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CD4+ T cell responses in Balb/c mice with food allergy induced by trinitrobenzene sulfonic acid and ovalbumin

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Abstract. The rapid increase in atopic diseases is potentially linked to increased hapten exposure, however, the role of haptens in the pathogenesis of food allergy remains unknown. Further studies are required to elucidate the cluster of differentiation 4 positive (CD4+) T cell response to food allergy induced by haptens. Dendritic cells were primed by trinitrobenzene sulfonic acid (TNBS) as a hapten or ovalbumin (OVA) as a model antigen, in a cell culture model. BALB/c mice were sensitized using TNBS and/or OVA. Intestinal Th1/Th2 cell and ovalbumin specific CD4+ T cells proliferation, intestinal cytokines (interleukin-4 and interferon-γ) in CD4+ T cells were evaluated. TNBS increased the expression of T cell immunoglobulin and mucin domain-4 and tumor necrosis factor ligand superfamily member 4 in dendritic cells. Skewed Th2 cell polarization, extensive expression of interleukin-4, reduced expression of interferon-γ and forkhead box protein P3 were elicited following concomitant exposure to TNBS and OVA, with reduced regulatory T cells in the mouse intestinal mucosa, whereas a Th1 response was detected when challenged by TNBS or OVA alone. This data suggests that TNBS, as a hapten, combined with food antigens may lead to a Th2 cell response in the intestinal mucosa.

Introduction

The prevalence of food allergies have increased in the last few decades (1), however, the pathogenesis remains unknown. The 'hygiene hypothesis' postulated that limited exposure to bacterial and viral pathogens during early childhood results in an insufficient stimulation of T helper (Th) 1 cells, which in turn cannot counterbalance the expansion of Th2 cells, leading to a predisposition towards allergy (2,3). However, multiple environmental factors with currently unrecognized interactions contribute to the atopic status (4). The prevalence of atopic diseases may be closely associated with increased environmental pollution and industrialization (5,6). In addition to classical allergy-triggering factors, toxic environmental agents are increasingly implicated as causal factors in allergic diseases (7). The 'hapten-atopy hypothesis' suggested that oral and cutaneous exposure to environmental chemicals, and in particular to haptens, may have additionally contribute to the increased prevalence of atopic disease (8,9).

In contrast with protein antigens, haptens are low molecular weight (usually <500 Da) chemicals, which are able to covalently bind to peptides and proteins and thus alter their immunogenic profiles, with these modifications resulting in atopic diseases when recognized by the immune system. Allergic reactions with different Th cell phenotypes may occur when chemical haptens come into direct contact with the skin, via binding with skin proteins (10). In addition, certain drugs, such as haptens, induce adverse reactions including drug hypersensitivity, with this occurring in approximately 7% of the population when drugs are absorbed and combined with self-protein (11,12). Trinitrobenzene sulfonic acid (TNBS), as a chemical hapten, and is frequently used in the induction of atopic disease in the laboratory (13), and has been demonstrated to result in a T cell-mediated immune response in the colonic mucosa in susceptible mice when administered rectally (14,15). With the increase of atopic diseases, dietary hapten exposure has additionally increased via different means, including processed food, oral antibiotics, formula milk and drug use (8,16). If haptens do bind to dermal proteins, then it may possible that these haptens may additionally bind to food antigens (8).

Cluster of differentiation (CD)4+ T cells have a pivotal role in the initiation of the allergic response by activating B cells to produce antigen specific IgE. Th2 cells and associated cytokines, including interleukin (IL)-4, IL-5 and IL-13, are considered as key factors in the production of IgE antibodies, which can be crossregulated by Th1 cytokines such as interferon (IFN)-γ and IL-12 (17). T regulatory cells (Treg) serve an important role in

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maintaining oral immune tolerance and suppressing allergic sensitization to food allergen (18). As the body's largest immunologic organ, the gastrointestinal tract receives multiple daily exposure to a variety of proteins, chemicals and microorganisms (19). It remains unknown whether haptens are associated with the pathogenesis of food allergy, and further studies are required to elucidate the CD4+ T cell response and the cytokines in the intestine associated with the hypersensitivity response that is induced by haptens and food antigens. We have previously established a mouse model of food allergy induced by TNBS in the presence of a food antigen, ovalbumin (OVA). The aim of the present study was to further elucidate the CD4+ T cell response and their cytokine profile in the intestine. The present study indicated that skewed Th2 polarization and higher IL-4 expression in the intestinal mucosa were involved in the hapten induced food allergy.

Materials and methods

Reagents. RNeasy Mini kit was obtained from Qiagen, Inc. (Valencia, CA, USA). IScript™ cDNA Synthesis kit and SYBR Green Supermix were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). X-ray films were obtained from Kodak (Rochester, NY, USA). Antibodies against IL-4, IFN-γ, forkhead box protein P3 (Foxp3) were from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), while rat phycoerythrin (PE)-Cy7-conjugated-anti-mouse IL-4, rat PE-conjugated-anti-mouse IFN-γ, rat allophycocyanin (APC)-anti-mouse CD4, rat APC-anti-mouse CD11c (cat. no. 17-0114; used at 1:100 dilution), rat fluorescein isothiocyanate-conjugated anti-mouse CD86 (cat. no. 11-0862; used at 1:200 dilution), rat PET-conjugated anti-mouse T-cell immunoglobulin and mucin domain-containing molecule (TIM4; cat. no. 12-5866; used at 1:100 dilution as well as rat APC anti-mouse tumor necrosis factor ligand superfamily member 4 (OX40L; cat. no. 17-5905; used at 1:200 dilution) were obtained from eBioscience, Inc. (San Diego, CA, USA). The remaining reagents in the current study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Bone marrow derived dendritic cells (BmDCs) generation. Two male BALB/c mice (20-24 g), 6-8 weeks old were provided by Guangdong Medical Laboratory Animal Center (Guangzhou, China) and housed in animal cages at 25±1˚C with 65±5% humidity for at least one week prior to experiments. The mice were anesthetized with isoflurane (2%; Sigma-Aldrich) prior to sacrification by cervical dislocation. Following sterilization with 75% alcohol, the hind legs were opened and the femurs were separated with tweezers and scissors in a biosafety cabinet. Bone marrow cells were obtained by flushing the femurs of BALB/c mice with phosphate-buffered saline (PBS). The bone marrow cells were resuspended in lysis buffer for 2-4 min to lyse red blood cells. The remaining bone marrow cells were washed twice in Roswell Park Memorial Institute (RPMI) 1640 medium. Bone marrow cells were then cultured at $1x10^6$cells/well in RPMI 1640 medium with 10 ng/ml of recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. The culture medium was refreshed with fresh medium supplemented with GM-CSF and IL-4 on days 3 and 5. The cells were harvested on day 7. The purity of CD11c+ DCs was greater than 85%.

