

TIM-4 Expressed by Mucosal Dendritic Cells Plays a Critical Role in Food Antigen–Specific Th2 Differentiation and Intestinal Allergy

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See editorial on page 1723.

Background & Aims: Food allergy accounts for significant morbidity. The etiology and immune mechanisms of food allergy, however, have remained poorly understood. In this study, we aimed to determine the role of T-cell immunoglobulin-domain and mucin-domain (TIM)-4, a recently identified member of cell surface molecules, in the pathogenesis of intestinal allergy in a murine model. **Methods:** We report that TIM-4 as well as costimulatory molecules were up-regulated in intestinal mucosal dendritic cells by in vitro or in vivo exposure to *Staphylococcus enterotoxin B* (SEB). SEB-conditioned intestinal dendritic cells loaded with a food macromolecule ovalbumin (OVA) induced potent OVA-specific T-helper (Th)2 lymphocyte responses in vitro and such Th2 responses were inhibited completely by TIM-4 blockade. **Results:** In vivo exposure to both SEB and OVA resulted in OVA-specific Th2 differentiation and intestinal allergic responses including increased serum immunoglobulin E and Th2 cytokine levels, activation of OVA-specific Th2 cells detected both ex vivo and in situ, and mast cell degranulation. Of importance, in vivo abrogation of TIM-4 or its cognate ligand TIM-1 by using a polyclonal antibody remarkably dampened Th2 differentiation and intestinal allergy. **Conclusions:** Our study thus identifies TIM-4 as a novel molecule critically required for the development of intestinal allergy.

As many as 15%–20% of populations have immunoglobulin (Ig)E-mediated hypersensitivity to foreign antigens.¹ Indeed, the prevalence of allergic diseases has increased rapidly across the world in the past few decades, with food allergies currently affecting 4%–6% of the population.² However, both the etiology and immune mechanisms of intestinal allergic diseases have remained poorly understood. It is believed that under normal circumstances the gut immune system is relatively hyporesponsive to food antigens and commensal bacteria and that this state of hyporesponsiveness (tolerance) is maintained by a number of important mechanisms including

the integrity of gut epithelium and the presence of tolerogenic dendritic cells (DCs) and regulatory T cells.³ Thus, conceivably any perturbation to the homeostasis between gut antigens and the gut immune system may lead to gut inflammation and food allergies. For instance, although food macromolecules such as chicken ovalbumin (OVA) are taken up by the intestinal mucosa only at a limited rate, an increased intestinal permeability can lead to the sensitization of the immune system to food antigens.^{3,4} Likewise, the intestinal mucosal DCs that, compared with the DCs at other sites, express lower levels of costimulatory molecules such as B7 required for T-cell activation,³ may become activated by increased exposure to bacterial toxin and enable food antigen–specific T-cell activation in the mesenteric lymph nodes.

The T cells of T-helper (Th)2 phenotype are known to be involved in the development of allergic disease. In contrast to Th1 cells, which release type 1 cytokines such as interferon (IFN)- γ , the major cytokines secreted by Th2 cells are interleukin (IL)-4, IL-5, and IL-13. These cytokines play an important role not only in the production of antigen-specific IgE by B cells⁵ but also in mucus secretion, muscle contractility, and eosinophilia.^{6,7} Mast cells activated by IgE cross-linking release allergic mediators to initiate the hypersensitivity reactions and allergic clinical symptoms on re-exposure to specific antigens.^{1,2} Thus, the activation of Th2 cells is a critical step in the immune mechanisms of food allergy but the factors involved in the initiation of food antigen–specific Th2 activation still remain unclear.

It is believed that nonspecific inflammatory responses in the gut may lead to the loss of mucosal immune tolerance.² Indeed, mounting evidence suggests that increased concurrent exposure of the mucosal immune

Abbreviations used in this paper: DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; OVA, ovalbumin; PGN, peptidoglycan; PT, pertussis toxin; qPCR, real-time reverse-transcription polymerase chain reaction; SEB, *Staphylococcal enterotoxin B*; Th, T helper; TIM, T-cell immunoglobulin-domain and mucin-domain.

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system to both bacterial components such as toxins and food antigens predisposes to food antigen sensitization and allergy.⁸ In this respect, we have observed previously that microbial products were required in synergy with food antigens for the induction of intestinal allergic sensitization.^{4,9} For instance, *Staphylococcus aureus*, one of the common commensal bacteria on the mucosal surface,¹⁰ or *Staphylococcal enterotoxin B* (SEB), was found by us and others to play a pathogenic role in a number of allergic diseases.^{11,12} However, the molecular mechanisms by which microbial products promote Th2 differentiation and the development of allergy, particularly food allergy, have remained poorly understood.

The T-cell immunoglobulin-domain and mucin-domain (TIM) gene family members including TIM-1, TIM-2, TIM-3, and TIM-4, recently have received increasing attention for their important regulatory role in Th1 and Th2 effector responses.¹³ Among these members, TIM-4, which is expressed in DCs in lymphoid organs and its ligand TIM-1, which is expressed by T cells, recently has been found to be a critical Th2 regulator.^{13,14} It has been shown that the interaction between TIM-4 and TIM-1 breaches the peripheral immune tolerance and leads to T-cell activation.^{13,14} The role of TIM-4 and its interaction with TIM-1 in the pathogenesis of allergy in general and intestinal allergic disease in particular still is unknown. However, based on the limited published information,^{13,14} it is plausible to hypothesize that TIM-4 plays a critical role in breaking gut mucosal tolerance toward food antigens, thus leading to the development of intestinal allergic disease.

We have investigated the role of TIM-4 in the development of Th2 cells and intestinal allergic disease by using in vitro and in vivo models of food allergy. We found that SEB exposure increased TIM-4 expression on intestinal DCs in vitro and in vivo. Co-exposure to both SEB and OVA led to the maturation of intestinal DCs and such conditioned intestinal DCs resulted in OVA-specific Th2 polarization, which was abrogated by blocking TIM-4. Of importance, concurrent exposure to SEB and OVA in vivo led to the sensitization of Th2 cells to OVA and the subsequent development of intestinal allergic responses, which largely were abolished by blocking TIM-4 or TIM-1. Our study thus reveals a critical role for TIM-4 in the pathogenesis of intestinal allergy.

Materials and Methods

Mice

Balb/c and C57BL/6 mice (male, 6–8 weeks old) were purchased from the Charles River Laboratory (Wilmington, MA). DO11.10 TCR transgenic mice that recognize an OVA peptide in the context of I-A^d major histocompatibility complex class II (MHC II) molecules were purchased from the Jackson Laboratory (Bar Har-

bor, ME). The experiments were approved by the Animal Research Ethics Board at McMaster University.

Murine Model of Intestinal Allergy

C57BL/6 mice were sensitized intraperitoneally with SEB (10 µg/mouse), OVA (20 µg/mouse), or both SEB and OVA on day 0. The mice were boosted twice with the same treatment on days 3 and 9, respectively. Mice were challenged with OVA (20 µg/mouse) in 0.25 mL of saline intragastrically twice on days 7 and 14. Mice then were killed 1 day after the last intragastric challenge (day 15). The blood was collected for serum preparation. Jejunal segments were excised and snap frozen for immunohistochemistry or fixed with 2% glutaraldehyde for electron microscopy or fixed with Carnoy solution for identification of mucosal mast cells. In separate experiments, intestinal segments, the spleen, and mesentery lymph nodes were collected for purification of DCs and T cells. Some mice were pretreated with goat anti-mouse TIM-4 polyclonal antibody (50 µg/mouse; R&D Systems, Minneapolis, MN; catalog number: AF2826; anti-TIM-4 extracellular domain) or rat anti-mouse TIM-1 polyclonal antibody (50 µg/mouse; R&D Systems; catalog number: MAB1817; clone 222414; anti-TIM-1 extracellular domain) intraperitoneally 30 minutes before the gavage.

