Disruption of T-cell immunoglobulin and mucin domain molecule (TIM)–1/TIM4 interaction as a therapeutic strategy in a dendritic cell–induced peanut allergy model

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Background: Recent reports indicate that dendritic cell (DC)–derived T-cell immunoglobulin and mucin domain molecule (TIM)–4 plays an important role in the initiation of Th2 polarization. This study aims to elucidate the mechanisms of peanut allergy mediated by microbial products and DCs and the relationship between peanut allergy and TIM4.

Methods: Mouse bone marrow–derived DCs (BMDCs) were generated and exposed to cholera toxin (CT) or peanut extract (PE) for 24 hours and then adoptively transferred to naive mice. After re-exposure to specific antigen PE, the mice were killed; intestinal allergic status was determined.

Results: Increased expression of TIM4 and costimulatory molecules was detected in BMDCs after concurrent exposure to CT and PE. Adoptively transferred CT/PE-conditioned BMDCs resulted in the increases in serum PE-specific IgE and skewed Th2 polarization in the intestine. Oral challenge with specific antigen PE induced mast cell activation in the intestine. Treating with Toll-like receptor 4 small interfering RNA abolished increased expression of TIM4 and costimulatory molecules by BMDCs. Pretreatment with anti-TIM1 or anti-TIM4 antibody abolished PE-specific Th2 polarization and allergy in the intestine.

Conclusion: Concurrent exposure to microbial product CT and food antigen PE increases TIM4 expression in DCs and promotes DC maturation, which plays an important role in the initiation of PE-specific Th2 polarization and allergy in the intestine. Modulation of TIM4 production in DCs represents a novel therapeutic approach for the treatment of peanut allergy. (J Allergy Clin Immunol 2008;122:55-61.)

Key words: Intestine, peanut allergy, Th2 cell, dendritic cell, bacteria

As much as 2% to 6% of the population has food allergy and food antigen-related disorders, and the prevalence has risen, especially in the last 20 years. The clinical symptoms of food allergy vary from slight abdominal discomfort to life-threatening anaphylactic shock. A common food allergy is peanut hypersensitivity, which can be life-threatening. However, no specific therapeutic remedies for food allergy are available currently, leaving avoidance of ingesting the offending food as the only preventive measure. Thus, it is important to elucidate the pathogenic mechanism involved to develop novel therapies for treating patients with this condition.

Growing clinical evidence indicates a correlation exists between bacterial infection and allergic diseases. For instance, Staphylococcus aureus colonization in the skin is associated with disturbances in the airway of patients with allergic disorders. Indeed, microbial products such as pertussis toxin, cholera toxin, and LPS are frequently used as immune adjuvant for elicitation of experimental allergic conditions. These models have proven instrumental to understanding how some microbial products are involved in the development of allergenic disorders and to elucidate the mechanisms involved.

Dendritic cells (DCs) are critical in initiating an immune reaction in the body. DCs capture and process exogenous antigens and transfer the antigenic information to T cells. DCs also produce costimulatory molecules to assist the process of antigen information transfer and T-cell activation. Subsequently, the naive CD4+ T cells develop to Th1 or Th2 cells according to the cytokine milieu. Th2 cells play a critical role in the pathogenesis of food allergy. However, the molecular mechanisms leading to the development of such food antigen-specific Th2 cells remain largely to be understood. Recent advances indicate that the interaction of T-cell immunoglobulin and mucin domain molecule (TIM)–1 and TIM4 plays an important role in Th2

Abbreviations used

- BMDC: Bone marrow–derived dendritic cell
- CFSE: Carboxyfluorescein diacetate succinimidyl ester
- C-mice: Mice in which bone marrow–derived dendritic cells were stimulated with cholera toxin alone
- CP-mice: Mice in which bone marrow–derived dendritic cells were stimulated with both cholera toxin and peanut extract concurrently
- CT: Cholera toxin
- DC: Dendritic cell
- LPFCM: Lamina propria–derived mononuclear cell
- PE: Peanut extract
- P-mice: Mice in which bone marrow–derived dendritic cells were stimulated with peanut extract alone
- RNAi: RNA interference
- TIM: T-cell immunoglobulin and mucin domain molecule
- TLR: Toll-like receptor