Effect of TNBS on the properties of DCs. BmDCs were cultured in the presence of TNBS (40 ng/ml), OVA (10 ng/ml) or lipopolysaccharide (LPS, 10 ng/ml) for 3 days. The cells were collected and analyzed by flow cytometry (BD FACSCanto II; BD Biosciences) for TIM4, CD86 and OX40L expression.

Mice and sensitization. A total of 42 male BALB/c mice (weight, 20-24 g; age, 6-8 weeks) were provided by the Guangdong Medical Laboratory Animal Center (Guangzhou, China) and were randomly divided into eight groups (16 mice for the establishment of the mouse model as shown in the schematic in Fig 2; 24 mice in another separate experiment, used for the analysis of $CD4^+$T-cell responses and the two mice remaining for isolation of bone marrow-derived dendritic cells as described above. Animals were fed on an OVA-free diet and kept at 25±1˚C with 65±5% humidity under a 12-h light/dark cycle (lights on between 07:30 and 19:30) for at least one week prior to experiments. All experimental procedures used in the present study were approved by the Ethics Committee for Animal Experimentation at Shenzhen Institute of Ear, Nose and Throat (Shenzhen, China). According to a previous study (20), the procedures to establish a food allergy model induced by TNBS and OVA are presented in Fig. 2a. The mixture of TNBS and OVA was prepared as follows: 1,000 μg TNBS and 1,000 μg OVA were dissolved in 10 ml sterile saline at 4˚C overnight using a magnetic stirrer. Following centrifugation, the supernatants were dialyzed to remove the extra TNBS. BALB/c mice (six mice/group) fed on an OVA-free diet were randomly divided into four groups: Control group; TNBS+OVAgroup; OVA group and TNBS group. Mice were sensitized by intraperitoneal injection (ip) with TNBS (1 mg/mouse), OVA (100 μg/mouse), or both TNBS and OVA on days 0, 1, 2, 3 and 4. Subsequently, mice were boosted with OVA (100 μg/mouse) in 0.1 ml of saline intragastrically on days 9, 11 and 13. Control groups were treated with normal saline (NS) by ip and then gavage. At 24 h following the last gavage, mice were sacrificed by cervical dislocation. Parameters of the intestinal hypersensitivity status were examined following previously described established protocols (21) which included: Levels of intestinal OVA-specific IgE antibody, intestinal histamine, numbers of mast cells, eosinophils and mononuclear cells in the lamina propria.

Assessment of intestinal CD4+ T cell phenotype and proliferation. Lamina propria mononuclear cells (LPMCs) were prepared following previously described procedures (10) with minor modifications. Briefly, jejunal segments were opened and washed with PBS, mucus was removed by the incubation with predigestion buffer for 20 min under rotation (40 x g) at 37˚C, following which the tissue was cut into small pieces and incubated with digestion buffer for 20 min under rotation (40 x g) at 37˚C. Following grinding into single cell suspensions with two sterile glass slides, cells were passed through a cell-strainer (100 μm). Resulting cells were centrifuged over a Percoll gradient to enrich for mononuclear cells. Cells ($1x10^6$/well) were cultured in the presence of OVA, and IL-4 and IFN-γ secreting cells were differentiated by flow cytometry. A proportion of cells ($1x10^6$/well) were also labeled with

Table I. Oligonucleotide sequences (5' to 3') of the forward and reverse primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward	Reverse
Interleukin-4	CCTCACAGCAACGAGAACA	ATCGAAAAGCCCGAAAGAGT
Interferon-γ	GGCCATCAGCAACAACATAA	TGAGCTCATTGAATGCTTGG
β-actin	CTGTCCCTGTATGCCTCTG	TGATGTCACGCACGATTT

carboxyfluoresceinsuccinimidyl ester (CFSE) (10 mmol/l) at 37˚C for 10 min. Labeling was stopped with 1 ml of autologous plasma and excess dye was washed away. CFSE-labeled cells ($1x10^6$/ml) were cultured with OVA (5 μg/ml) in RPMI 1640 media for 4 days. Following culture, cells were labeled with rat APC-conjugated anti-mouse CD4 (cat. no. 17-0041, 1:200 dilution; eBioscience, Inc.), rat PE-cy7-conjugated anti-mouse IL-4 (cat. no. 25-7042-82, 1:200 dilution; eBioscience, Inc.), rat PE-conjugated anti-mouse IFN-γ (cat. no. 12-7311; 1:200 dilution; eBioscience Inc.) and Rat Alexa Fluor® 647 anti-mouse Foxp3 (cat. no. 560401; 1:200 dilution; BD Biosciences, Franklin Lakes, NJ, USA), and Th1, Th2 and Treg cellular proliferation was assessed by flow cytometry (BD FACSCanto II; BD Biosciences).

Flow cytometry. BmDCs and LPMCs were collected from the culture and fixed with 1% formaldehyde and 0.1% Triton X-100) and permeabilization buffer if necessary for 30 min at 4˚C, washed with 1% bovine serum albumin (BSA)/PBS 3 times, and blocked for 30 min at 4˚C with 1% BSA. Cells were incubated with the indicated fluorescein-conjugated antibodies (10 μg/ml; rat PE-Cy7-conjugated-anti-mouse IL-4, rat PE-conjugated-anti-mouse IFN-γ, rat APC-anti-mouse CD4, rat APC-anti-mouse CD11c, rat fluorescein isothiocyanate-conjugated anti-mouse CD86, rat PET-conjugated anti-mouse TIM4 or rat APC anti-mouse OX40L) for 1 h at 4˚C. Following three washes, cells were resuspended in 400 μl PBS. The mean intensity of fluorescence was determined for 10,000 cells using a FACScan flow cytometer (BD Biosciences). An isotype IgG was used as a negative control. All experiments were performed a minimum of 3 times.

