

GASTROENTEROLOGY

Vagal activities are involved in antigen-specific immune inflammation in the intestine

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Key words

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Abstract

Background and Aims: The mechanism of intestinal immune inflammation, such as food allergy, remains to be further understood. The present study aims to investigate the role of the vagal nerve in the pathogenesis of skewed T-helper 2 (Th2) responses in the intestine.

Methods: The expression of the immunoglobulin E (IgE) receptor on the vagus nerve in the mouse intestine was observed by immunohistochemistry. Vagus ganglion neurons (VGN) were isolated from mice and cultured in vitro. The IgE receptor/IgE complex on vagus neurons was examined by immune precipitation assay. A food allergy mouse model was developed; the effect of the partial removal of the vagal nerve (PRVn) via surgery or administration with anticholinergic agents on the suppression of Th2 inflammation was evaluated.

Results: The high-affinity IgE receptor was detected on the intestinal vagus nerve. An increase in the expression of the IgE receptor on the vagus nerve was observed in the intestines of mice with intestinal immune inflammation. Isolated mouse VGN express IgE receptor I, which could form complexes with IgE. Re-exposure to specific antigens activated the sensitized VGN, manifesting the release of transmitter glutamate that could activate dendritic cells by increasing the expression of CD80 and major compatibility complex class II and suppressing interleukin-12. The PRVn suppressed Th2 inflammation in the intestine.

Conclusions: The intestinal vagus nerve in mice expresses a high-affinity IgE receptor. An antigen-specific immune response can activate the vagus nerve in the intestine and induces the release of transmitters to modulate dendritic cell phenotypes that facilitate the development of skewed Th2 polarization in the intestine.

Introduction

The prevalence of intestinal inflammation, such as food allergy and inflammatory bowel disease (IBD), keeps rising especially in the past two to three decades.^{1,2} The mechanisms are poorly understood. The therapeutic effect of intestinal inflammation is not satisfactory currently. Therefore, it is important to have fresh ideas and novel therapeutic remedies for the treatment of intestinal inflammatory disorders.

Partial removal of the vagal nerve (PRVn) is initially performed for peptic ulcer disease.³ PRVn is currently being researched as a less invasive alternative weight loss procedure to gastric bypass surgery.⁴ It is proposed that the hypersensitivity of the vagus nerve plays a critical role in the pathogenesis of intestinal immune inflammation⁵ and therefore, PRVn is also proposed to treat intricate intestinal inflammation, such as IBD;⁶ however, the mechanism is not fully understood as yet. In addition to the surgical removal of the vagal nerve, chemical agents have also been employed in experimental vagotomy.

The vagus nerve is the 10th of the 12 cranial nerves. Upon leaving the medulla between the olivary nucleus and the inferior cerebellar peduncle, it extends through the jugular foramen, and then passes into the carotid sheath between the internal carotid artery and the internal jugular vein down below the head, to the neck, chest, and abdomen, where it contributes to the innervation of the viscera. It is the major parasympathetic nerve in the gastrointestinal tract. Acetylcholine is the major transmitter. Activating the vagus nerve increases the digestive function of the gastrointestinal tract.

Recent publications indicate that the vagus ganglion neurons (VGN) express high-affinity immunoglobulin (Ig) E receptors.^{7,8} Antigen-specific immune reactions are involved in immune inflammation in the intestine.⁹ Based on this information, we hypothesize that antigen-specific allergic reactions on the vagus nerves play a role in the pathogenesis of the intestine. Therefore, in this study, using a mouse food allergy model and a cell culture model, we observed the expression of the IgE receptor on the intestinal vagus nerves, identified an IgE receptor/IgE complex on

the surface of VGN, and observed the effect of PRVn on the suppression of antigen-specific immune inflammation in the intestine.

Methods

Reagents

A glutamate ELISA kit was purchased from Usen Life Science (Wuhan, China; sensitivity = 10 pg/mL). An OVA-specific IgE ELISA kit was purchased from AbD Serotec (Shanghai, China; sensitivity = 2 ng/mL). Biotinylated antimouse FcεRI antibody was purchased from eBioscience (San Diego, CA, USA), and antimouse choline acetyltransferase (ChAT) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipopolysaccharide (LPS) and atropine were purchased from Sigma Aldrich (Shanghai, China). The HotStarTaq Master Mix kit was purchased from QIAGEN (Hong Kong, China). The iScript cDNA Synthesis kit was purchased from Bio-Rad (Shanghai, China).