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polarization.13,14 TIM1 is constitutively expressed on CD4+ T cells14; its ligand TIM4 is expressed in DCs.15 The interaction of TIM1 and TIM4 promotes TLR2 cell polarization.15 Our recent findings indicate that staphylococcal enterotoxin B exposure enhances TIM4 expression by intestinal mucosal DCs, thus facilitating the onset of ovalbumin-specific TLR2 responses in the intestine.6,7 However, the role of DC-expressed TIM4 in the pathogenesis of a clinically relevant food-allergic disease such as peanut allergy remains to be investigated.

With the goal of elucidating the immunogenesis of peanut-specific TLR2 responses and allergy, we established a murine model of peanut allergy by using bone marrow-derived DCs (BMDCs). BMDCs were first exposed to cholera toxin (CT) and a food antigen peanut extract (PE)9 concurrently and adoptively transferred to naive mice. A strong hypersensitivity reaction was induced in the intestine on specific antigen PE challenge. We further found that RNA interference (RNAi) for Toll-like receptor (TLR)-4 and TIM4 gene expression in DC or pretreatment with anti-TIM1 antibody abolished peanut-specific TLR2 response and allergy in the gut.

METHODS

Peanut antigen preparation

Peanut extract from crude peanut was prepared according to previous reported procedures8 and used as a specific antigen in this study.

Stimulating BMDCs with CT and/or PE in vitro

Bone marrow-derived DCs were prepared from Balb/c mice (The Jackson Laboratory, Bar Harbor, Maine) and cultured in RPMI-1640 media. On day 7, CD11c+ cells were isolated from the cultured cells with magnetic cell sorting (Miltenyi, Auburn, Calif) and were exposed to (1) CT (Sigma, Oakville, Ontario, Canada) at a dose of 20 μg/mL, or (2) PE (20 μg/mL), or (3) both CT (20 μg/mL) and PE (20 μg/mL) for 24 hours.

Flow cytometry

Cells were fixed with 1% paraformaldehyde for 30 minutes on ice. Cells used to detect cytokines (such as IL-4 and IFN-γ) were permeabilized with a reagent kit (BD Bioscience, Mississauga, Ontario, Canada). Cells were then stained with fluorescence-labeled antibodies for 30 minutes on ice and analyzed with a flow cytometer (FACScan, BD Bioscience).

RNAi

Mouse siRNA of TLR4 and TIM4 was designed and synthesized by Invitrogen (Burlington, Ontario, Canada). The sequence of TLR4 was 5′-gca-ttagagttgctca-3′ (NM_021297); TIM4 was 5′-ggaaatcctgttgaataa-3′ (NM_178759); control siRNA sequence was 5′-aac gaa gca act aag ctc g-3′ that did not target any genes. Transfection was performed following the manufacturer’s instructions as well as our previous studies.15,16 Briefly, TLR4 or TIM4 siRNA was added to culture of BMDCs (106 cells/mL) at a concentration of 60 pmol. Transfection was performed with oligofectamine following the manufacturer’s instructions. The efficiency of siRNA transfection was determined by Western blotting, which showed naive BMDCs naturally express TLR4 and TIM4 that could be inhibited by treating the cells with TLR4 or TIM4 siRNA; the peak inhibitory effect was reached 48 hours posttransfection and sustained for another 48 hours and declined thereafter; the expression of TLR4 or TIM4 in these BMDCs returned to normal levels 96 hours after transfection. The control siRNA did not affect TLR4 or TIM4 expression in BMDCs.