Isolation and Culture of Intestinal DCs

The intestinal DC population was obtained using a well-established method.^{15,16} Briefly, jejunal segments were cut into small fragments and digested with collagenase D (1 mg/mL). Cell suspensions were passed through a Falcon cell strainer (70-µm mesh) (BD Biosciences, Mississauga, ON, Canada). Resulting cells were centrifuged over a Percoll gradient to enrich for mononuclear cells. DC culture was referred to in a previous report^{15,16} with modification. CD11c⁺ DCs were separated further from the mononuclear cells by CD11c MicroBeads using AutoMACS (Miltenyi Biotec). The purity of purified DCs was more than 95% as examined by fluorescence-activated cell sorting (FACS). DCs then were cultured in the presence of 1000 U/mL IL-4 and 800 U/mL granulocyte-macrophage colony-stimulating factors with or without graded doses of SEB for 48 hours.

Co-Culture of Intestinal DCs and Transgenic T Cells

For DC and T-cell co-culture experiments, on day 3, the resulting DCs were washed 3 times with phosphate-buffered saline (PBS) before co-culture with purified naive OVA transgenic CD4 T cells. For isolation of transgenic CD4 T cells, the mesentery lymph node cells of DO11.10 mice were isolated, passed through a Falcon cell strainer (70-µm mesh) (BD Biosciences), washed in RPMI 1640, and resuspended in MACS buffer (BD Biosciences). DO11.10 CD4 T cells were separated with anti-CD4-MACS beads (Miltenyi Biotec). Purified OVA trans-

genic CD4 T cells (10^5 cells/well) then were co-cultured with the DCs (see previously; 10^5 cells/well) pretreated with or without SEB (see previously) in RPMI 1640 containing 10% fetal calf serum and 800 U/mL granulocyte-macrophage colony-stimulating factor in the presence of SEB (at gradient doses of 0–200 ng/mL) and/or OVA peptide (OVA_{323–339}: ISQAVHAAHAEINEAGR; 5 μ g/mL) for 2 days at 37°C. For T-cell proliferation assay, cells were pulsed for the last 16 hours with [^{3} H] thymidine (1 μ Ci/well). Incorporation of [^{3} H] thymidine was analyzed with liquid scintillation counting. For cytokine assay, cells were cultured in the presence of OVA or 50 ng/mL phytohemagglutinin (PHA) and cell supernatants then were collected for the measurement of IL-4, IL-13, and IFN- γ by enzyme-linked immunosorbent assay (ELISA).

Methods of isolation and culture of splenic and intestinal mucosal lymphocytes, real-time reverse-transcription polymerase chain reaction (qPCR), flow cytometry, ELISA, and immunohistochemistry are detailed in the

Supplementary materials (see supplementary material online at www.gastrojournal.org).

Statistics

Data were expressed as the means $\pm$ SD. Differences between groups were determined with a Student *t* test (2 groups) or analysis of variance (3 or more groups); a *P* value of less than .05 was considered to be statistically significant.

Results

Localization of TIM-4 Expression to DCs in Intestinal Mucosa

Apart from its high expression in the thymus, recent evidence has indicated that TIM-4 also is expressed by DCs located in the spleen and lymph nodes and only weakly is expressed in nonlymphoid tissues such as the lung and liver.¹³ To begin investigating the role of TIM-4

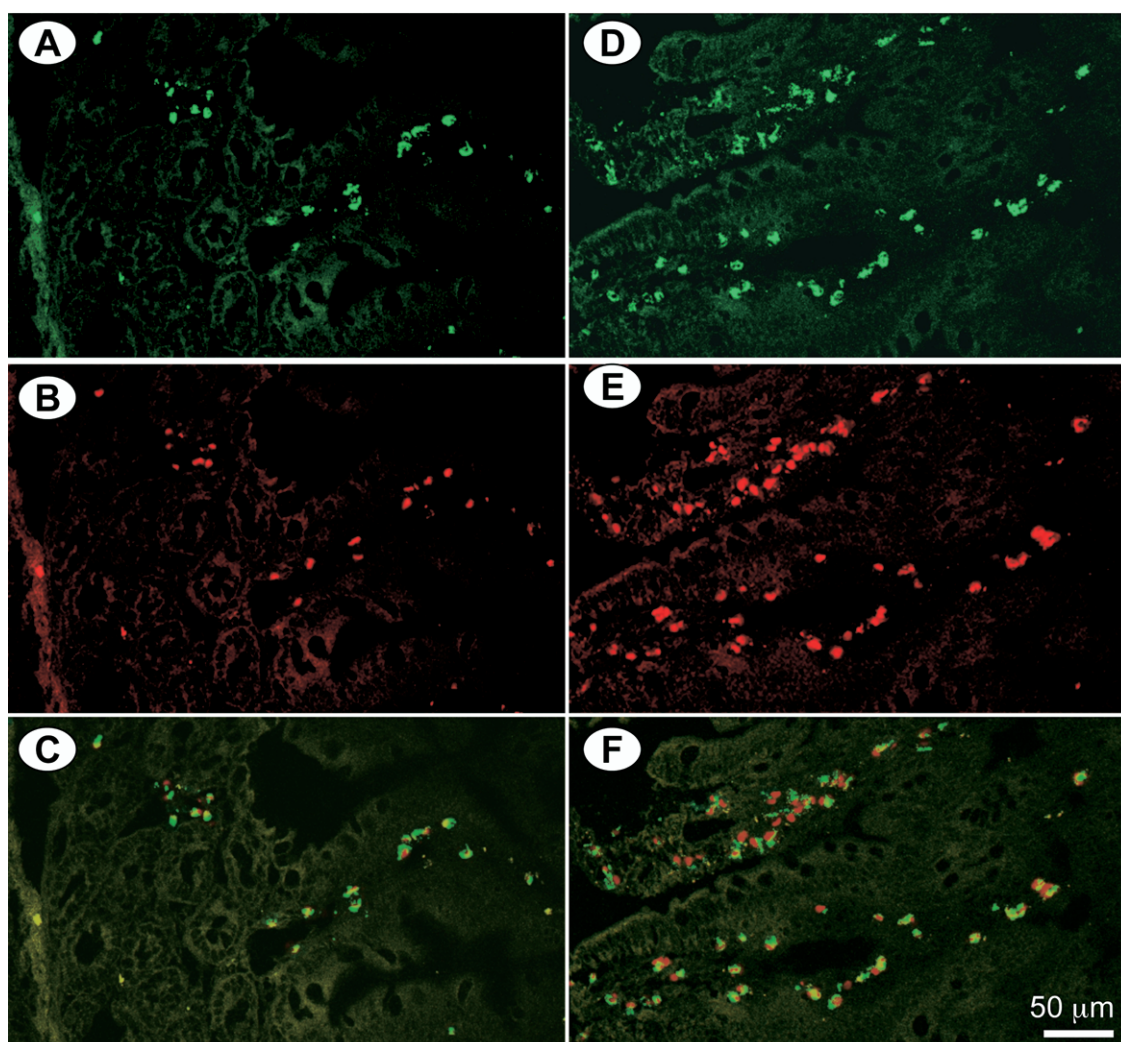


Figure 1. TIM-4-expressing DCs within intestinal mucosa and enhanced by SEB. (A–C) Intestinal tissues were from mice treated with saline. (D–F) Intestinal tissues were from mice treated with SEB. The red color indicates TIM-4-positive staining; the green color indicates CD11c-positive staining; the yellow color resulted from merged green and red colors, indicating a colocalization of CD11c and TIM-4 in the intestinal mucosa.