Animals

Balb/c mice (male, body weight: 25–30 g, 4–6 weeks old) were purchased from Beijing Experimental Animal Institute (Beijing, China). The mice were maintained in a pathogen-free environment and had free access to food and drink. The procedures in the animal study were approved by the Animal Care Committee at The Third Military Medical University (Chongqing, China).

Mouse model of intestinal inflammation

Balb/c mice were subcutaneously injected with ovalbumin (OVA, 0.1 mg/mouse) mixed with 0.1 mL alum on days 0 and 3. The mice were administered 0.3 mL saline (1 mg/mouse) via gavage feeding on days 9, 11, and 13. The mice were killed on day 14.

Evaluation of intestinal immune inflammation

Clonic segments were excised and processed to cut paraffin sections. Sections were stained with hematoxylin–eosin. Cell numbers of mast cells, eosinophils, and mononuclear cells were counted on each section under a light microscope. Levels of interleukin (IL)-4 and OVA-specific IgE in colonic protein extracts were assessed by ELISA. Antigen-specific lymphocyte proliferation was evaluated by carboxyfluorescein succinimidyl ester (CFSE) dilution assay.

PRVn

Following published procedures,¹⁰ a group of mice underwent laparotomy under general anesthesia. The subdiaphragmatic branches of the vagal nerve were identified at the abdominal esophagus and cut. The group was designated as the PRVn group. In the sham operation, the subdiaphragmatic vagal nerve was dissected free, but not cut. Another group of mice was treated with cholinergic antagonist, atropine (saline was used in the control group), at a dose of 10 μg/kg body weight (ip), 30 min prior to each exposure to the specific antigens.

Nodose ganglion neuron culture

Mouse nodose ganglion neurons were isolated and cultured following published procedures.¹¹ Briefly, after decapitation, nodose ganglia were excised and incubated for 30–50 min in phosphate-buffered saline (PBS) containing 0.3% trypsin and 0.1% collagenase at 32°C. Throughout the entire procedure, the medium was continuously saturated with a 95% O₂/5% CO₂ gas mixture (pH 7.4). Neurons were isolated by trituration, plated on Petri dishes, and cultured with Dulbecco's modified Eagle's medium, containing 10% heat-inactivated fetal bovine serum and 5 mg/mL insulin.

Immunohistochemistry

Intestinal cryosections were fixed with acetone for 20 min, blocked by 1% bovine serum albumin for 30 min, incubated with the first antibodies (biotinylated antimouse FcεRI [1:300] and goat antimouse ChAT [1:100]) overnight at 4°C; after washing with PBS, the sections were incubated with streptavidin–fluorescein isothiocyanate (1:300) and anti-goat IgG–Cy5 (1:200) for 1 h at room temperature. The sections were washed with PBS, mounted with coverslips, and observed under a confocal microscope. The positive immune staining on each image was quantitatively analyzed by Adobe Photoshop CS5 (San Jose, CA, USA) and normalized to the percentage of the naïve group.

ELISA

Levels of IgE/IL-4 in the intestinal protein extracts and the level of glutamate in the culture supernatant were evaluated by ELISA with commercial reagent kits. The procedures followed the manufacturers' instructions.

Immune precipitation and Western blotting

In immune precipitation, the VGN were collected and lysed in lysis buffer (10 mM Tris-HCl, pH 8.0; 1 mM ethylenediaminetetraacetic acid [EDTA]; 140 mM NaCl; 1% Triton; 0.1% sodium dodecylsulfate [SDS]; and 0.1% Na-Deoxycholate). Protein lysates were incubated overnight at 4°C with either anti-FcεRI or anti-IgE. Protein G beads were then added for 6 h at 4°C. After several washes in lysis buffer, the beads were resuspended in loading buffer, and SDS–polyacrylamide gel electrophoresis (PAGE) was performed under non-reducing conditions. Goat IgG antibodies were used as the control. The membrane was again incubated with either anti-FcεRI or anti-IgE. The immune blots were processed, as described later.

In Western blotting, cellular extracts were prepared. Equal amounts of protein (50 μg/well) were separated by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were incubated with biotin-labeled primary antibody against FcεRI at a concentration of 1 μg/mL overnight at 4°C. Peroxidase–avidin was added at a concentration of 10 ng/mL to the membranes for 1 h at room temperature. The membranes were then developed by ECL Plus Western blotting detection reagents (GE Healthcare Life Science, Baie d'Urfe, QC, Canada), and the signal was detected by X-ray film.

Reverse transcription–polymerase chain reaction

Total cellular RNA was extracted using the iScript cDNA Synthesis kit from 1×10^7 vagus neurons. cDNA was synthesized from 2 µg RNA using the HotStarTaq Master Mix kit. The primers and conditions of FcεRIα were: forward, ccttcctgctatgggaaca; reverse, gtctgaaggagcagccaatc (NCBI: NM_010184). The annealing temperature was 60°C, the time was 30 s, and it ran for 35 cycles.