Western blot analysis. The total proteins were extracted from jejunal segments in protein extraction buffer, which consisted of 20 mmol/l Tris-Cl buffer (pH 7.5), containing 1 mmol/l ethylenediaminetetraacetic acid (EDTA), a protease inhibitor cocktail (complete, Mini, EDTA-free, 1 tablet in 10-ml buffer), 1% sodium dodecyl sulfate (SDS), 10% Triton X-100 and 2 mol/l dithiothreitol. Following 30 min on ice, the samples were centrifuged (17,600 x g, 10 min, 4˚C), and protein concentration of the resulting supernatant was measured using the Bradford method with BSA as a standard. Sample proteins were denatured in a 250 mmol/l Tris-Cl loading buffer (pH 6.8), containing 100 mmol/l EDTA, 2% SDS, 10% glycerol, 1% β-mercaptoethanol and bromphenol blue, heated at 100˚C for 10 min. Each aliquot was loaded in duplicate onto a 10% SDS-polyacrylamide gel and proteins were separated by electrophoresis, prior to transfer to nitrocellulose membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline (pH 8) for 1 h at room temperature and then incubated overnight at 4˚C with the following primary antibodies: Goat polyclonal anti-mouse IL-4 (1:200 dilution; cat. no. sc-1260), rat monoclonal anti-mouse IFN-γ (1:300 dilution; cat. no. sc-69910), or rat polyclonal anti-mouse Foxp3 (1:200 dilution; cat. no. sc-28705) (all from Santa Cruz Biotechnology, Inc.). Subsequently, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig)G (cat. no. sc-2006) or donkey anti-goat IgG (cat. no. sc-2020) (both at 1:5,000 dilution and from Santa Cruz Biotechnology, Inc.) secondary antibodies for 1 h at room temperature. Following washing, the hybridized bands were detected using enhanced chemiluminescence detection kits and Hyperfilm ECL reagents (cat. nos. GERPN2134 and GE28-9068-35, respectively; Sigma-Aldrich).

Reverse-transcription quantitative polymerase chain reaction(RT-qPCR). Jejunal mucosa was removed from intestinal tissue and total RNA was extracted using an RNeasy mini kit (Qiagen, Inc.). A total of 1 μg RNA was reverse transcribed into cDNA using the IScript™ cDNA Synthesis kit (cat no. 170-8891, Bio-Rad Laboratories, Inc.) according to the manufacturer's recommended protocol. The resulting complementary DNA was then subjected to RT-qPCR using the iQ™ SYBR® Green Supermix (cat. no. 1708880; Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. The primers used for the RT-qPCR amplification of IL-4, IFN-γ are presented in Table I, β-actin was used as an internal control. RT-qPCR reactions were conducted using a MiniOpticon thermal cycler (Bio-Rad Laboratories, Inc.) in triplicate. The amplification protocol was a follows: 1 cycle at 98˚C for 1 min followed by 40 cycles at 98˚C for 10 sec, 55˚C for 20 sec, 72˚C for 30 sec. A standard curve was generated for the determination of the linear range and amplification efficiency. The relative cytokine gene expression compared to a house keeping gene was analyzed by using the comparative quantification cycle method (22) according to the standard curve.

Statistical analysis. All values were presented as the mean ± standard deviation of a minimum of three independent experiments. The values were analyzed by one-way analysis of variance, followed by Tukey's test for multiple comparisons. SPSS 18.0 (International Business Machines, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

OX40L and TIM4 expression on DCs was regulated by TNBS. DCs serve a key role in the initiation and induction of T cell