Adaptive transfer of BMDCs and experimental groups

Male Balb/c mice were maintained on peanut-free chow under specific pathogen-free conditions. The experimental procedures were approved by the Animal Ethical Committee at McMaster University. Mice were adaptively transferred with BMDCs (107 cells/mouse) via tail vein injection in 100 μL saline on day 0 and boosted on days 3, 6, 9, and 12. The following experimental groups were designed on the basis of conditions applied to the adaptively transferred BMDCs. CP-mice: BMDCs were stimulated with both CT and PE concurrently; C-mice: BMDCs were stimulated with CT alone; P-mice: BMDCs were stimulated with PE alone; TLR4–mice: BMDCs were transfected with TLR4 siRNA before exposure to CT and PE; TIM4–mice: BMDCs were transfected with TIM4 siRNA before exposure to CT and PE; αTIM1-mice: 30 minutes before the adoptive transfer with CT/PE-conditioned BMDCs, mice were peritoneally injected with anti-TIM1 antibody (50 μg/mouse; or nonspecific rat IgG as a control; R&D Systems, Burlington, Ontario, Canada).

Oral challenge with specific antigen PE

Mice were challenged with PE (5 mg/mouse) via gavage 14 days after adoptive transfer. Control mice were challenged with saline or an irrelevant protein ovalbumin (5 mg/mouse).

Assessment of antigen specific T-cell proliferation

Lamina propria–derived mononuclear cells (LPMCs) were isolated from excised jejunal segments, as we reported previously.6 The LPMCs (106 cells/well) were cultured in RPMI-1640 media in the presence or absence of specific antigen PE (20 μg/mL) for 96 hours. [3H]-thymidine incorporation was assessed with a scintillation counter.

Statistical analysis

Data are presented as the means ± SDs. Differences between 2 groups were evaluated with the Student t test; data among 3 or more groups were evaluated with ANOVA. A P < .05 was accepted as a significant criterion.

Methods of real-time RT-PCR, Western blotting, ELISA, general anaphylactic response assessment, and observation of eosinophils/mast cells are detailed in this article’s Online Repository at www.jacionline.org.

RESULTS

CT promotes TIM4 production by DCs

Cholera toxin is used as an immune adjuvant in animal models of food allergy,17,18 but its mechanisms are not well understood. To study the potential role of TIM4 in the process, in vitro experiments were performed. As depicted in Fig 1A, BMDCs had baseline expression of TIM4 mRNA that was enhanced significantly by exposure to CT alone; the exposure to PE alone did not increase the expression of TIM4 mRNA in BMDCs compared with naive control cells. The concurrent exposure to both CT and PE did not further enhance TIM4 mRNA expression in BMDCs compared with that treated by CT alone. Western blotting experiments resulted in a 42-kd band that appeared clearly in naive BMDCs; exposure to CT or both CT and PE (CP) increased the band size and density but not in those exposed to PE alone (Fig 1, B). These data demonstrated that TIM4 expression by BMDCs could be enhanced by exposure to CT. To elucidate whether expression of TIM4 in BMDCs could be released to the microenvironment, culture media of BMDCs were collected at the end of experiments and subjected to assessment of TIM4. As expected, TIM4 was detected in culture media of naive BMDCs at low levels that were increased significantly by exposure to CT or both CT and PE (Fig 1, C).

CT promotes DC maturation

We further examined whether CT exposure could also promote DCs’ maturation. We assessed the indicators of DC maturation,
including the expression of CD80, CD86, and MHC II, in BMDCs after stimulation with CT and/or PE. The results show that exposure to CT or CT1 PE promoted the expression of CD80, CD86, and MHC II in BMDCs significantly more than treatment with PE alone. PE also increased these molecules slightly, but did not reach the significance criteria. Transfection with TLR4 siRNA, but not control siRNA, inhibited the enhancement of these costimulatory molecules induced by CT or CT1 PE (Fig 2, CP).

Adoptively transferred BMDCs populating the intestinal lamina propria

As the first step toward establishing a murine model of peanut allergy by using BMDCs, we examined whether adoptively transferred BMDCs would populate the intestinal mucosa. To this end, a batch of CD11c CD BMDCs were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; 5 μmol/L) for 15 minutes and adoptively transferred to naive mice via tail vein injection at 107 cells/mouse. One day after the injection, mice were killed; the intestinal segments were excised and processed for confocal microscopy. CFSE-stained cells were localized in the lamina propria (Fig 3), demonstrating that the BMDCs transferred as such could reach the intestine.