Preparation of the lamina propria mononuclear cells

The small intestinal segments were excised from the mice after they were killed and cut into $2 \times 2 \times 2$ mm pieces. The tissue was treated with 1 mmol/L EDTA for 1 h at 37°C with stirring, and then treated with type IV collagenase (1 mg/mL) for 2 h at 37°C with stirring. The resulting cell suspension was filtered through a 100-µm cell strainer first, then through a 40-µm cell strainer. After centrifugation, gradient density centrifugation with Percoll was performed on the cells. The cells were then washed twice, and resuspended in RPMI-1640 medium containing 10% fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The viability of the cells was checked with the trypan blue exclusion method; only those with viability exceeding 90% were used in the experiments.

T-cell proliferation assay

CD4⁺ T cells were first positively separated by magnetic cell sorting (MACS) (Miltenyi Biotech, Auburn, CA, USA) (purity was >95%, as verified by flow cytometry) from isolated lamina propria mononuclear cells (LPMC) and stained with CFSE. CD11c⁺ dendritic cells (DC) were isolated from LPMC by MACS, and co-cultured with CD4⁺ T cells (DC : T cell = 1:10) for 3 days in the presence of specific antigen, OVA. The CFSE-dilution assay was performed by flow cytometry.

Generation of bone marrow-derived DC

The bone marrow was taken from the femurs of naïve Balb/c mice. The cells were treated with red blood cell lysis buffer (8.3 g NH₄Cl, 1 g KHCO₃, and 1.8 mL of 5% EDTA in 1000 mL distilled water). The mononuclear cells were cultured in RPMI-1640 medium containing 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 20 ng/mL granulocyte macrophage colony-stimulating factor, and 20 ng/mL IL-4. The unattached cells were removed from the culture on day 2; the attached cells continued to be cultured until the sixth day when the cells were collected, checked by flow cytometry for the phenotypes first, and then used for further experiments. As shown by flow cytometry, over 90% cells were CD11c and major histocompatibility complex class II (MHCII) positive. The levels of CD80, CD86, and MHCII were much lower than control bone marrow-derived DC (BmDC) that were stimulated by LPS (added at the beginning of culture).

Spleen mononuclear cell preparation

Spleen cell suspensions were obtained from 8- to 10-week-old male mice. Red blood cells were lysed with 0.15 M NH₄Cl lysis

buffer, and the remaining mononuclear cells were washed with culture medium for further experiments.

Statistical analysis

Parametric data are expressed as mean $\pm$ standard deviation. Means were analyzed by Student's *t*-test between two groups, and ANOVA analysis in more than two groups. *P* < 0.05 was set as the significant criterion.

Results

PRVn inhibits mouse intestinal T-helper 2 immune inflammation

After re-exposure to specific antigen, OVA, the sensitized mice showed immune inflammation in the intestines. The inflammatory phenomena included abundant inflammatory cell infiltration in the jejunal mucosa (Fig. 1), increases in the levels of IL-4 and antigen-specific IgE in jejunal protein extracts, and marked CD4 T-cell (isolated from the small intestine) proliferation upon exposure to specific antigen, OVA, in culture. Mice treated with PRVn, or pretreatment with atropine, showed a much less extent of inflammation in the jejunum (Fig. 1). The results indicate that vagal activities are involved in the skewed T-helper (Th) 2 responses in the intestine.

Vagus expresses IgE receptors in the intestine

A recent publication indicated that the high-affinity IgE receptor can be expressed by neural tissue.⁸ Thus, the PRVn that inhibits intestinal immune inflammation might be via a reduction of the number of IgE receptors in the intestine. To test the hypothesis, we stained the jejunal sections with antibodies against the cholinergic marker ChAT and IgE receptor FcεRI. Even in naïve mice, the colocalization of immune-positive staining of both ChAT and FcεRI could be seen in the jejunum. The expression of FcεRI on the vagus was markedly increased in the jejunum of mice with food allergies. The immune-positive products of ChAT and FcεRI were significantly reduced in mice treated with PRVn/sensitization (Fig. 2). The results indicate that intestinal vagus expresses the IgE receptor FcεRI, which can be attenuated by PRVn.

