Foxp3-sufficient DCs or Foxp3-deficient DCs (Fig. S8). The mice were sensitized to OVA, and then treated with SEB and SIT. The results showed that DC-deficient mice reconstituted with Foxp3-deficient DCs (Fig. S9) were induced allergic inflammation in the intestine, while those reconstituted with Foxp3-sufficient DCs did not show appreciable allergic responses (Fig. 5).

Discussion

The phenotyping of DC is an important event in determining the types of immune responses in the body. To understand factors regulating DC phenotyping is of significance. It is generally regarded that exposure to microbial products, such as LPS, induces IL-12 production by DCs and initiates Th1 dominant immune responses in the

body to fight against the invading microbes. The present data reveal a previously unknown feature of the DC phenotyping. DCs expressed Foxp3 in response to SEB stimulation, which was suppressed by relatively higher doses of SEB. Foxp3 is the transcriptional factor of TGF- β . TGF- β -producing DCs have immune tolerogenic properties by promoting the generation of Tregs (20, 21), which may also be induced by exposure to microbial products (22). The results showed that the regulatory effects of SEB on TGF- β production by DCs was induced at low doses of SEB. When the SEB doses reached and beyond 100 ng/ml, the expression of STAT6 was induced in DCs; the STAT6 bound to Foxp3 at the TGF- β promoter locus to interfere with the gene transcription of TGF- β .

SEB is one of the enterotoxins of *S. aureus*. *S. aureus* is a common bacterium distributing extensively on the surface of the body (23) as well as in the airway (24) and the digestive tract (25). In healthy conditions, the epithelial barrier prevents the products of *S. aureus*, such as SEB, from being absorbed. However, in the condition of epithelial barrier dysfunction (26), SEB may be absorbed into the deeper region of the mucosa to contact immune cells such as DCs. The serum-specific IgE to SEB can be detected in patients with allergic rhinitis and bronchial asthma (27), indicating that the absorbed SEB may maneuver naive DC's phenotyping. The present data have expanded the previous findings by showing that SEB induced the expression of Foxp3 or STAT6 in DCs, depending on the concentrations of SEB in the micro-environment.

Our previous work also indicates that the components of SEB serve as a hapten (25) or induce TIM4 expression (28) to promote the development of intestinal allergy.

It has been extensively studied that the IL-12-producing DCs induce Th1 dominant immune responses, while the phenotypes of DC initiating the Th2 dominant immune response are less defined. In the recent ten years, it has been cumulatively reported that TIM4-producing DCs play an important role in the initiation of Th2 dominant immune responses (3, 29), such as intestinal allergy (28), airway allergy (29) and allergic dermatitis (30). The TIM4 binds TIM1 on CD4⁺ T cells to drive these T cells to differentiate into Th2 cells (3). The present data uncover another functional aspect of SEB that exposure of naive DCs to SEB at a dose of 100 ng/ml or higher doses induced TIM4 expression in a mechanism of inducing STAT6 expression. STAT6 is required in the development of Th2 polarization, and plays a central role in exerting IL-4 mediated biological responses by serving as a gene transcriptional factor (31).

Our data demonstrate that STAT6 binds Foxp3, the gene transcriptional factor of TGF- β , to interfere with the TGF- β gene transcription in DCs. Others also found that STAT6 was involved in the suppression of Treg development, which promoted allergic responses (32). On the other hand, the data also show that SEB promotes TIM4 expression. The data are in line with previous reports, such as we and others showed that exposure to flagellin, another common microbial product, increased both TIM4 and STAT6 expression in mast cells (12). Takaki et al identified a silencer region

containing a STAT6 binding site in the T cell's Foxp3 promoter, which reduced TGF- β 1-mediated Foxp3 promoter activation and chromatin modification (31).

The data show that SEB increased the expression of Foxp3 in DCs. Since Foxp3 is the transcriptional factor of TGF- β , TGF- β is an important molecule in the immune tolerance (33), the data implicate that SEB can contribute to the immune tolerance at given concentrations. It is in line with previous reports, such as Zhang et al reported that SEB pre-treatment could promote mixed chimerism, which inhibited allogeneic cornea transplant rejection, possibly via deleting CD4⁺ and CD8⁺ T cells and acquiring donor-specific immunosuppression (11). Kurella et al reported that SEB could induce anergy of CD4⁺ T cells via transcriptional modulation of TCR, Notch and Wnt signaling pathways (34). However, opposite results against the present data were also reported. Administration of SEB together with antigens successfully induced allergy like reactions in animal models reported by our group (28) as well as by other investigators (35). The present data provide the explanation for this phenomenon.

The induction of immune tolerance by SEB only occurred in exposure to low doses of SEB. When the SEB doses were raised, it induced the expression of STAT6; the latter repressed the function of Foxp3 and increased the expression of TIM4 in DCs. The data and the reasoning are supported by that mixing SEB in the specific allergen vaccines enhanced SIT efficacy to inhibit the allergic reactions in the mouse intestine.

The data show that after exposure to lower doses of SEB, DCs showed lower levels of CD80 and CD86, which is the feature of immature DC. Immature DCs contribute to the development and maintenance of immune tolerance. Thus, the present data also indicate that lower doses of SEB contribute to maintaining DCs' immature status. In summary, the present data show that DCs have the capacity to express Foxp3. Exposure to SEB increases the expression of Foxp3 and TGF- β by DCs to confer DCs the tolerogenic property. Using as an adjuvant, SEB is capable of enhance the effect of SIT.