DC-induced peanut-specific Th2 differentiation and activation in the intestine

By using the adoptive BMDC transfer approach, we determined whether PE-loaded BMDCs could lead to the generation of PE-specific T cells of Th2 phenotype in the intestine. Thus, LPMCs were prepared from excised intestinal segments, and the mesentry lymph nodes and the cells were cultured in the presence or absence of specific antigen PE. As evaluated by [3H]-thymidine incorporation, significant proliferation was observed in cells from CP-mice, but not in cells from C-mice, or P-mice, or naive mice. Serving as controls, some LPMCs from each group were not stimulated with PE in culture, and these cells did not show a significant difference in [3H]-thymidine incorporation among different groups (Fig 4, A; see this article’s Fig E1 in the Online Repository at www.jacionline.org).

To identify the cells undergoing enhanced proliferation, LPMCs were cultured in the presence or absence of PE; IL-4 and IFN-γ-secreting cells were then differentiated by flow cytometry. As depicted in Fig 4, B, a significant increase in the number of IL-4–secreting CD4 T cells was detected in samples from CP-mice and only slightly increased in those from C-mice, P-mice, and naive mice. On the other hand, the number of IFN-γ–secreting cells decreased in CP-mice compared with those not stimulated with PE, but no significant changes were observed among the remaining groups.

To quantify the level of cytokine release by these cells, levels of IL-4 and IFN-γ in culture media were assessed by ELISA. Higher IL-4 levels and lower IFN-γ levels were observed in culture media of cells from CP-mice; however, levels were not changed significantly in naive mice, C-mice, or P-mice (Fig 4, C). In the absence of PE, levels of IL-4 and IFN-γ in culture media did not show a difference from naive mice.

DC-induced peanut-specific antibody and Th2 cytokine responses in the intestine

Antigen-specific antibodies and Th2 cytokines play a critical role in intestinal allergic reactions19 and are important diagnostic
parameters in diagnosing and evaluating therapeutic efficiency in patients with allergic diseases. Therefore, we next assessed PE-specific antibodies of IgE, IgG1, and IgG2 in the sera and extracts of intestinal tissue. The results showed that PE-specific IgE and IgG1 increased significantly in both sera and intestinal extracts in CP-mice; PE-specific antibodies were not detected in C-mice and naive mice, but a low level was detected in P-mice (Tables I and II; see this article’s Fig E2 in the Online Repository at www.jacionline.org).

To examine further the profile of T cells, we assessed levels of TH1 (IFN-γ) and TH2 (IL-4) cytokines in the sera and intestinal extracts of CP-mice; PE-specific antibodies were not detected in C-mice and naive mice, but a low level was detected in P-mice (Fig 5, A). In comparison, levels of IFN-γ were significantly lower in CP-mice than in other control groups (Fig 5, B), in sharp contrast with high IL-4 levels detected in CP-mice.

Role of TLR4 in DC-induced peanut-allergic responses

It is accepted that TLR recognizes microbial stimulation and induces a pathogenic response in the host. CT is the major toxin in the Gram-negative bacterium Vibrio cholerae and is used in the development of food allergy animal models, and TLR4 could recognize Gram-negative bacterial products. Because we have found that both CT and PE are required to enable BMDC to elicit PE-specific TH2 allergic responses in the intestine, we examined the role of TLR4 in the process. We first found that BMDCs expressed TLR4 at the mRNA and protein levels (data not shown).
We then set out to knock down TLR4 gene expression from BMDCs by using an RNAi approach. We found that as a result of TLR4 knockdown, DC expression of TIM4 in response to CT stimulation was completely blocked, whereas control siRNA did not show any inhibitory effect (Fig 1). Decreased TIM4 expression by these DCs was associated with markedly reduced DC maturation markers (Fig 2). When such DCs were treated with CP and subsequently used to sensitize mice, they failed to induce a PE-specific TH2 response (Figs 4 and 5) and peanut-specific hypersensitivity reactions in the intestine (Fig 6). These results indicate that TLR4 is required in CT-involved initiation of peanut allergy by DCs.