VGN express FcεRI that can be bound by the IgE antibody

To further characterize the expression of FcεRI in the intestinal vagus nerve, VGN were isolated and cultured. The VGN constitutively express FcεRI on the surface, as shown by confocal immune microscopy; the results were confirmed by real-time reverse transcription–polymerase chain reaction and Western blotting (Fig. 3). To see if FcεRI on the VGN is involved in an antigen–antibody reaction, mouse sera (obtained from mice sensitized to OVA; IgE titer: 1:512, as tested by passive cutaneous anaphylactic assay) was added to the VGN culture at a dose of 0.1 mL/mL media for 6 h. As shown by immune precipitation assay, an immune complex of FcεRI and IgE was formed on the VGN (Fig. 3). The results imply that VGN have an anatomical basis to be involved in the antigen–antibody reaction.

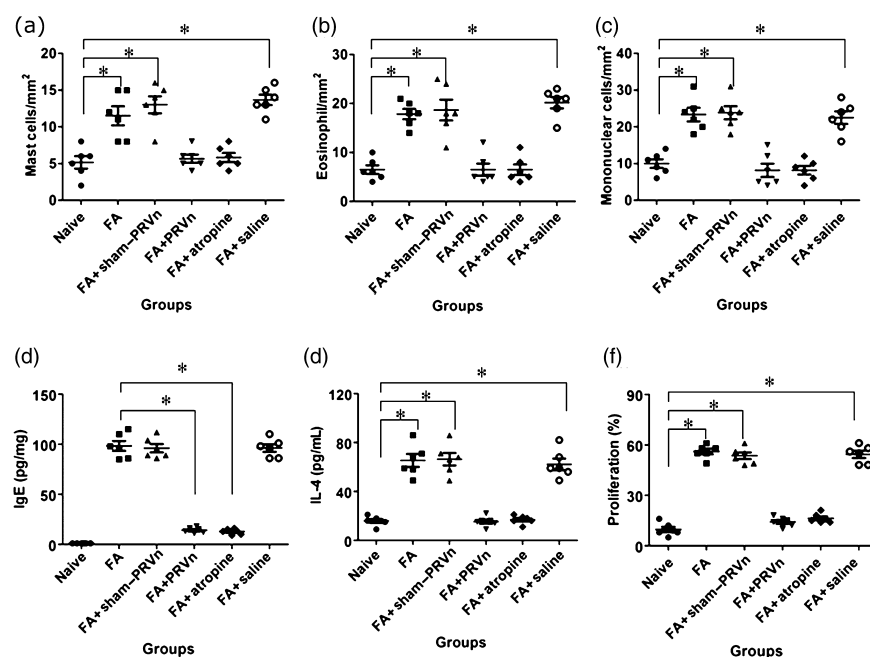


Figure 1 Partial removal of the vagal nerve (PRVn) or blocking cholinergic receptor inhibits immune inflammation in the intestine. (a–c) Scatter dot plots show cell counts in the intestinal sections. Each dot represents a single datum from one mouse that is an average number from the counts of 20 high-power fields ($\times 200$). (d–e) Scatter dot plots indicate the levels of antigen-specific immunoglobulin E (IgE) (d) and interleukin-4 (IL-4) (e) in the intestinal protein extracts. (f) Scatter dot plots indicate the proliferation rate (%) of CD4 T cells from the small intestine. Atropine, mice pretreated with atropine (250 ng/mouse; ip, 30 min prior to each exposure to specific antigen); FA, mice with food allergy; naïve, naïve mice; PRVn, mice treated with PRVn; saline, mice treated with saline; sham, mice treated with sham operation. $*P < 0.01$.