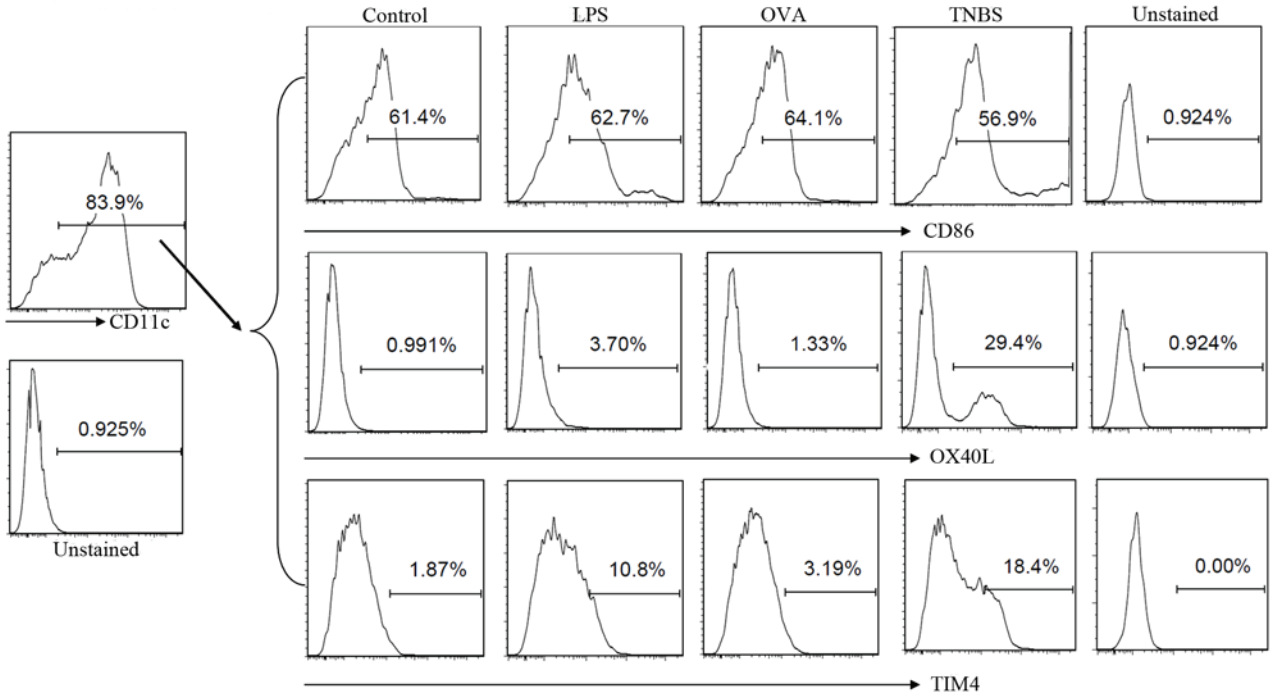

Figure 1. TNBS increased the expression levels of TIM4 and OX40L in BmDCs. BmDCs were prepared and exposed to saline (control), LPS, OVA or TNBS in culture for 72 h. Cells were collected and analyzed by flow cytometry. CD11c+ BmDCs were gated for further analysis. The histograms indicate the levels of CD86, OX40L and TIM4 expression in each group. Data are from 3 three independent experiments. TNBS, trinitrobenzene sulfonic acid; TIM4, T-cell immunoglobulin and mucin domain-4; OX40L, tumor necrosis factor ligand superfamily member 4 ; BmDCs, bone marrow derived dendritic cells; LPS, lipopolysaccharide; OVA, ovalbumin; CD, cluster of differentiation.

proliferation and differentiation. Following generation and cultured with LPS, OVA or TNBS, the CD11c+ BmDCs were gated by flow cytometry for further analysis. The expression of OX40L and TIM4 in the BmDCs increased following the addition of TNBS to the culture compared with the control. However, the CD86 expression was only slightly affected by the stimulation with TNBS (Fig. 1).

Skewed Th2 polarization was detected in the intestinal mucosa sensitized by TNBS and OVA. The skewed Th2 polarization in the intestine is considered as an important factor in the pathogenesis of food allergy, however, the CD4+ T cell response in hapten induced food allergy remains unclear. To elucidate the Th1/Th2 phenotypic response and the cytokine profile in the intestine following challenge with TNBS and OVA, a model of food allergy was generated (Fig. 2A). As presented in Fig. 2B-D, hypersensitivity status was induced in the intestinal mucosa, resulting in significant higher IgE (Fig. 1B), histamine expression levels (Fig. 2C) and increased infiltration of mast cells, eosinophils and mononuclear cells (Fig. 2D) following exposure to TNBS and OVA simultaneously, while there was no significant difference compared with control group when challenged with TNBS or OVA alone. The CD4+ IL-4+ T cells and CD4+ IFN-γ+ T cells in the LPMCs were detected by flow cytometry (Fig. 3). The frequency of the CD4+ IL-4+ T cells in mouse LPMCs from the TNBS+OVA group was increased 2-fold compared with the OVA and TNBS alone groups (Fig. 3A and C). The frequency of the CD4+ IFN-γ+ T cell phenotype in the mouse LPMCs from the TNBS+OVA group was greater than 3-fold lower compared with the OVA and TNBS alone groups (Fig. 3B and D). These results may indicate that there is a Th2 polarization in the intestine mucosa induced by TNBS combined with OVA.

TNBS combined with OVA promotes OVA specific Th2 proliferation in the intestine. To further identify the OVA specific Th2 proliferation in the intestinal mucosa, a population of the isolated LPMCs from each group was stained with CFSE and cultured for 4 days in the presence of the specific antigen (OVA, 5 μg/ml). Cells were collected following culture and analyzed by flow cytometry. The histograms show the results of the CFSE dilution, the gated portion is the proliferated cells (Fig. 4A). CD4+ IL-4+ or CD4+ IFN-γ+ T cells were gated for further proliferation analysis. The frequency of OVA specific CD4+ IL-4+ T cell proliferation was significantly increased in cells from the TNBS+OVA group (66.3%) compared with the OVA group (17.5%) or the TNBS group (16.8%; Fig. 4B and C), while the frequency of CD4+ IFN-γ+ T cell proliferation in the TNBS+OVA group showed a significant reduction (greater than 3-fold) in the LPMCs compared with the other two groups (Fig. 4B and D). Together, these results suggest that TNBS combined with OVA as a specific antigen may facilitate the differentiation of the Th2 phenotype in the intestine, while differentiation of Th1 cells can be elicited by TNBS or OVA alone.

Cytokine profiles were altered in the intestinal mucosa when challenged with both TNBS and OVA. IL-4 was selected to represent Th2-type cytokines and IFN-γ to represent Th1 cytokines. To further investigate the cytokine profile of CD4+ T cells in the intestine, the expression levels of IL-4 and IFN-γ