Critical role of TIM1/TIM4 interaction in the induction of peanut allergy in the intestine

That CT exposure markedly enhanced TIM4 expression by DCs (Fig 1) and that TLR4 knockdown inhibited TIM4 expression in these cells, which was associated closely with abrogation of DC-induced peanut-allergic responses (Fig 2, Figs 4–6), strongly suggest the involvement of TIM1−TIM4 pathway in peanut allergy. To investigate this further, we either knocked down the TIM4 gene from BMDCs by RNAi or pretreated recipient mice with anti-TIM1 antibody before DC transfer. As shown in Figs 4, 5, and 6, the treatment successfully inhibited the skewed TH2 responses in recipient mice manifesting as reduced IL-4 levels and an enhancement of abdominal skin microvascular permeability. Blocking the interaction of TIM1-TIM4 with anti-TIM1 or TIM4 RNAi abolished the response (Fig E5).

DISCUSSION

The current study indicates that microbial product CT and food antigen PE promote the expression of TIM4 and costimulatory and MHC II molecules in DCs. Such DCs conditioned by CT and PE, on adoptive transfer, could populate the intestinal mucosa and subsequently induce TH2 peanut antigen–specific allergic responses in the intestine after oral exposure to PE proteins. Of importance, we have identified both TLR4 and TIM4 to be 2 critical DC-associated molecules required for the development of peanut allergy in our model. These results suggest that the concurrent exposure to intestinal microbial products and food antigens is an important step to enable antigen-presenting cells to initiate peanut allergy in the intestine.

Dendritic cells capture, process, and present antigen to T cells and dictate the subsequent immune responses. In the current study, we observed that the same BMDCs captured both CT and PE (data not shown), indicating that these DCs have an opportunity to process these 2 molecules simultaneously to produce mediators to direct subsequent immune reactions. We also observed that CT upregulated costimulatory molecules CD80 and CD86, and MHC II on DCs, in agreement with previous reports. Thus, these PE antigen–loaded DCs would have acquired the capability to generate the necessary signals for PE-specific TH2 cell activation.

Recent reports indicate that the interaction of TIM1 and TIM4 plays a critical role in TH2 polarization. TIM1 is constitutively expressed on CD4+ T cells and is a positive regulator of TH2 cells, and activation of this molecule was found to promote TH2 cell proliferation. On the other hand, TIM4 is the ligand of TIM1 and is expressed by DCs. In our current study, we found that CT was particularly potent in upregulating TIM4 expression in DCs. CT is produced by bacterium *Vibrio cholerae*, one of the bacteria in the intestine. Accumulated evidence indicates that bacterium-derived factors correlate with the prevalence of allergic diseases. The colonized *V. cholerae* secrete toxins

### TABLE I. PE specific antibody (OD 492 nm) in intestinal extracts

<table>
<thead>
<tr>
<th>BMMCs treated with</th>
<th>Media</th>
<th>CT</th>
<th>PE</th>
<th>CP</th>
<th>CP TLR4−</th>
<th>CP αTIM1</th>
<th>CP TIM4−</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE</td>
<td>0</td>
<td>0</td>
<td>0.21 ± 0.11</td>
<td>1.85 ± 0.38*</td>
<td>0.29 ± 0.08</td>
<td>0.25 ± 0.04</td>
<td>0.19 ± 0.12</td>
</tr>
<tr>
<td>IgG1</td>
<td>0</td>
<td>0</td>
<td>0.52 ± 0.12</td>
<td>2.88 ± 0.71*</td>
<td>0.66 ± 0.14</td>
<td>0.51 ± 0.11</td>
<td>0.61 ± 0.15</td>
</tr>
<tr>
<td>IgG2</td>
<td>0</td>
<td>0</td>
<td>0.25 ± 0.08</td>
<td>0.06 ± 0.07*</td>
<td>0.18 ± 0.08</td>
<td>0.24 ± 0.06</td>
<td>0.29 ± 0.16</td>
</tr>
</tbody>
</table>

CP, CT and PE; αTIM1, mice were treated with anti-TIM1 antibody. TLR4−, BMMCs were pretreated with TLR4 siRNA transfection before exposure to CT and PE. *P < .05 compared with PE group.