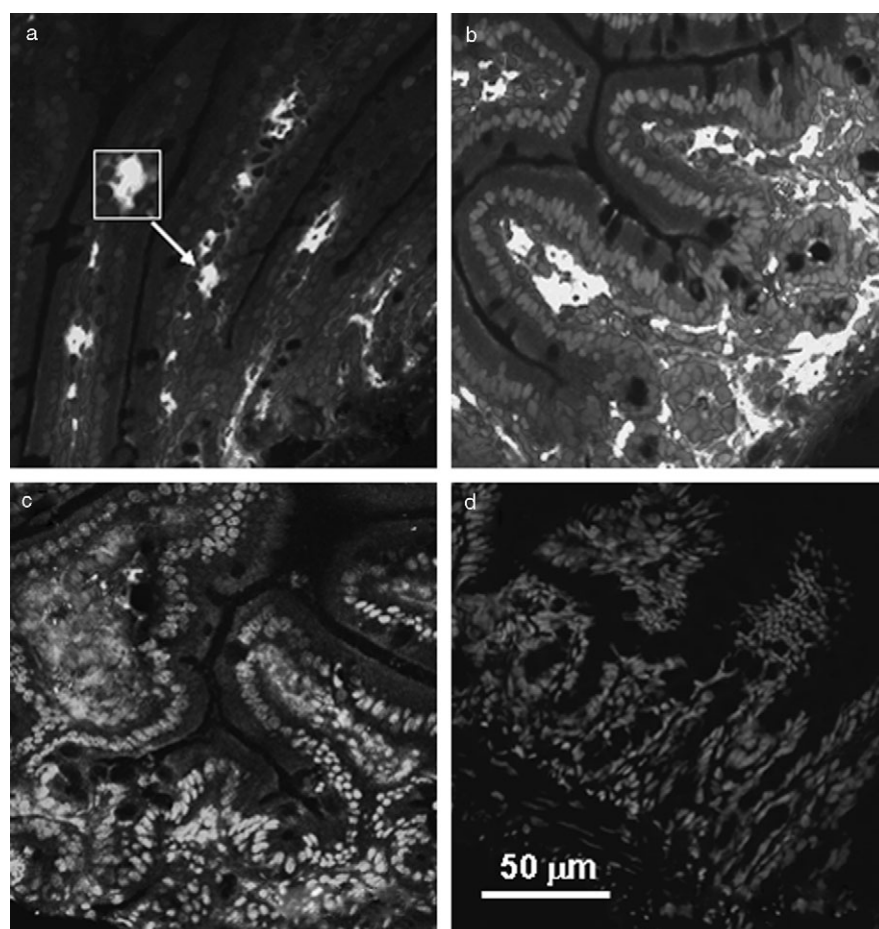


Figure 2 Immunoglobulin (Ig) E receptor expression on vagal neurons and fibers is increased in the intestines of mice with food allergies. (a–c) Representative confocal images show immune positive staining of IgE receptor I and choline acetyltransferase in the jejunal tissue. (a) naïve; (b) food allergy (FA); (c) FA/partial removal of the vagal nerve; (d) isotype IgG. (d) Staining with isotype IgG used as a negative control.

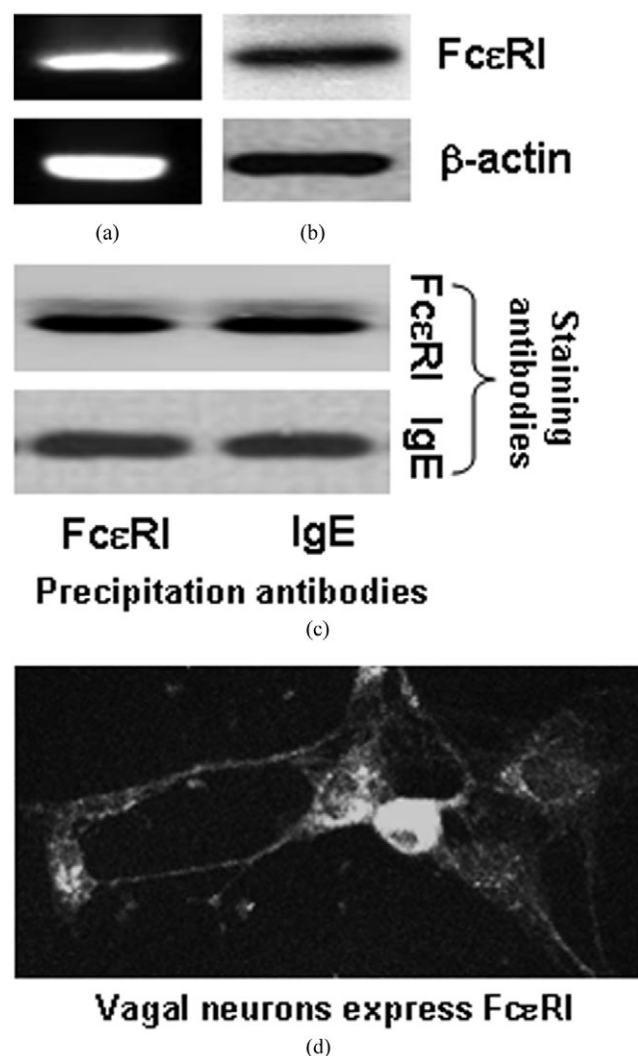


Figure 3 Vagus neurons express the immunoglobulin E (IgE) receptor. Vagus neurons were isolated and cultured for 2 weeks. (a) Agarose gel bands show the mRNA of FcεRI expression in cultured vagus neurons. (b) Western blotting gel bands show the FcεRI protein from cultured vagus neuron extracts. (a,b) β-actin is used as the internal control. (c) Immune blots show that the complex of FcεRI/IgE on vagus neurons was detected by immunoprecipitation assay. (d) Representative confocal image shows the FcεRI immune-positive staining (white) in vagal neurons.