in the immune regulation of intestinal allergy, we first examined its expression and regulation within the jejunal intestinal mucosa by immunofluorescence microscopy. We found that jejunal mucosa of naive mice had easily identifiable CD11c⁺ cells but only a small number of these DCs expressed TIM-4 (Figure 1A–C). Because *Staphylococcus aureus* represents one of the common commensal bacteria on the mucosal surface¹⁰ and this bacterium or its SEB was found to be involved in the pathogenesis of allergic diseases,^{11,17} we examined whether in vivo exposure to SEB may modulate TIM-4 expression by intestinal DCs. Indeed, exposure to SEB for 48 hours resulted in an increase in the number of CD11c⁺ DCs in the subepithelial region of jejunal mucosa and more importantly the majority of these mucosal DCs expressed TIM-4 (Figure 1D–F). These data suggest that TIM-4 is a highly inducible molecule on intestinal mucosal DCs, thus implicating this molecule in the regulation of intestinal immunologic conditions.

Regulation of TIM-4 Expression in Intestinal DCs by SEB Exposure In Vitro and In Vivo

Having localized TIM-4 expression to intestinal mucosal DCs and provided the evidence of its up-regulation in these cells on SEB exposure by using immunohistochemistry, we further examined the regulation of its expression in detail by using qPCR and ELISA. To this end, we first examined the regulation of TIM-4 expression in vitro. CD11c⁺ DCs were prepared and purified (the purity was >90% as examined by FACS) from the jejunum of naive mice and cultured in the presence of gradient doses of SEB for 48 hours. Intestinal DCs expressed low levels of TIM-4 before in vitro SEB stimulation (Figure 2A). The expression of TIM-4 increased in

DCs at both messenger RNA (mRNA) and protein levels in a SEB dose-dependent manner (Figure 2A and D). To examine whether TIM-4 expression also was SEB dose-dependent in vivo, mice were treated intraperitoneally with various doses of SEB and killed 48 hours later. DCs then were isolated from the small intestine. Because splenic DCs were found previously to express TIM-4,¹³ we also isolated splenic DCs and used them as a reference comparison for the relative expression level of TIM-4 in intestinal mucosal DCs. Indeed, in vivo SEB treatment up-regulated TIM-4 mRNA and protein in a dose-dependent manner (Figure 2B and E). Importantly, both constitutive and inducible levels of TIM-4 expression by intestinal mucosal DCs were very much comparable with those by splenic counterparts (Figure 2C and F). These findings suggest that intestinal mucosal DCs are similar to DCs of lymphoid organs in that low constitutive TIM-4 expression in DCs is prone to induction by bacterial toxins such as SEB.

Enhancement of Intestinal DC Maturation by SEB and OVA Exposure In Vitro

Having shown the ability of SEB exposure to enhance TIM-4 expression on intestinal DCs, we examined whether SEB exposure also could promote DC maturation. To this end, isolated DCs were cultured in the presence of SEB at gradient doses for 48 hours and surface expression of CD80, CD86, and MHC class II molecules were examined by FACS. CD80 (Figure 3A and E), CD86 (Figure 3B and F), and MHC II (Figure 3C and G) were increased significantly by SEB exposure in a dose-dependent manner. Because OVA is one of the common food antigens that constantly is taken up by intestinal epithelial cells, although normally only at a limited

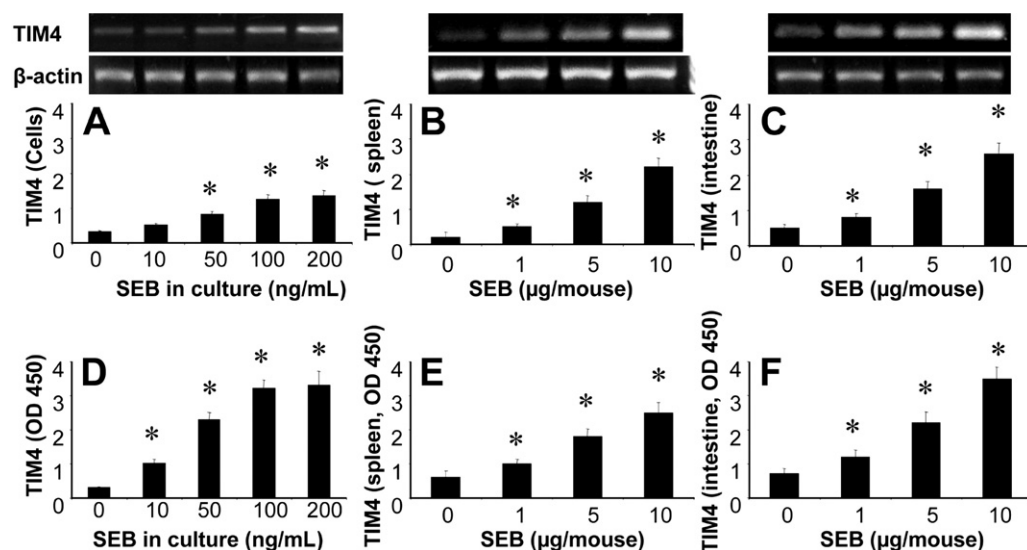


Figure 2. SEB promotes TIM-4 expression in intestinal DCs. TIM-4 expression in DCs was determined by qPCR (A–C, row 1) and ELISA (D–F, row 2). (A) DCs were prepared from the jejunum and cultured in the presence of SEB at gradient doses for 48 hours. (B and C) Mice (10 mice per group) were treated with gradient doses of SEB. DCs were prepared from the (B) spleen and the (C) jejunum and directly subjected to TIM-4 expression evaluation by qPCR and ELISA. Values are expressed as means ± SD from 3 separate experiments. **P* < .05 compared with dose 0.