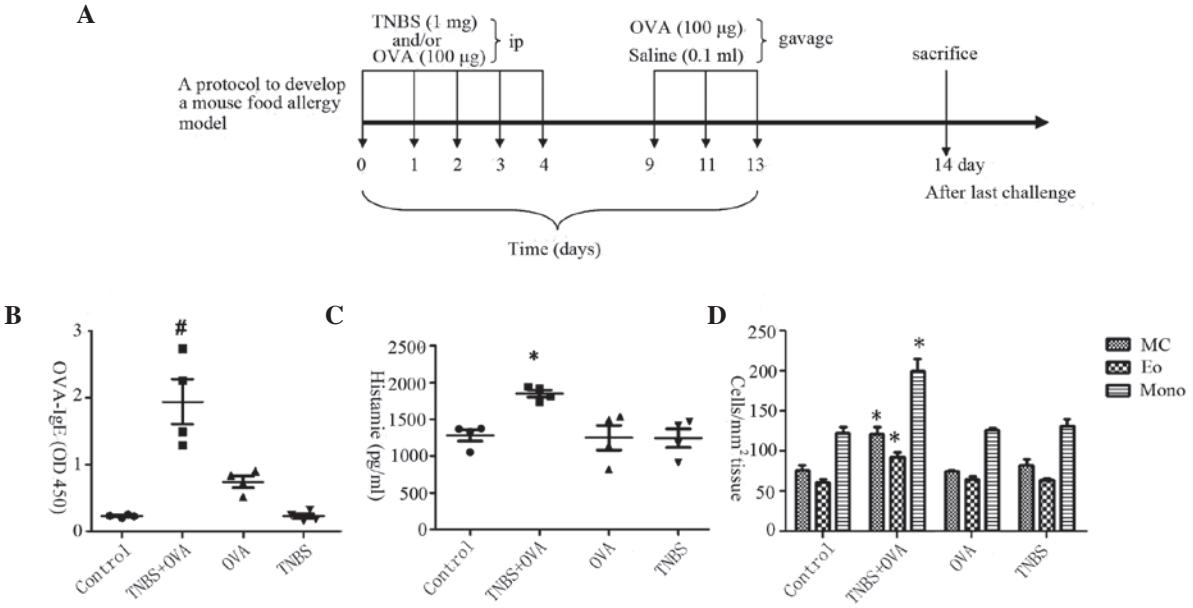

Figure 2. Hypersensitivity response induced in the small intestine. (A) Protocol to develop food allergy inflammation in the intestine. BALB/c mice (4 per group) fed on an OVA-free diet were randomly divided into four groups: Control; TNBS+OVA; OVA; and TNBS. Mice were sensitized by ip with TNBS (1 mg/mouse), OVA (100 μg/mouse), or both TNBS and OVA on days 0, 1, 2, 3 and 4. Mice were then boosted with OVA (100 μg/mouse) in 0.1 ml of saline intragastrically on days 9, 11 and 13. Control groups were treated with normal saline by ip and then gavage. At 24 h following the last gavage, mice were sacrificed by cervical dislocation. (B) Graphs presenting the levels of OVA-specific IgE in the intestinal tissue as measured by ELISA. (C) Graphs presenting the intestinal levels of histamine as determined by ELISA. (D) Numbers of MC, Eo and Mono cells in the intestinal mucosa. *P<0.05 vs. the control group, #P<0.01 vs. the OVA group. OVA, ovalbumin; TNBS, trinitrobenzene sulfonic acid; ip, intraperitoneal injection; IgE, immunoglobulin E; MC, mast cells; Eo, eosinophils; Mono, mononuclear cells.

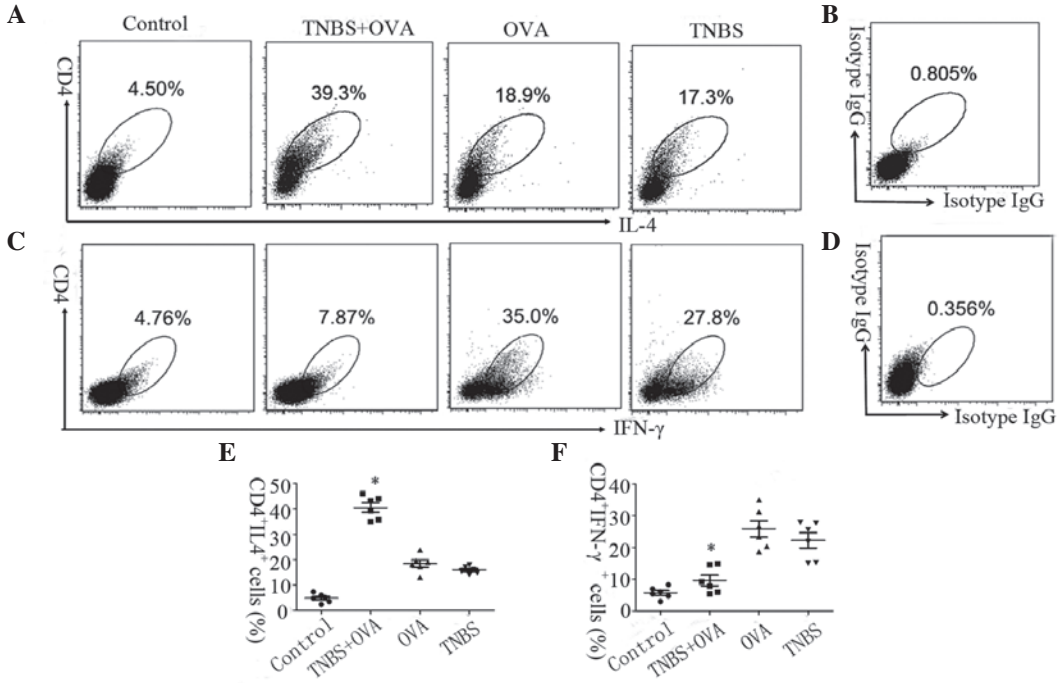

Figure 3. OVA specific Th2 and Th1 phenotype in the intestine. LPMCs were isolated from the small intestine of the mice and analyzed by flow cytometry following culture in the presence of OVA for 4 days. (A) Graphs the frequency of CD4+ and IL-4+ T cells. (B) The isotype control. (C) Graphs show the frequency of CD4+ and IFN-γ+ T cells in the LPMCs. (D) The isotype control. (E) Graphs show the individual data points of A. (F) Graphs show the individual data points of C. The data represent six separate experiments. *P<0.05 vs. the OVA or TNBS group. Th, T helper; LMPCs, lamina propria mononuclear cells; OVA, ovalbumin; CD, cluster of differentiation; IL, interleukin; IFN, interferon; TNBS, trinitrobenzene sulfonic acid.

in the intestinal extracts were measured by western blotting and RT-qPCR. The results indicated that the protein expression levels of IL-4 increased significantly in TNBS+OVA group compared with the OVA, TNBS and control groups (Fig. 5A). In comparison, the levels of IFN-γ were significantly lower in the TNBS+OVA group compared with the other groups