Interaction of antigen–IgE–FcεRI triggers VGN to release mediators

As VGN express FcεRI (Fig. 3), we determine whether an IgE–FcεRI interaction can induce VGN to release mediators. To this end, we generated VGN, primed the cells with OVA-specific IgE, and exposed the cells to OVA in the culture. As expected, there was a marked increase in the level of glutamate detected in the culture medium (Fig. 4). The results indicate that antigen–antibody-specific responses can activate VGN.

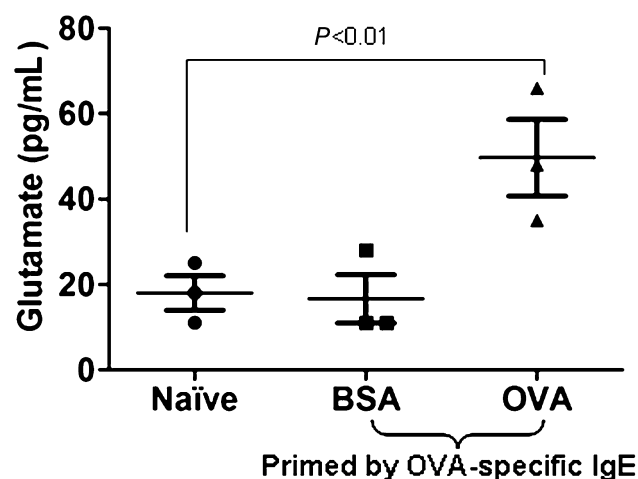


Figure 4 Antigen–antibody interaction induces vagus ganglion neurons (VGN) to release glutamate. VGN were prepared and primed by ovalbumin (OVA)-specific immunoglobulin E (IgE). Primed VGN were exposed to OVA (10 µg/mL) or bovine serum albumin (10 µg/mL) in culture for 6 h. Level of glutamate in the culture supernatant was assessed by ELISA (sensitivity = 10 pg/mL). Scatter dot plots show the glutamate level in the culture supernatant. Each dot represents a single datum from one experiment.

Activation of VGN modulates DC function

We then investigated the mechanism by which the vagal nerve regulates Th2 response. We generated BmDC and co-cultured DC and OVA-specific IgE-primed VGN. After exposure to OVA, the cells were analyzed by flow cytometry. The results showed that significant increases in CD86 and MHCII were detected in DC, whereas the level of IL-12 was much lower in DC than those stimulated by LPS. The changes in DC were abolished by pretreatment with the antiglutamate antibody (Fig. 5). Further experiments showed that OVA-specific, IgE-primed VGN significantly promoted the development of Th2 cells (Fig. 6). The results indicate that activated vagal neurons can modulate DC phenotype status that enables DC to induce Th2 responses.

Discussion

The role of enteric neuro-immunity in the pathogenesis of intestinal immune inflammation, such as food allergy, has caught attention of both scientists and clinicians. In line with recent publications,⁸ the present study presents evidence that the intestinal vagus nerve expresses IgE receptor FcεRI, which could be bound by antigens and specific IgE to form antigen–IgE complexes on VGN that enable VGN to be sensitized. Re-exposure to specific antigens causes VGN to release neural transmitter glutamate; the latter modulates DC phenotyping and further facilitates the development of Th2 cells. Furthermore, excised vagus nerves inhibit experimental immune inflammation in the intestine.

The present data are in line with a recently published study, in which van der Kleij *et al.*⁸ reported that VGN express IgE receptor I α and β chains, but not the γ chain. Although a different antibody was used in the present study, we confirmed that the existence of