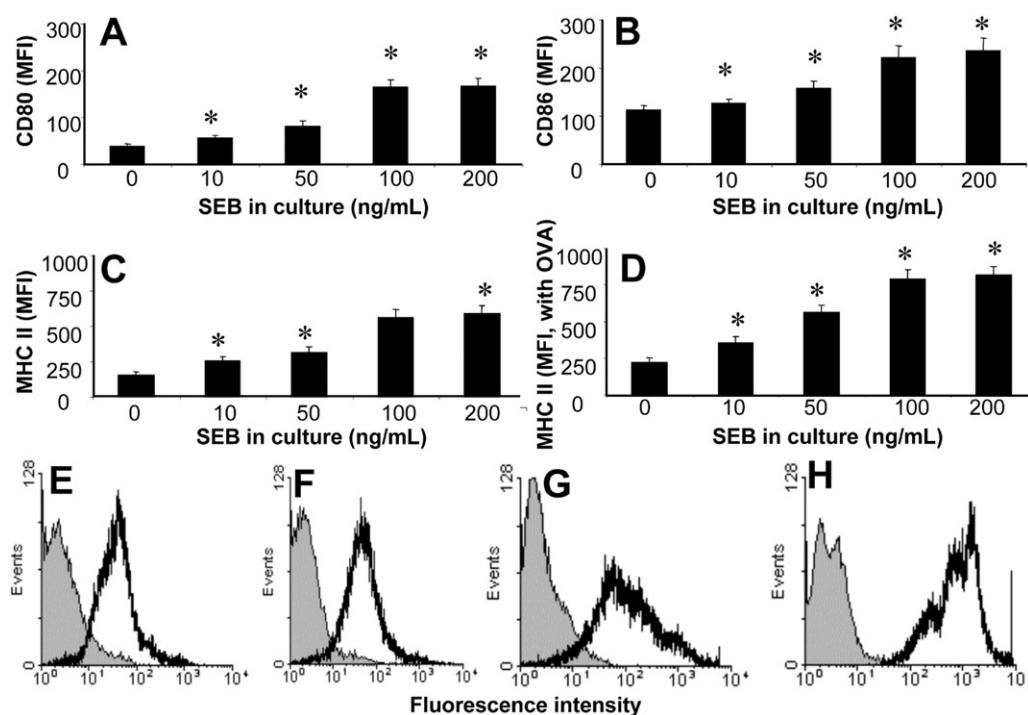


Figure 3. Enhanced maturation of intestinal DCs by SEB. Isolated intestinal DCs were cultured in the presence of SEB at gradient doses or in the presence of both SEB and OVA for 48 hours. Expression of CD80, CD86, and MHC II on DCs was stained with fluorescence-labeled antibodies, and analyzed by flow cytometry. Bars indicate the median fluorescence intensity (MFI) (means $\pm$ SD; from 3 separate experiments). (A and E) CD80, (B and F) CD86, (C and G) MHC II, and (D and H) MHC II expression on cells exposed to SEB and OVA. (E–H) Representative fluorescence histograms show counts of positively stained cells. * $P < .05$ compared with no SEB treatment.

rate,⁴ we examined whether co-exposure to both SEB and OVA might modulate expression of intestinal DC maturation molecules further. We found that co-exposure to SEB and OVA further increased MHC II expression (Figure 3D and H). These results together suggest that exposure to intestinal bacterial toxins such as SEB is a critical activating signal of intestinal mucosal DCs and co-exposure to both bacterial toxins and food antigens such as OVA may engender the type of intestinal DCs capable of sensitizing and activating food antigen-specific T cells.

Role of TIM-4 in OVA-Specific Th2 Polarization Stimulated by SEB-Conditioned Intestinal DCs In Vitro

Having observed the up-regulation of TIM-4 expression on intestinal mucosal DCs both in vitro and in vivo by SEB exposure, we next sought to investigate whether TIM-4 from intestinal DCs could play a role in the development of food antigen-specific Th2 cells by using an in vitro transgenic T-cell model.¹⁸ To this end, the mesenteric CD4 T cells were isolated from DO11.10 mice and co-cultured with SEB-conditioned intestinal DCs in the presence of OVA_{323–339} peptides for 2 days. We first examined the proliferation rate of DO11.10 T cells. The results showed that DO11.10 CD4 T-cell proliferation increased significantly on co-culture with OVA_{323–339} loaded, SEB-conditioned jejunal DCs in a SEB dose-dependent manner (Figure 4A). The peak levels of

DO11.10 T-cell proliferation were comparable with that by PHA stimulation (Figure 4A). To evaluate the phenotype of these transgenic CD4 T cells, we examined the secretion of Th2 and Th1 cytokines, IL-4, IL-13, and IFN- γ . We found that the secretion of Th2 cytokines IL-4 (Figure 4B) and IL-13 (Figure 4C) increased significantly in a SEB dose-dependent manner. In contrast, while under these conditions, there was a detectable level of Th1 cytokine IFN- γ production, it decreased when higher doses of SEB were used to condition intestinal DCs (Figure 4D), which was in sharp contrast to a high level of IFN- γ induction by PHA.

To examine the role of TIM-4 in OVA-specific Th2 polarization driven by SEB-conditioned intestinal DCs, some of the DC–T-cell cultures were treated with anti-TIM-4 antibodies. We found that abrogation of TIM-4 on DCs completely abolished DO11.10 T-cell proliferation (Figure 4A) and Th2 cytokine IL-4/IL-13 secretion (Figure 4B and C). In comparison, abrogation of TIM-4 allowed increased Th1 IFN- γ secretion by a high-dose SEB exposure (Figure 4D). This suggests that TIM-4 is critically required for OVA-specific Th2 differentiation in this in vitro model of food allergy. Because TIM-1 is the ligand for TIM-4 expressed by T cells, we also examined the effect of anti-TIM-1 antibodies. Indeed, the use of this antibody in our co-culture system had an effect similar to that by anti-TIM-4 antibodies (Figure 4A–D).