### TABLE II. PE specific antibody (OD 492 nm) in the sera

<table>
<thead>
<tr>
<th>BMMCs treated with</th>
<th>Media</th>
<th>CT</th>
<th>PE</th>
<th>CP</th>
<th>CP TLR4−</th>
<th>CP αTIM1</th>
<th>CP TIM4−</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE</td>
<td>0</td>
<td>0</td>
<td>0.14 ± 0.09</td>
<td>2.31 ± 0.56*</td>
<td>0.16 ± 0.07</td>
<td>0.27 ± 0.08</td>
<td>0.31 ± 0.12</td>
</tr>
<tr>
<td>IgG1</td>
<td>0</td>
<td>0</td>
<td>0.63 ± 0.23</td>
<td>3.04 ± 0.56*</td>
<td>0.71 ± 0.23</td>
<td>0.97 ± 0.76</td>
<td>0.75 ± 0.56</td>
</tr>
<tr>
<td>IgG2</td>
<td>0</td>
<td>0</td>
<td>0.16 ± 0.05</td>
<td>0.03 ± 0.05*</td>
<td>0.21 ± 0.05</td>
<td>0.16 ± 0.03</td>
<td>0.21 ± 0.11</td>
</tr>
</tbody>
</table>

CP, CT and PE; αTIM1, mice were treated with anti-TIM1 antibody. TLR4−, BMMCs were pretreated with TLR4 siRNA transfection before exposure to CT and PE. *P < .05 compared with PE group.
such as CT that may communicate with the immune system to modulate immune function. Because DCs are localized at the first defense line by their distribution in the subepithelial region in the body, they have the highest probability of encountering CT from the colonized *V. cholerae* and food antigens compared with other immune cells, and such probability increases even further when the integrity of the intestinal epithelium is compromised.

In the current study, the transferred DCs, conditioned by CT and PE, led to increases in peanut antigen–specific T<sub>H2</sub> cells, which was accompanied by the enhancement of TH2 function and allergic reaction in the body. This is in line with the observation that DC-induced peanut antigen–specific T<sub>H2</sub> responses and hypersensitivity reactions in the intestine can be abolished by treating the recipient mice with anti-TIM1 antibody or by abolishing TIM4 expression in DCs with RNAi. Indeed, we observed that intestinal CD<sub>4</sub><sup>+</sup> T<sub>H2</sub> cells expressed TIM1 molecules (data not shown). Our findings suggest that blockade of either TIM1 on T cells or TIM4 on DCs could disrupt the TIM4-TIM1 signaling pathway and accomplish a similar therapeutic outcome for peanut allergy. Compared with other proposed therapies such as gene therapy for peanut allergy, we believe that disrupting TIM1/TIM4 interaction may represent an even better strategy.

Finally, it is proposed that TLR4 recognizes CT and mediates CT effect on immune cells. We also observed this phenomenon in the current study. After knocking down TLR4 gene in DCs by RNAi, these DCs did not respond to CT nor change functionally by increasing production of costimulatory molecules, MHC II and TIM4, although these DCs did respond after the exposure to CT and PE concurrently. These DCs may disrupt the TIM4-TIM1 signaling pathway and accomplish a similar therapeutic outcome for peanut allergy. Compared with other proposed therapies such as gene therapy for peanut allergy, we believe that disrupting TIM1/TIM4 interaction may represent an even better strategy.

In summary, we report that concurrent exposure to CT and PE promotes the production of costimulatory molecules, MHC II, and TIM4 in DCs and enables these antigen-presenting cells, on adoptive transfer, to activate peanut antigen–specific T cells of T<sub>H2</sub> phenotype in the intestine. This activation process could be blocked by inhibiting TIM1 and TIM4 interaction.
We thank Dr Mary H. Perdue for her enthusiastic support of this study.

**Key messages**

- Concurrent exposure to microbial product CT and food antigen PE drives DCs to acquire TIM4 and costimulatory molecules required for peanut-specific Th2 responses in the intestine.
- Pretreatment with anti-TIM1 or anti-TIM4 antibody blocks the peanut-specific allergic response in the intestine, thus holding therapeutic implications for the treatment of allergic diseases.