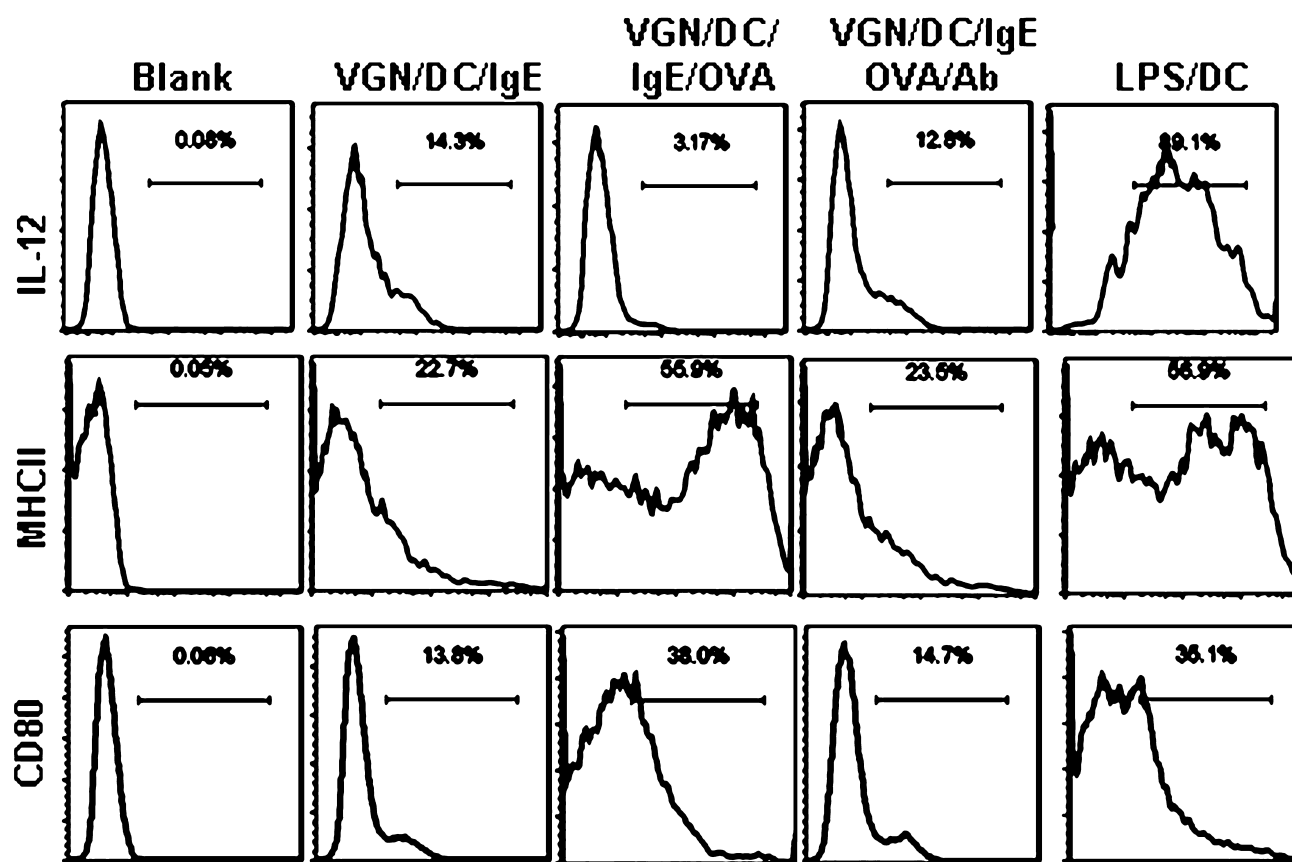


Figure 5 Vagus ganglion neurons (VGN) regulate dendritic cell (DC) phenotyping. VGN and bone marrow-derived DC (BmDC) were prepared. VGN were primed by exposing to ovalbumin (OVA)-specific immunoglobulin E (IgE). BmDC were cultured to day 6 (at immature status, as checked by flow cytometry). Cells were further treated according to the following procedures, and analyzed by flow cytometry. Blank, cells were not stained; VGN/DC/IgE, VGN were primed by exposing to OVA-specific IgE and co-cultured with DC. Ratio of VGN : DC = 1:10; VGN/DC/IgE/OVA, procedures were the same as VGN/DC/IgE, except that OVA (10 µg/mL) was added to the culture; VGN/DC/IgE/OVA/albumin (Ab), procedures were the same as VGN/DC/IgE/OVA, except that cells were pretreated with an antiglutamate antibody (1 µg/mL); lipopolysaccharide (LPS), DC were cultured in the presence of LPS (100 ng/mL). Data represent three separate experiments. IL-12, interleukin-12, MHCII, major histocompatibility complex class II.

FcεRI was expressed by VGN. Furthermore, the present study demonstrates a previously undescribed phenomenon that the intestinal vagus nerves express FcεRI, the high-affinity receptor of the IgE antibody. FcεRI is expressed mainly by mast cells that are involved in immediate allergic inflammation. Other investigators have also mentioned that DC also express FcεRI¹² which might enhance their ability to capture and internalize antigens for presentation to T lymphocytes. Our data indicate that the vagus nerve also has the potential to be involved in antigen–antibody reaction-induced immune inflammation based on such an anatomical feature, as shown in the present study.

Glutamate is one of the neural transmitters of vagal neurons. It can be released upon nervous excitation. Although the physiological function of glutamate is beneficial to the body, overproduction of this transmitter might be pathogenic. Our findings have shown a novel phenomenon that antigen–antibody responses can activate VGN to enable them to release glutamate to the microenvironment. The event implies that during allergic reaction, the vagus

might be activated and over-release glutamate; the latter further contacts immune cells to modulate their function, as demonstrated by the present data.