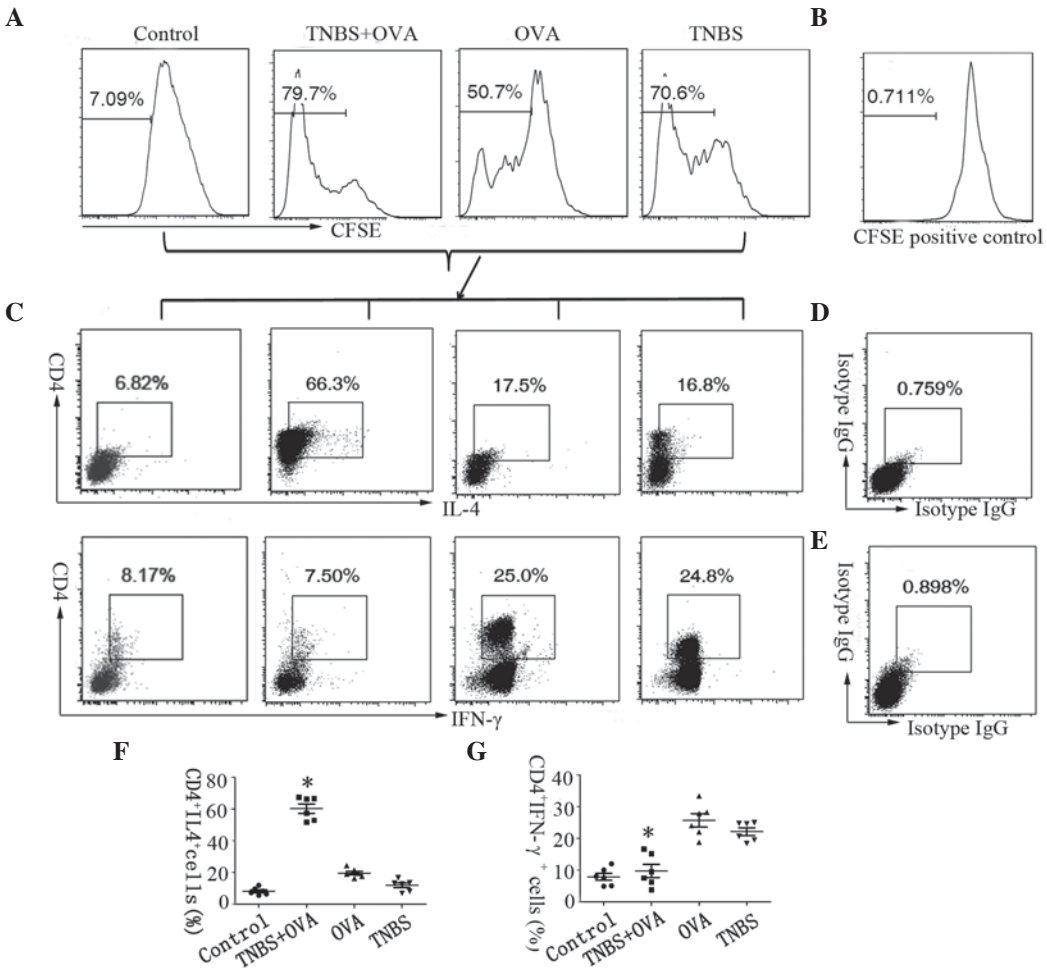

Figure 4. OVA specific Th2 and Th1 proliferation in the intestine. LPMCs were isolated from the small intestine of the mice and analysed by flow cytometry. Cells were stained with CFSE and cultured in the presence of OVA for 4 days. (A) Graphs showing the proliferation rate of LPMCs, (B) shows the CFSE positive control. (C) Plots showing the frequency of CD4+ and IL-4+ cells and CD4+ and IFN-γ+ cells in the LPMCs, (D and E) show the isotype controls. (F) Graph showing CD4+ IL-4+ cells. (G) Graph showing CD4+ IFN-γ+ cells. The data represent six separate experiments. *P<0.05 vs. the OVA or TNBS alone group. OVA, ovalbumin; Th, T helper; LMPCs, lamina propria mononuclear cells; CFSE, carboxyfluoresceinsuccinimidyl ester; CD, cluster of differentiation; IL, interleukin; IFN, interferon; TNBS, trinitrobenzene sulfonic acid; IgG, immunoglobulin G.

(Fig. 5B). There was increased IL-4 mRNA expression and reduced expression of IFN-γ mRNA in the intestinal mucosa of the mice in the TNBS+OVA group, with this difference significant (P<0.05) when compared with the control group (Fig. 5C and D). This may indicate that there was a skewed Th2 phenotype cytokine response in the intestine when challenged with TNBS combined with OVA, while this response was not elicited when challenged with TNBS or OVA alone.

OVA specific Treg proliferation and Foxp3 expression in the intestine. Tregs are a subpopulation of T cells which maintain oral-tolerance and downregulate the immune system. The Foxp3 gene is identified as the master transcriptional factor of Tregs, and serves an important role in the development and function of regulatory T cells (23). Therefore, the present study determined the Treg proliferation in the intestinal mucosa by FACS and measured Foxp3 expression in the intestinal extracts by western blotting. The histograms show the CFSE dilution and the proportion of proliferated cells gated (Fig. 6A). CD4+ Foxp3+ cells were gated for further proliferation analysis. The rate of proliferation of OVA specific Tregs was significantly reduced in LPMCs from the TNBS+OVA group compared with the control, OVA and TNBS alone groups (Fig. 6B and C). The expression of Foxp3 in the mouse intestinal tissues from the TNBS+OVA group was significantly reduced compared with the other groups (Fig. 6D). This may indicate that there is a functional deficiency in Tregs in the intestine induced by TNBS and OVA.

Discussion

With the increase in the incidence of atopic diseases, there has additionally been an increase in dietary hapten exposure (16). The 'hapten-atopy hypothesis' suggests that oral and cutaneous exposure to environmental haptens may contribute to the increase of atopic disease (24). Further studies are required to elucidate the role of haptens in the pathogenesis of food allergy. A previous study indicated that in an established mouse model of food allergy induced by TNBS, as a typical hapten, in the presence of a food antigen OVA, there was a hypersensitive status in the intestine, with higher OVA specific IgE, histamine expression and increased infiltration of inflammatory cells. In the present study, the CD4+ T cell response and the cytokine profile in the intestine were investigated. The results indicated that TNBS is able to facilitate the expression of TIM4 and