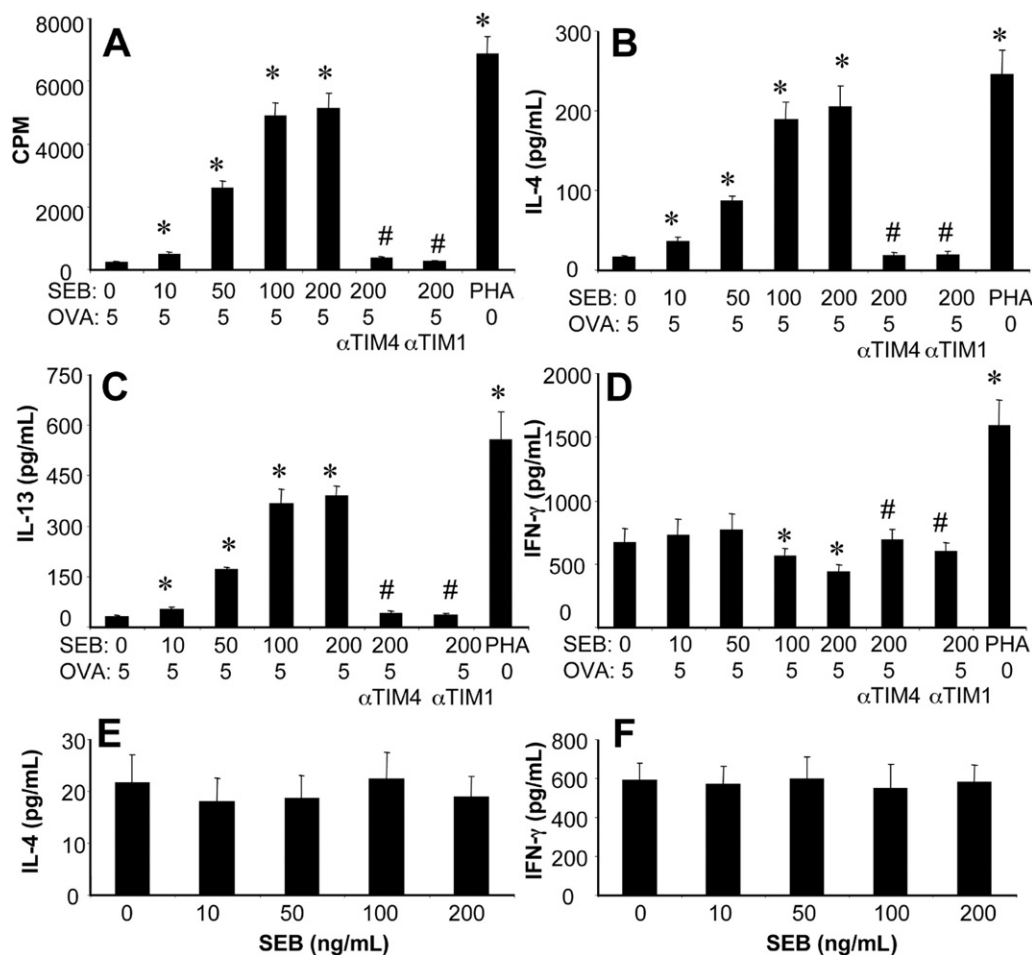


Figure 4. Antigen-specific Th2 polarization by SEB-conditioned intestinal DCs and its inhibition by TIM-4 blockade in vitro. Isolated intestinal DCs were treated with gradient doses of SEB (ng/mL) with or without OVA (μg/mL) in culture. The SEB-conditioned DCs were co-cultured with separated OVA transgenic CD4 T cells. (A) DO11.10 CD4 T-cell proliferation. Bars indicate [3H] thymidine incorporation in CD4 T cells. (B) Bars indicate the levels of IL-4 in culture media. (C) Bars indicate the levels of IL-13 in culture media. (D) Bars indicate the levels of IFN-γ in culture media. Data are expressed as mean ± SD. **P* < .05, compared with group 0. #*P* < .05, compared with the group treated with both SEB and OVA in the absence of anti-TIM-4 or anti-TIM-1. αTIM-4: the group of DCs was exposed to anti-TIM-4 antibody (5 μg/mL), SEB, and OVA concurrently. αTIM-1: the group of CD4 T cells was pretreated with anti-TIM-1 antibody (5 μg/mL) and then exposed to SEB-conditioned and OVA-loaded DCs.

Concurrent Exposure to SEB and OVA Elicits Intestinal Th2 Polarization and Allergic Responses In Vivo

Having identified a critical role of TIM-4 in food antigen-specific Th2 polarization in vitro, we set out to investigate its role in vivo. To approach this question, we first developed and fully characterized a novel in vivo model of intestinal food allergy. To this end, Balb/c mice were primed intraperitoneally with SEB alone, OVA alone, or both SEB and OVA on day 0, boosted intraperitoneally on days 3 and 9, and locally challenged in the gut by gavage on days 7 and 14. Twenty-four hours after the last gavage, the mice were killed and subjected to immunologic analyses (Figure 5A). We first examined the circulating levels of IL-4, IL-13, and IFN-γ by ELISA. We found that although Th2 cytokines IL-4 (Figure 5B) and IL-13 (Figure 5C) were somewhat increased in the circulation of mice treated with SEB or OVA alone, they were

increased most dramatically in the mice treated with both SEB and OVA (Figure 5B and C). In contrast, the level of Th1 cytokine IFN-γ was decreased under these conditions (Figure 5D).

Because markedly increased circulating levels of Th2 cytokines in SEB-/OVA-treated mice suggest the activation of Th2 cells in this model, we examined the phenotype of T-cell responses in the intestinal mucosa and systemic lymphoid organs. To this end, we isolated both jejunal mucosal mononuclear cells and splenocytes from the mice and cultured them in the presence of OVA (5 μg/mL) for 4 days for examination of OVA-specific T-cell proliferation and cytokine responses. The results show that only the T cells isolated from the mice treated with both SEB and OVA, but not with SEB or OVA alone, showed significantly higher levels of proliferation (Figure 5E) or released significantly higher levels of Th2 cytokine IL-4 (Figure 5F) on OVA stimulation. At the same time a