**REFERENCES**

METHODS
Real-time quantitative RT-PCR
Total RNA was extracted from the BMDCs by using an RNeasy Mini kit (Qiagen, Mississauga, Ontario, Canada). cDNA was synthesized from 1 μg RNA by using the iScript Tm cDNA Synthesis Kit (Bio-Rad, Mississauga, Ontario, Canada). The resulting cDNA was subjected to real-time quantitative RT-PCR (qPCR) that was performed with a LightCycler using a SuperScript III Platinum SYBR Green Two-Step qPCR Kit (Invitrogen, Burlington, Ontario, Canada). The amplified product was detected by the presence of a SYBR green fluorescent signal. The standard curve was designed with β-actin cDNA. The resulting TIM4 products were quantitated with the standard curve. The primers and qPCR conditions included TIM4, forward: 5’-ggaaggctcgcatttggaa-3’; reverse: 5’-tctcagcttggtggtgaag-3’ (annealing temperature: 60°C, for 30 seconds, 39 cycles, 272 bp, PubMed, NM_178759); β-actin: forward: 5’-acccatagctgactccatcc-3’; reverse: 5’-ttcagcttggtggtgaag-3’ (227 bp, PubMed, NM_007393).

Western blotting
Equal amounts of protein (40 μg/well) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with biotin-labeled primary antibodies at a concentration of 1 μg/mL overnight at 4°C. Peroxidase-avidin was added at a concentration of 1 μg/mL to the membranes for 1 hour at room temperature. The membranes were then developed by ECL Plus Western Blotting Detection Reagents (GE Healthcare membranes for 1 hour at room temperature. The membranes were then developed by ECL Plus Western Blotting Detection Reagents (GE Healthcare Life Science, Baie d’Urfe, Quebec, Canada) and signal detected by x-ray film.

Eosinophil and mast cell observation
Jejunal segments were excised and fixed with 4% formalin and stained with hematoxylin and eosin, for eosinophil counting, or with Carnoy solution and stained with 0.5% toluidine blue, for mast cell counting or with 2% glutaraldehyde and processed for electron microscopy to determine mast cell degranulation with the procedures we reported previously. The numbers of mast cells and eosinophils in intestinal sections were counted with light microscopy; 10 fields (×200) were randomly selected from each mouse (60 fields per group). The degranulation of mast cells and eosinophils was observed with electron microscopy. Degranulation was defined as the absence of all or a portion of granular contents or reduced density of granular matrices. Ten mast cells were randomly selected from each mouse; 60 mast cells in total were analyzed for each group. The samples were coded; the observer did not know which groups the samples were from to avoid bias during observation.

ELISA
The blood was collected immediately at the sacrifice, and the sera were separated by centrifugation. Total proteins were extracted from the excised jejunal segments. Levels of IL-4 and IFN-γ in the extracts were assessed with commercial reagent kits (R&D Systems, Burlington, Ontario, Canada) following the manufacturer’s instructions. PE-specific antibodies against IgE, IgG1, and IgG2 in the sera and intestinal tissue were also evaluated by ELISA following the methods we described previously with modification. PE-specific-antibody levels were expressed as OD (492 nm) values. The procedures of TIM4 ELISA were the same as for IgE except that (1) the plates were coated with culture supernatants, (2) the primary antibody was goat antimouse TIM4 polyclonal antibody (1 μg/mL; R&D Systems), and (3) the second antibody was peroxidase conjugated goat IgG. The OD value was recorded to indicate levels of TIM4 in culture supernatants.

Body temperature
Rectal temperature was recorded by a rectal probe digital thermometer (VWR, Mississauga, Ontario, Canada) from each mouse every 10 minutes, 4 times.