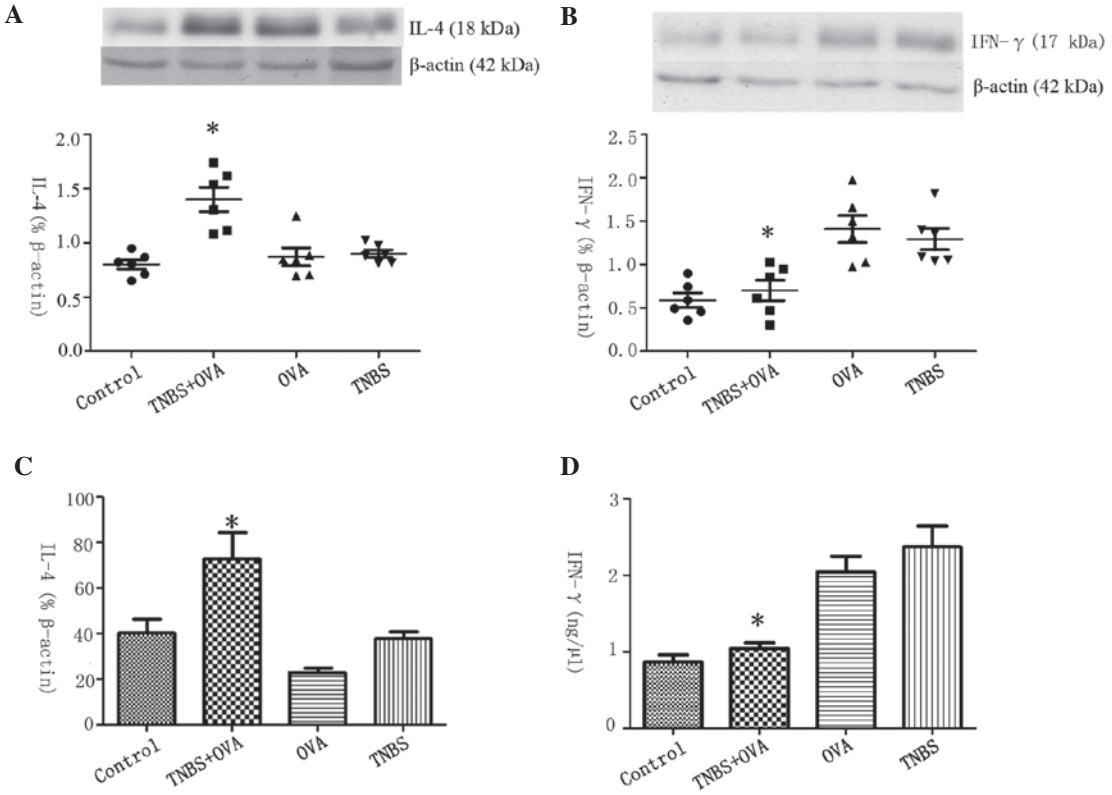

Figure 5. Protein and mRNA expression levels of IL-4 and IFN-γ in the intestine. Protein was extracted from tissue from the small intestine. IL-4 and IFN-γ expression levels were measured by western blotting. The immunoblots indicate the protein levels of (A) IL-4 and (B) IFN-γ. The dot plots show the integrated density of the protein bands normalized to β-actin. Total RNA was extracted from the intestinal tissue. (C) IL-4 and (D) IFN-γ relative expression levels were measured by reverse transcription-quantitative polymerase chain reaction. Values are presented as the mean ± standard deviation. Data are the mean of six separate experiments. *P<0.05 vs. OVA or TNBS alone group. IL, interleukin; IFN, interferon; OVA, ovalbumin; TNBS, trinitrobenzene sulfonic acid.

OX40L on DCs, with skewed Th2 polarization and increased IL-4 expression in the intestinal mucosa observed to be induced by TNBS in the presence of OVA, with a reduction in Tregs and the expression of Foxp3. This may indicate that haptens combined with food antigens may facilitate Th2 phenotypic differentiation in the intestinal mucosa, which is different from a Th1 response caused by TNBS alone.

Haptens are small molecules that are able to elicit an immune response only when attached to a large carrier such as a protein (25,26). Hapten sensitization is common in 2-15% of allergic emergencies that are caused by chemicals (25). Cutaneous exposure to haptens may produce immune responses with different Th cell phenotypes according to different hapten exposure regimen and doses (27). Allergic contact dermatitis is generally regarded as a delayed-type hypersensitivity reaction which is mediated by selective Th1 cells (28). Atopic dermatitis is characterized by preferential Th2 cell responses with a Th2 cytokine shift (29). The identity of the T cell response and cytokine profile following hapten exposure via other surfaces, such as the intestinal tract or airways, remains unknown. The results of the present study demonstrate that there was a skewed Th2 phenotype, with proliferation of OVA-specific Th2 cells in the intestinal mucosa when concurrently exposed to TNBS and OVA as a food antigen, while the skewed Th2 response in the intestine was not elicited following exposure to TNBS or OVA alone. This may provide evidence for the first time that haptens may also covalently bind to food antigens and thus alter their immunogenic profile and serve an important role in initiating a Th2 response in the intestine. In addition, the mechanism of TNBS-induced Th2 polarization and whether DCs can be activated by TNBS remain unclear. The present study indicates that TNBS is able to stimulate DCs to express TIM-4 and OX40L, which are the ligands of TIM1 and CD134, respectively, on T cells that enable the amplification of Th2 cell differentiation.

A key feature of food allergy is a Th2-predominant allergen-specific immune response, with the production of allergen specific IgE antibodies (30). As a Th2 cytokine, IL-4 has the effect of regulating B cell growth, T cell growth and function, and thus is a critical factor for the development of Th2 type responses (31), while it can be antagonized by the Th1 type cytokine, IFN-γ. In the present study, the cytokine expression levels in the intestine were investigated, with the results indicating increased IL-4 expression and reduced IFN-γ expression levels following exposure to TNBS and OVA simultaneously. This may indicate that TNBS can facilitate the intestinal sensitization to luminal antigens. As with hapten-induced atopic dermatitis, the results of the current study show that the exposure of haptens in the presence of food antigens in the intestinal tract may additionally be characterized as a Th2 response with increased Th2 cytokines. Tregs serve a critical role in the maintenance of immune homeostasis in the body. Reductions in Treg numbers and the impairment of Treg function have been noted in patients with allergic diseases by an unknown mechanism (32). Tregs may prevent immunopathological reactions and maintain peripheral tolerance to haptens by acting via a cell-to-cell contact mechanism (33). In the present study, the results