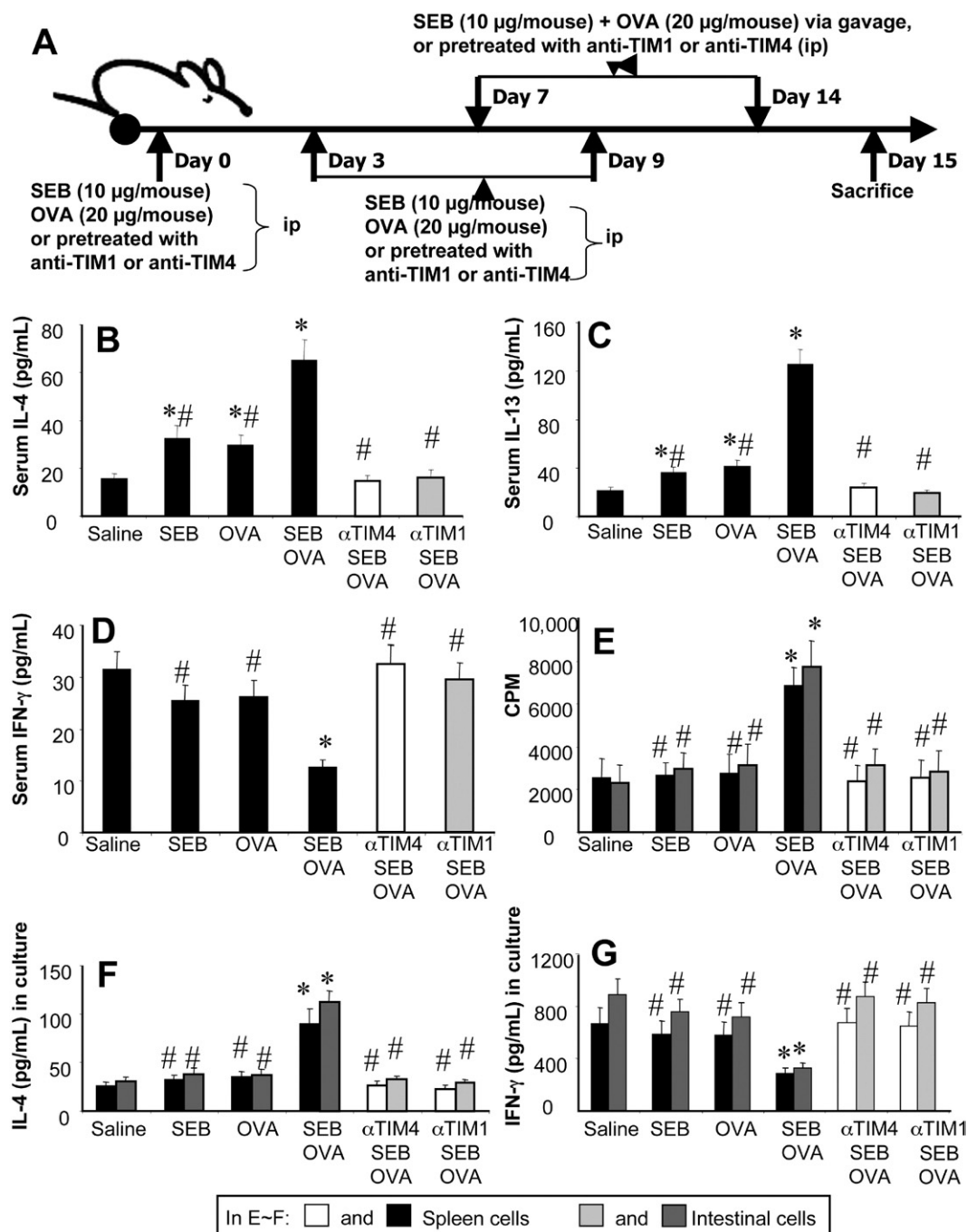


Figure 5. Elicitation of OVA-specific Th2 responses by SEB and OVA exposure and their inhibition by TIM-4 blockade in vivo. (A) Experimental protocol. Balb/c mice were primed intraperitoneally with SEB alone, OVA alone, or both SEB and OVA on day 0, boosted intraperitoneally on days 3 and 9, and challenged locally in the gut by gavage on days 7 and 14. Twenty-four hours after the last gavage, the mice were killed and subjected to immunologic analyses. In separate experiments, mice were treated intraperitoneally with α TIM-4 or α TIM-1 (50 μ g/mouse) 30 minutes before injection of SEB and OVA. (B–D) Bars indicate the levels (mean $\pm$ SD) of (B) IL-4, (C) IL-13, and (D) IFN- γ in the serum of mice. (E–G) Splenic and intestinal mononuclear cells were separated from mice and cultured in the presence of OVA for 4 days. (E) Splenic and intestinal T-cell proliferation in response to OVA stimulation. (F and G) Levels of (F) IL-4 and (G) IFN- γ in culture supernatants. Data are presented as means $\pm$ SD. * P < .05, compared with saline controls. # P < .05, compared with the group treated with both SEB and OVA in the absence of anti-TIM-4 or anti-TIM-1. Each experiment was repeated 3 times.

decreased Th1 response was seen, as exemplified by significantly decreased IFN- γ production by both intestinal mucosal and splenic T cells (Figure 5G). These results together suggest that concurrent in vivo exposure to both

microbial toxin and food antigen is a critical requirement for the development of food antigen-specific Th2 cells.

To further strengthen our observation, we performed immunohistochemistry to localize Th2 cells in the intes-

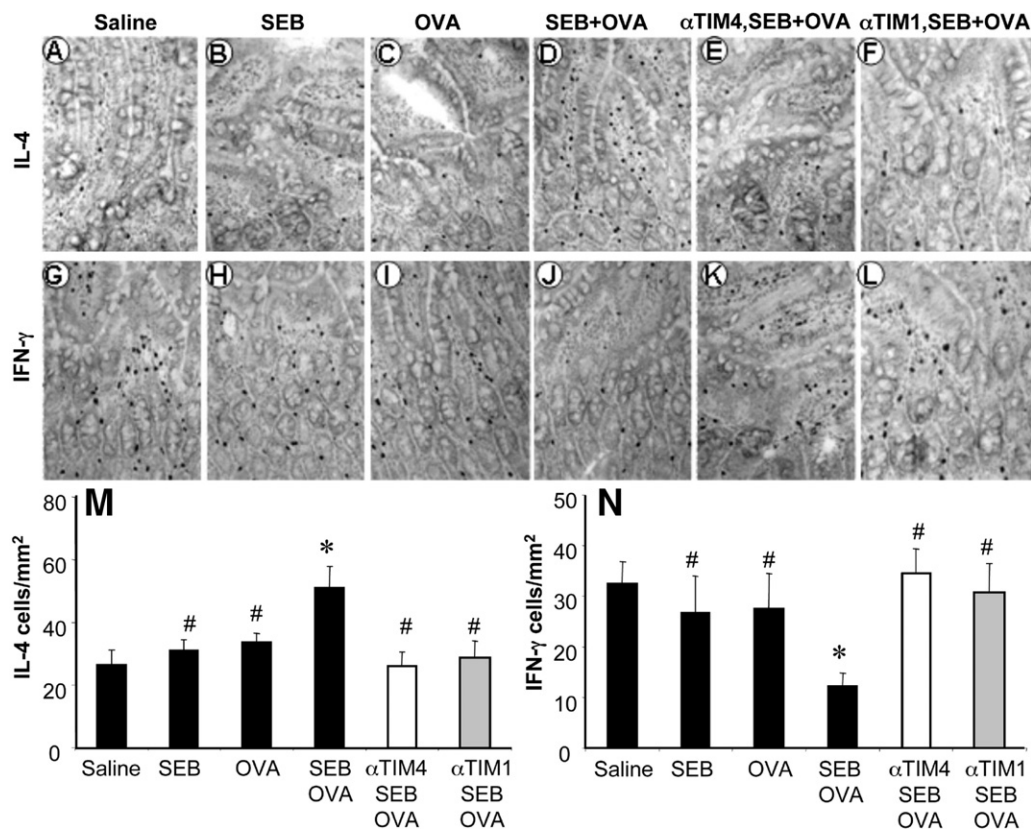


Figure 6. IL-4- and IFN- γ -producing cells in the intestinal mucosa and its modulation by SEB and OVA exposure and TIM-4 blockade. (A–L) Representative immunohistochemistry images from mice treated with (A and G) saline, (B and H) SEB alone, (C and I) OVA alone, (D and J) both SEB and OVA, pretreated with anti-TIM-4 antibody, then treated with (E and K) both SEB and OVA and pretreated with anti-TIM-1 antibody, then treated with (F and L) both SEB and OVA. Cryosections of jejunal tissues were stained with (A–F) anti-IL-4 or (G–L) anti-IFN- γ antibodies. Positively stained cells (dark staining) were counted under a light microscope (40 fields per mouse; 10 mice/group). (M and N) Bars indicate the cell numbers per mm² jejunal tissue. Data are expressed as the means $\pm$ SD. * $P < .05$, compared with saline controls. # $P < .05$, compared with the group treated with both SEB and OVA in the absence of anti-TIM-4 or anti-TIM-1.

tinal mucosa. Thus, cryosections were prepared from jejunal segments and stained with anti-IL-4 and anti-IFN- γ monoclonal antibodies. The cells positively stained for IL-4 and IFN- γ were observed in the mucosa of all samples from different groups of mice (Figure 6A–L). More specifically, compared with saline controls, the density of IL-4-positive cells was slightly higher in mice exposed to SEB or OVA alone but was increased significantly in mice exposed to both SEB and OVA (Figure 6M). In comparison, although IFN- γ -positive cells were observed in saline control mice and were decreased slightly in mice treated with SEB or OVA alone, the number of such cells was decreased significantly in the sections from mice exposed to both SEB and OVA (Figure 6N), thus in keeping with ex vivo observations using isolated intestinal mucosal T cells (Figure 5).