RESULTS
Irrelevant antigen does not induce PE-specific T<sub>H2</sub> response
For a comparison, we stimulated BMDCs with both CT (20 μg/mL) and ovalbumin (20 μg/mL), and then adoptively transferred to 6 Balb/c mice with the same procedures used in BMDCs treated with CT and PE (detailed in the main article). Mice were challenged with PE. Samples of the blood and jejunal segments were collected. No PE-specific IgE was detected in the serum; instead, high levels of ovalbumin-specific IgE (122.8 ± 23.6 ng/mL) were determined in the serum. Mast cell numbers were significantly higher in the jejunum (68.6 ± 15.6/mm<sup>2</sup>) than that in naive control mice (22.4 ± 10.4/mm<sup>2</sup>; P < .05). The ratio of mast cell degranulation did not show a significant difference between the ovalbumin group and the naive group. The T-cell proliferation response in the ovalbumin group (cpm, 1156.4 ± 225.3) was essentially similar to those from naive group (cpm, 1356.4 ± 353.6; P > .05).

A general anaphylactic response was triggered in mice by PE challenge. Compared with naive mice, PE-challenged mice showed that rectal temperature dropped markedly (Fig E5, A), and serum histamine levels increased (Fig E5, B) and significantly enhanced the microvascular permeability as indicated by Evans blue extravasations (Fig E5, C). Pretreatment with antibodies against TIM1 (or mice transferred with TIM4-deficient BMDCs) abridged the pathophysiological changes induced by PE challenge.

To elucidate whether the expression of TIM4 in intestinal DCs was increased in peanut allergy, an in vivo study was performed. A group of Balb/c mice was sensitized to PE with CT as an adjuvant with reported procedures. DCs in the jejunum did express TIM4 that was significantly increased after sensitized by PE + CT (Fig E6).

REFERENCES
FIG E1. PE-specific T-cell proliferation (a dose-response study). LPMCs were isolated from mice treated with anti-TIM1 antibody (A) or anti-TIM4 antibody (B), or BDMCs were transfected with TLR4 siRNA (C); cells were cultured in the presence of specific antigen PE; the [3H] incorporation was detected by a liquid scintillation counter. Bars indicate the cpm (means ± SDs from 6 mice/group). *P < .05 compared with dose 0 group.
FIG E2. Serum PE-specific IgE levels. Mice received CT/PE pulsed BMDCs. Before each transfer, mice also received antibody against TIM1 (A) or TIM4 (B) at graded doses. Some mice received TLR4 siRNA–treated BMDCs (C). Serum PE-specific IgE were determined by ELISA. Bars indicate serum IgE (means ± SDs from 6 mice/group). *P < .05 compared with dose 0 group.
FIG E3. Mast cell degranulation (a dose-response study). Mice were treated with anti-TIM1 (A) or anti-TIM4 (B), or BDMCs were transfected with TLR4 siRNA (C) prior to the procedures described in the article. On killing, a piece of jejunum was processed for electron microscopy with the procedures described in the main article and Fig 6. Bars indicate the ratio of degranulated granules (means ± SDs from 6 mice/group). *P < .05 compared with dose 0 group. Naïve, Naive mice served as controls.
FIG E4. Serum mouse mast cell protease (MMCP)-1 levels (a dose-response study). Mice were treated with anti-TIM1 (A) or anti-TIM4 (B), or the BDMCs were transfected with TLR4 siRNA (C) prior to the procedures described in the article. Serum MMCP-1 levels were determined by ELISA. Bars indicate serum MMCP-1 levels (means ± SDs from 6 mice/group). *P < .05 compared with dose 0 group. Naive, Naive mice served as controls.
FIG E5. Peanut allergy induces a general anaphylactic response. A, Bars indicate the changes of rectal temperature. B, Bars indicate serum histamine. C, Bars indicate Evans blue extravasations in the abdominal skin or jejunal tissue. Data are expressed as the means ± SDs. *P < .05 compared with naive group. CP, BMDCs were pulsed with CT and PE and adoptively transferred to mice.
FIG E6. PE + CT increases the expression of TIM4, CD80, and CD86 in intestinal DCs. Isolated intestinal CD11c<sup>+</sup> DCs were stained with antibodies against TIM4, or CD80, or CD86, respectively. The stained cells were analyzed by flow cytometry. Representative histograms show cell populations positively stained by TIM4 (A and B), CD80 (C and D), and CD86 (E and F).