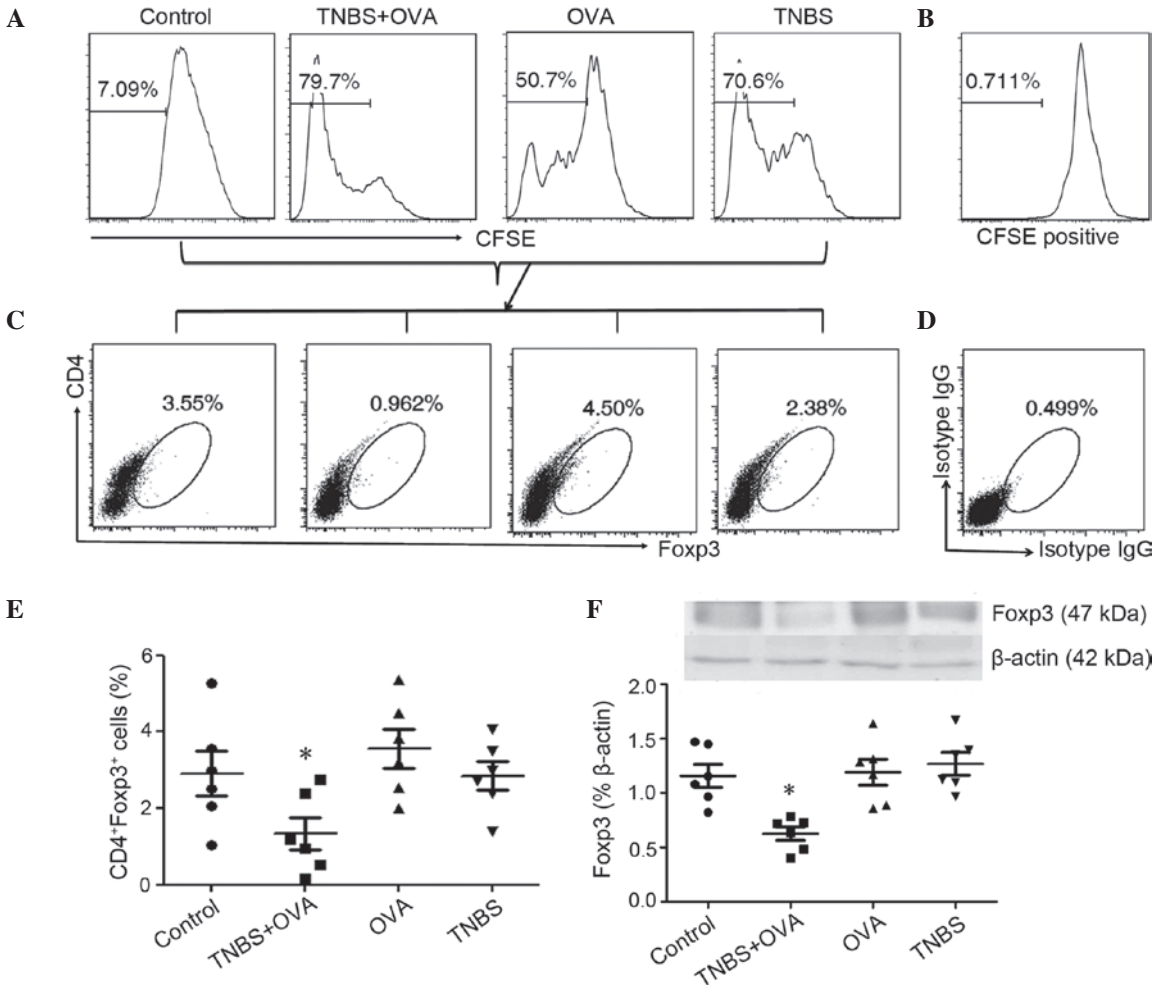

Figure 6. OVA specific Treg proliferation and Foxp3 expression in the intestine. LPMCs were isolated from the small intestines of the mice and analysed by flow cytometry. Cells were stained with CFSE and cultured with OVA for 4 days. (A) The graphs show the proliferation rate in LPMCs, (B) shows the CFSE positive control. (C) The plots show the frequency of CD4+ and Foxp3+ cells in the LPMCs, (D) shows the isotype controls. (E) Graph showing the CD4+ Foxp3+ cells. (F) Foxp3 expression levels in the intestinal tissue was measured by western blotting. Values are presented as the mean ± standard deviation. The data represent six separate experiments. *P<0.05 vs. the OVA or TNBS alone group. OVA, ovalbumin; Treg, regulatory T cells; Foxp3, forkhead box protein P3; LMPCs, lamina propria mononuclear cells; CFSE, carboxyfluoresceinsuccinimidyl ester; CD, cluster of differentiation; TNBS, trinitrobenzene sulfonic acid; IgG, immunoglobulin G.

indicated that OVA-specific Treg proliferation was reduced, and, as a marker of Tregs, Foxp3 expression was reduced in the intestine following the challenge with TNBS in the presence of OVA compared with the other groups. This suggests that there is a functional deficiency in Tregs in the intestinal mucosa when concurrently exposed to haptens and food antigens.

TNBS-induced colitis is generally regarded as Th1 cell-mediated inflammation (14,15), while another previous study reported that hapten-induced colitis may additionally display features of intestinal hypersensitivity which may be observed during food allergy (34). Consistent with previous studies, a Th2 cell-mediated hypersensitivity in the intestine was elicited in the present study following the mice being challenged with TNBS and OVA via intraperitoneal injection and subsequent treatment with OVA as a specific food antigen via gavage. These data imply that TNBS may possess an adjuvant-like effect on the intestinal allergic reaction to food antigens when treated via intraperitoneal injection, while it may result in a Th1 response in the intestinal mucosa by itself. This may be supported by that haptens may act as immune response-stimulating adjuvants and immune response-steering adjuvants to alter the type of Th1/Th2 cellular response (35). Further studies are required to reveal the mechanisms associated with the adjuvant effect.

In summary, the data indicated that a skewed Th2 polarization and higher IL-4 expression with reduced Tregs in the intestinal mucosa were involved in the TNBS-induced food allergy. This may provide novel insight into the study of the pathogenesis of food allergy occurring as a result of environmental haptens.

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