To provide further evidence of intestinal allergy in our model, we examined the circulating total and OVA-specific IgE levels by ELISA and the extent of mast cell degranulation by electron microscopy, which has been shown by us and others previously to be a sensitive readout of intestinal allergy.¹⁸ We found that mice

treated with both SEB and OVA showed much higher titers of both total IgE (Figure 7A) and OVA-specific IgE (Figure 7B) in the sera. As expected, saline control or SEB alone-treated mice did not have any measurable amounts of OVA-specific IgE, whereas their serum total IgE levels were very low (Figure 7B). To measure IgE-mediated mast cell activation/degranulation adequately, mice were treated in vivo as described previously and challenged with OVA by gavage before the removal and processing of their jejunal tissues for both light and electron microscopy. By using light microscopy, although mast cells were observed in jejunal samples from all groups of mice, the intestinal mucosa of mice treated with both SEB and OVA had the greatest number of mast cells (Figure 7C). By using electron microscopy, few degranulated granules in mast cells were observed in saline control mice (Figure 7D). However, extensively degranulated mast cells were observed in mice treated with both SEB and OVA (Figure 7E and F), but few were observed in mice treated with SEB alone or OVA alone (Figure 7F). We also found a significant extent of jejunal tissue edema in mice that were treated with SEB and OVA and subsequently chal-

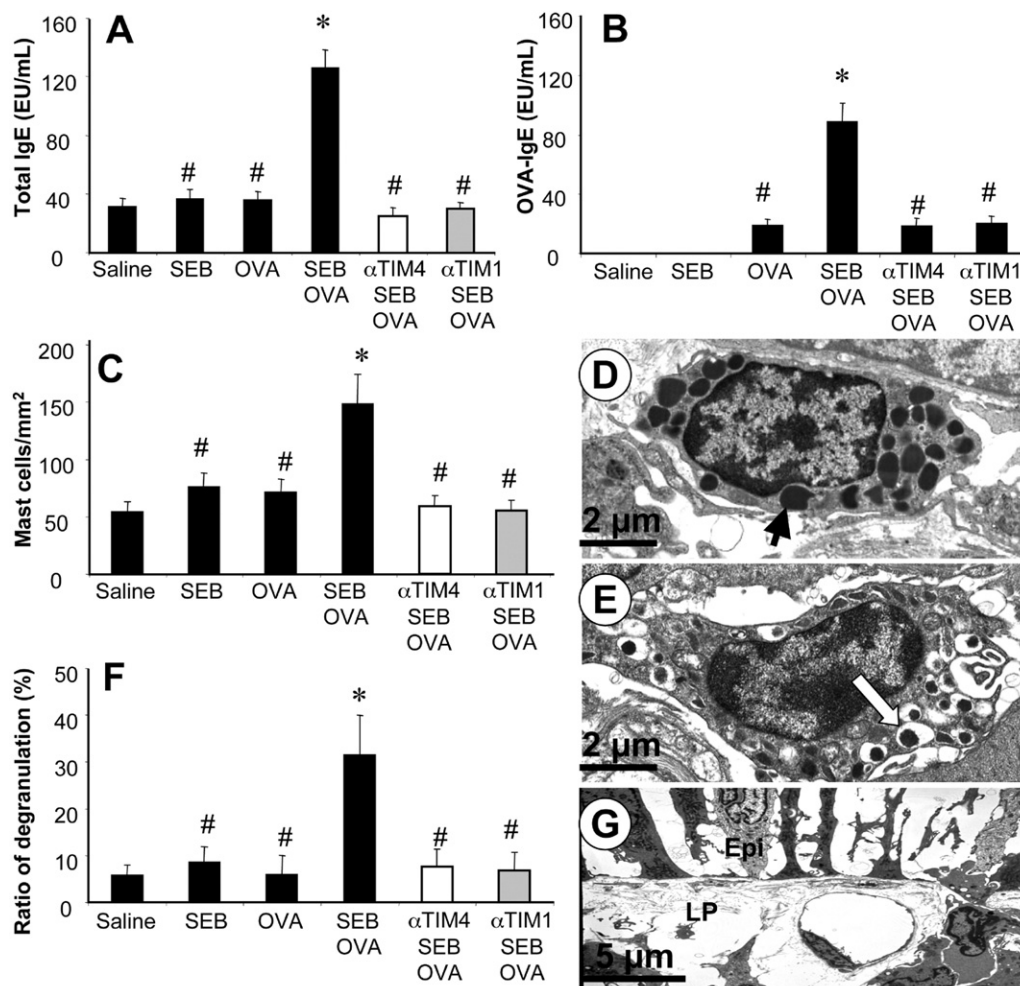


Figure 7. Elicitation of OVA-specific IgE and intestinal allergic responses by SEB and OVA exposure and their inhibition by TIM-4 blockade in vivo. Mice were exposed to SEB, OVA, or both SEB and OVA with or without α TIM-4 and α TIM-1 pretreatment. (A and B) Bars indicate values of (A) serum total IgE and (B) OVA-specific IgE. (C) Mast cell density in jejunal tissue determined by specific staining and light microscopy. (D) Representative electron photomicrograph of a mast cell from saline control group with numerous cytoplasmic granules (solid arrow) of high electron density. (E) Representative electron photomicrograph of a mast cell from SEB- and OVA-treated mice, showing extensive degranulation (open arrow). (F) Qualification of mast cell degranulation in jejunal tissue expressed as the ratio of degranulated vs granulated mast cells determined under an electron microscope. (G) Low-magnification image of jejunal section from SEB- and OVA-treated mice showing the empty areas indicative of tissue edema. Epi, epithelium; LP, lamina propria. Data are expressed as means $\pm$ SD (10 mice/group). * P < .05, compared with (A, F, and G) saline controls or with (B) group OVA. # P < .05, compared with the group treated with both SEB and OVA in the absence of anti-TIM-4 or anti-TIM-1.

lenged by gavage with OVA (Figure 7G). By measuring the changes in short circuit current of segments of the jejunum mounted on Ussing chambers and challenged by OVA, as a physiologic index of gut allergy, we observed a significant increase in short-circuit current in the gut tissue of mice treated with SEB and OVA ($34.5 \pm 6.6 \mu\text{A}/\text{cm}^2$), but not in the tissue of those treated with SEB ($8.5 \pm 2.6 \mu\text{A}/\text{cm}^2$) or OVA alone ($7.6 \pm 2.9 \mu\text{A}/\text{cm}^2$) or saline control ($9.1 \pm 2.8 \mu\text{A}/\text{cm}^2$). The earlier-described results together suggest that concurrent in vivo exposure to both microbial toxin and food antigen leads to the development of food antigen-specific Th2 cells and intestinal food allergy. As a specificity control, a group of mice were exposed to SEB and OVA concurrently as depicted in Figure 5A and challenged with a bystander

antigen bull serum albumin (50 $\mu\text{g}/\text{mouse}$). These mice did not show any hypersensitivity reactions in the intestine (data not shown).

TIM-4 Is Required for the Development of Intestinal Th2 Activation and Allergic Responses Triggered by Concurrent SEB and OVA Exposure In Vivo

Our observation that TIM-4 was up-regulated on intestinal mucosal DCs treated with SEB and was required for Th2 polarization in an OVA DO11.10 T-cell model, suggests that this molecule also may be required for food antigen-specific Th2 polarization and intestinal allergy in vivo. Indeed, by using our model of SEB-OVA-induced intestinal food allergy and in vivo anti-TIM-4

monoclonal antibody treatment (Figure 5A), we found that pretreatment with anti-TIM-4 diminished the OVA-specific Th2 cytokine responses in the sera (Figure 6B and C) and ex vivo cultured T cells (Figure 6F), and blocked OVA-specific T-cell proliferation (Figure 6E), and effectively restored the inhibited Th1 responses (Figure 6D and G). Likewise, immunohistochemistry revealed that the numbers of IL-4-secreting (Figure 7E) as well as IFN- γ -secreting T cells (Figure 7K) in the intestinal mucosa were back to control levels. Consequently, the inhibited Th2 responses were accompanied with diminished OVA-specific IgE levels (Figure 7A and B) and reduced mast cell activation (Figure 7C–G). The similar effects were accomplished by using a monoclonal antibody against the TIM-4 cognate ligand TIM-1 (Figures 6 and 7). These in vivo results thus lend further support that TIM-4 plays an important role in the induction of Th2 polarization and intestinal food allergy.