The data show that FcεRI⁺ VGN distribute in the lamina propria, the residential area of variable immune cells, including DC. DC are the first point of immune responses. Activated DC dictate certain immune reactions depending on the stimuli.¹³ Our data show that after exposure to glutamate, DC show high levels of CD80 and MHCII, but low levels of IL-12, since IL-12 is the key cytokine to initiate Th1 response.¹⁴ The major factor from DC in the initiation of Th2 response is unclear and needs to be further understood; the lack of IL-12 in activated DC is proposed as one of the plausible mechanisms to induce skewed Th2 response.¹⁵ The data are in line with previous studies, such as Liu *et al.*'s report that DC in airway mucosa express cholinergic muscarinic receptor. Cholinergic agent-primed DC have the capacity to modulate T cell function.¹⁶ Our data imply that glutamate-primed DC might initiate Th2 immune response.

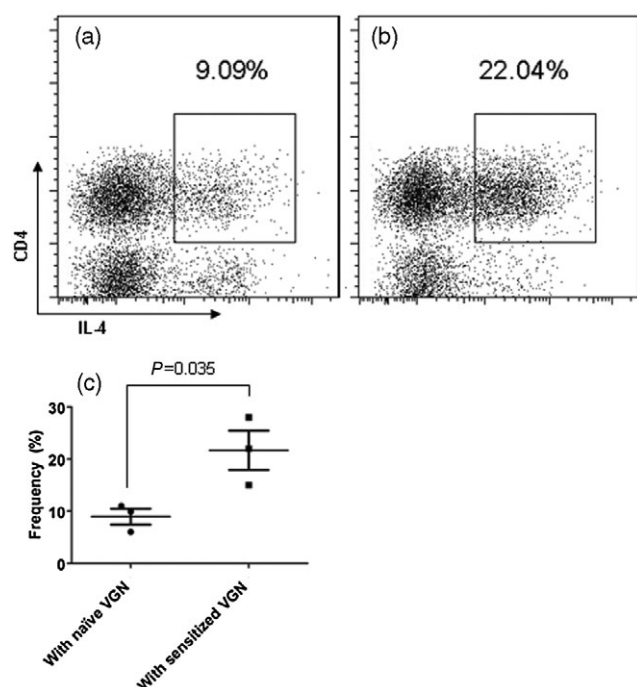


Figure 6 Sensitized vagus ganglion neurons (VGN) promote T-helper 2 cell development. Mouse VGN were prepared. VGN were primed with or without ovalbumin (OVA)-specific immunoglobulin E (IgE). Spleen mononuclear cells (SpMo) were prepared and cultured with naïve VGN (a) or VGN primed with OVA-specific IgE (b). Ratio of SpMo : VGN was 50:1. Culture media and reagents were changed every 3 days. Cells were harvested on day 10, and stained with anti-CD4 and anti-interleukin-4 (IL-4) antibodies. Cells were analyzed by flow cytometry. Representative dot plots show frequency of CD4⁺ IL-4⁺ T-cell population (gated cells). (c) Scatter dot plots show individual datum; each dot represents a datum from a separate experiment.

The use of PRVn to inhibit intestinal immune inflammation has been reported previously.³ The present data provide further information to show that PRVn plays a role in the inhibition of skewed Th2 response, with evidence that the overexpression of the IgE receptor FcεRI was found on the intestinal vagus nerve of mice with immune inflammation. Furthermore, the data show that the food allergy-related intestinal inflammation is almost extinguished after treatment with PRVn or cholinergic antagonists; the study implies that blocking vagal function might have therapeutic potential in food allergy-related immune inflammation in the intestine. However, the vagus nerve is the most important nerve for maintaining function in the gastrointestinal system. It is not wise to remove the vagus nerve to treat immune inflammation in the intestine. The present data indicate that administration of cholinergic antagonists might benefit patients with food allergy inflammation; however, this needs to be further investigated.

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Supporting information

Additional Supporting Information may be found in the on line version of this article:

Figure S1 High affinity IgE receptor is localized on intestinal vagus nerve. (a) Naïve; (b) food allergy (FA); (c) FA, partial removal of the vagal nerve (PRVn); (d) isotype immunoglobulin G. ChAT, choline acetyltransferase; IgER1, immunoglobulin E receptor-1. ■, IgER1; □, ChAT.

Figure S2 Vagus neurons express IgE receptor. (a) FcεRI; (b) propidium iodide; (c) merging of (a) and (b); (d) isotype immunoglobulin G staining.

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