Declare of conflict of interest: None to declare.

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Author contribution:

XYL, LZX, XQL, XRG, ZQL, LTY, GY, SC, ZGL and LTY performed the experiments, analyzed data and reviewed the manuscript. HBL, TGL and PCY organized the study and supervised the experiments. PCY designed the project and wrote the paper.

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Figure legends

Fig 1. Effect of SEB on phenotyping CD4⁺ T cells in the intestine. A, the gated histograms indicate the frequency of proliferating CD4⁺ T cells after challenged with OVA or BSA in the culture for 4 days, which were isolated from the intestine of OVA/SEB (the SEB doses are denoted above each panel)-immunized mice. B, the bars indicate the phenotypes of the gated cells of panel A. C-E, the bars indicate the

protein levels of TGF- β (C), IL-4 (D) and IFN- γ (E) in extracts of mouse intestinal tissue.

The data of bars are presented as mean $\pm$ SD. *, $p < 0.01$, compared with the dose 0 group. Samples from individual mice were analyzed separately. Each group consisted of 6 mice. The data represent 6 independent experiments.

Figure 2. SEB modulates DC's phenotypes. A-C, The gated cells are TGF- β^+ DCs (A), CD11c $^+$ Foxp3 $^+$ DCs in the gated cells of panel A (B), and TIM4 $^+$ DCs (C) in LPMCs, which were isolated from 6 mice treated with SEB (similar to that in Fig. 1). D-G, the bars indicate the mRNA levels of TGF- β , Foxp3 and TIM4 in BMDC extracts (D); the Western blots indicate the protein levels of TGF- β and TIM4 in the DC extracts (E); the flow cytometry histograms indicate the frequency of CD80 $^+$ (F) and CD86 $^+$ (G) BMDCs. The BMDCs were cultured in the presence of SEB at gradient doses (0-400 ng/ml) for 4 days. &400: DCs isolated from STAT6-deficient mice were treated with SEB at 400 ng/ml. The data of bars are presented as mean $\pm$ SD. *, $p < 0.01$, compared with the dose 0 group. The data are representatives of 3 independent experiments.

Figure 3. Interaction between the SEB-induced STAT6 and Foxp3 in DCs. DCs were cultured in the presence of SEB at gradient concentrations for 48 h. A-B, DCs were collected and analyzed by ChIP assay. The bars indicate the levels of Foxp3 (A) and STAT6 (B) at the TGF- β promoter locus. C, DCs were treated with SEB at 50 ng/ml and 400 ng/ml respectively for 48 h. The DC extracts were analyzed by

immunoprecipitation (IP). The immune blots indicate the levels of the complex of Foxp3 and STAT6. D-E, wild and STAT6^{-/-} DCs (from the spleen of STAT6^{-/-} mice) were transfected with a luciferase-tagged TGF- β gene vector (or a control vector, #) and stimulated by SEB (400 ng/ml) in the culture for 48 h. The bars indicate the luciferase activities in the DC extracts (D) and TGF- β levels in the culture supernatant (E). F, wild and STAT6^{-/-} DCs were cultured with naive CD4⁺ T cells (DC: T cells = 1:1) and stimulated with SEB (400 ng/ml) in the culture for 3 days. The cells were analyzed by flow cytometry. The gated cells are CD25⁺ Foxp3⁺ T cells. The data of bars are presented as mean $\pm$ SD. *, p<0.01, compared with the dose 0 group (A, B) or saline group (D, E). The data are representatives of 3 independent experiments.

Figure 4. SEB-primed Foxp3-deficient DCs do not induce Foxp3⁺ TGF- β ⁺ T cells. A, the gated cells are CD4⁺ T cells after cultured with DCs (The types of DCs are denoted above each panel; T cell:DC = 1:1) for 72 h. B, the gated cells are Foxp3⁺ TGF- β ⁺ cells in the gated cells of panel A. C-D, the bars indicate the mRNA levels of Foxp3 (C) and TGF- β (D) in CD4⁺ T cells isolated from the cocultured cells of panel A. E-F, the immune blots indicate the protein levels of Foxp3 (E) and TGF- β (F) in CD4⁺ T cells isolated from the cocultured cells of panel A. Foxp3-d DC: Foxp3-deficient DC (prepared by RNAi). Control DC: DCs were treated with control shRNA. Data of bars are presented as mean $\pm$ SD. *, p<0.01, compared with the naïve DC group. The data are representatives of 3 independent experiments.

Figure 5. SEB-primed DCs modulate allergic reactions in the intestine. Sensitized mice were treated as denoted on the X axis of the bar graphs. After a challenge with the specific antigen OVA, the food allergy parameters in the mice were evaluated. The bars indicate the serum Th2 cytokines (A), serum specific IgE (B), intestinal mast cell/eosinophil infiltration in the intestinal mucosa (C), intestinal CD4⁺ T cell proliferation in the culture in the presence of the specific antigen OVA (D), decrease in the core temperature in the 30 min after the specific antigen challenge (E) and diarrhea mice number (F). DC-Foxp3 RNAi: DC-deficient mice were reconstituted with Foxp3-sufficient DCs. DC-control RNAi: DC-deficient mice were reconstituted with DCs treated with control RNAi. The data of bars are presented as mean $\pm$ SD. *, $p < 0.01$, compared to the naive mice group. #, $p < 0.01$, compared with the sensitized/saline group. Samples from individual mice were processed separately. Each group consisted of 6 mice.