TIM-4 Expression and Th Polarization by Concurrent Exposure to OVA and a Bacterial Product Other Than SEB

Having established a critical role of SEB in induction of TIM-4 on intestinal mucosal DCs and subsequent Th2 sensitization to OVA, we also examined the effects of other relevant intestinal microbial products including lipopolysaccharide (LPS), staphylococcal peptidoglycan (PGN), and pertussis toxin (PT) on DC TIM-4 expression and Th2 polarization. LPS, PGN, and PT were found to have a differential effect on DC TIM-4 expression. Up-regulation of TIM-4 on intestinal DCs by PGN and PT was associated with in vivo Th2 polarization on co-exposure to OVA, whereas lack of TIM-4 up-regulation in DCs by LPS was associated with OVA-specific Th1 polarization. Detailed experimental design, observations, and relevant discussion can be found in Supplementary Figure 1.

Discussion

Although the development of Th2 cells is believed to be a critical step in the immune mechanisms of food allergy, the factors and mechanisms involved in the initiation of food antigen-specific Th2 activation remain poorly understood. Recent evidence suggests that increased exposure of the mucosal immune system to both bacterial toxins and food antigens predisposes the host to food antigen sensitization and allergy.^{8,9} However, little is known about the molecular mechanisms by which microbial products promote Th2 differentiation and the development of food allergy.

In the current study, we have found that exposure to the bacterial toxin SEB increases TIM-4 expression on intestinal DCs in vitro and in vivo. Co-exposure to both SEB and a food macromolecule OVA can further mature intestinal DCs. Such conditioned intestinal DCs are able to cause OVA-specific Th2 polarization and activation in

vitro. Furthermore, concurrent exposure to SEB and OVA leads to the sensitization of Th2 cells to OVA and the subsequent development of intestinal allergic responses in vivo. Importantly, blockade of TIM-4 in vitro or in vivo inhibits OVA-specific Th2 activation and intestinal allergy. Our study reveals that activation of intestinal mucosal DCs by microbial toxins is critical to the development of food antigen-specific allergic responses, and that TIM-4 expressed on DCs, via its interaction with TIM-1 on T cells, represents an important molecular mechanism in this process.

Recent studies have indicated that the interaction between TIM-4 and TIM-1 breaches the peripheral immune tolerance and leads to T-cell activation.^{13,14} Emerging evidence suggests a role of TIM family members in the immunopathogenesis of certain allergic diseases.^{19,20} Indeed, administration of anti-TIM-1 antibodies suppresses Th2 allergic airway inflammation and conjunctivitis.^{19,20} However, the role of TIM-4 and its interaction with TIM-1 in the pathogenesis of intestinal allergic disease is completely unknown.

The pioneer work performed by Meyers et al¹³ and Umetsu et al¹⁴ has shown that TIM-4 ligation of TIM-1 on CD4 T cells specifically induces Th2 cell proliferation. TIM-4 expression has been found primarily on DCs located within classic lymphoid organs such as the spleen and lymph nodes,²⁰ whereas TIM-4 expression in DCs located within the mucosal tissues is unclear. In the present study, we have found that although TIM-4 normally is expressed weakly in the intestinal mucosal DCs, it is highly inducible by exposure to a relevant microbial product such as SEB both in vitro and in vivo. We previously developed rodent models of intestinal sensitization using pertussis toxins,^{4,9} and cholera toxins also were used in the development of peanut allergy models.²¹ It would be of interest to understand whether microbial products other than SEB also can modulate TIM-4 expression in mucosal DCs and contribute to Th2 polarization in the intestine. Our current study provides further evidence that similar to SEB, bacterial PGN and PT are able to induce TIM-4 expression in intestinal DCs and when co-delivered with OVA in vivo, can induce OVA-specific intestinal Th2 polarization. In sharp contrast, LPS failed to induce TIM-4 expression in intestinal DCs and on co-delivery with OVA, it instead induced a Th1 polarization (Supplementary materials). These results suggest that only some, but not all, microbial products promote Th2-type intestinal conditions via TIM-4 expression by DCs.

In our current study, we found that SEB-conditioned intestinal mucosal DCs were particularly conducive to food antigen-specific Th2 polarization, as exemplified by Th2 cytokine production by OVA-specific CD4 T cells on co-culture with SEB-conditioned, OVA antigen-loaded intestinal DCs. This effect was mediated via TIM-4 expression on these antigen-presenting cells induced by

SEB exposure. Our observation provides a novel molecular mechanism to explain how the primed DCs specifically may promote Th2 sensitization toward normally otherwise harmless food antigens. Our findings also provide a mechanistic explanation for previous studies showing that SEB exposure was found to facilitate DC-mediated Th2 activation.²² We further observed in our current study that SEB-conditioned intestinal DCs led to a concomitantly decreased Th1 cytokine response. This could be owing to the inhibitory effect of increased IL-4 on the activities of STAT-6 and T-box expressed in T cells (Tbet), and subsequently on IFN- γ gene expression.²³ It is noteworthy that our results differ from those of previous studies^{13,14} in that we observed the inability of SEB-conditioned DCs on their own to trigger a robust Th2 response in T cells in the absence of food antigen OVA in vitro. Our in vivo observations also indicate that only co-exposure to both SEB and OVA can induce robust Th2 activation, OVA-specific IgE, and mast cell degranulation. We believe that this is unique to the properties of intestinal mucosal DCs because under normal circumstances these antigen-presenting cells are positioned to be tolerogenic^{3,4} and the full activation of these cells requires at least 2 hits. Indeed, we observed that although SEB exposure up-regulated the expression of CD80, CD86, and MHC II in intestinal DCs, in accord with the published data,^{14,24} concurrent exposure to both SEB and OVA further increased MHC II expression in these DCs. In support, Okano et al¹⁷ reported that exposure to SEB alone could not induce lung allergic responses whereas concurrent exposure to both SEB and a protein antigen could.

Together our findings suggest that Th2 sensitization to food antigens may occur when the intestinal mucosal integrity is disrupted, resulting in increased exposure to both microbial products and food macromolecules. The consequence of such increased exposure to intestinal luminal substances is the activation of mucosal DCs, which via TIM-4 expression and its interaction with TIM-1, activates food antigen-specific Th2 cells and thus leads to the development of intestinal allergic reaction. Our study identifies a critical role of TIM-4 on mucosal DCs in the mechanisms of intestinal allergy and thus suggests that abrogation of TIM-4 or TIM-1 or both would represent a powerful way to dampen allergic responses not only in the gut but elsewhere.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1053/j.gastro.2007.08.006](https://doi.org/10.1053/j.gastro.2007.08.006).

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