

Intestinal epithelial cells express galectin-9 in patients with food allergy that plays a critical role in sustaining allergic status in mouse intestine

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Abstract

Background and aims: Mechanisms in sustaining the allergic hypersensitivity status in the body are unclear. Galectin-9 (Gal-9) has strong immune regulatory capacity. The present study aims to elucidate the role of Gal-9 in sustaining allergic status in the intestine.

Methods: Duodenal biopsies were obtained from 20 patients with peptic ulcer and food allergy (FA). The expression of Gal-9 in intestinal tissue was examined at both protein level and mRNA level. Two coculture systems with intestinal epithelial cells (IEC) and mast cells, or dendritic cells (DC) and T cells were established to investigate the source of Gal-9 in the intestine and the mechanism by which Gal-9 modulated DC's phenotyping and sustained the T helper 2 polarization.

Results: Normal IEC showed mild expression of Gal-9 that was markedly enhanced in patients with FA. Mast cells had the capability to induce IEC to produce Gal-9 via releasing tryptase that activated the proteinase-activated receptor 2 on IEC. Gal-9 activated DC to produce TIM4 (T-cell immunoglobulin mucin domain) via ligating TIM3 on DC via activating the cyclic guanosine monophosphate (cGMP) pathway. In a mouse FA model, blocking Gal-9 inhibited the allergic hypersensitivity status and the antigen-specific Th2 response in the intestine.

Conclusions: IEC-derived Gal-9 contributes to sustaining the allergic status in the intestine.

Skewed T helper type 2 (Th2) polarization plays a critical role in the pathogenesis of a broad array of allergic disorders such as food allergy (FA), allergic asthma, and allergic dermatitis. Once established, the allergic status in most adult cases usually sustains in a long term or for the rest of the life. The mechanism that sustains allergic hypersensitivity status is poorly understood.

The galectin family is defined by conserved peptide sequence elements in the carbohydrate recognition domain (1). Fifteen members have been identified in mammals (2). Galectin-9 (Gal-9) is a member of the large family of lectins

that has affinity for beta-galactosides (2, 3). Galectins exhibit various biological functions, such as cell aggregation and adhesion, proliferation, cell death, modulation of inflammation, and metastasis. Gal-9 exhibits regulatory functions for activated eosinophils (4). Gal-9 also regulates immune cells' function via ligating TIM3 (T-cell immunoglobulin and mucin domain molecule-3) (5, 6). Therefore, it seems that Gal-9 has the capability to regulate immune response especially on allergic status. Recent studies support this hypothesis by showing that Gal-9 acts as an eosinophil recruiter in airway allergic inflammation (7) and plays a role in allergic

conjunctivitis (8). On the other hand, Gal-9 has the capacity to suppress Th1 and Th17 pattern inflammation (9). However, the role of Gal-9 in the pathogenesis of FA remains to be investigated.

TIM is a recently described molecular group that includes four subtypes: TIM1, TIM2, TIM3 and TIM4. TIM1 and TIM4 play a critical role in the pathogenesis of skewed Th2 responses (10, 11). TIM1 is mainly expressed by activated Th2 cells. TIM4 is expressed by activated dendritic cells (DC) (12, 13). Interaction between TIM1 and TIM4 triggers Th0 cells to differentiate to Th2 cells (10, 11). Inhibition of TIM1 abolishes allergic responses (14). TIM3 is expressed on DC and Th1 cells; activation of TIM3 suppresses Th1 response (15, 16). Thus, it is of significance to elucidate whether the overexpression of Gal-9 inappropriately suppresses Th1 response via ligation of TIM3, which consequently results in skewed Th2 polarization in the body.

The prevalence of FA is as high as 2–8% in the world (17). The pathogenic feature of FA is skewed Th2 pattern inflammation in the intestine. As Gal-9 suppresses Th1 response and modulates DC function (5, 6, 17), we hypothesize that Gal-9 is involved in the pathogenesis of FA. Therefore, in the present study, we investigated the role of Gal-9 in sustaining the allergic hypersensitivity in FA. Indeed, the data show that Gal-9 plays an important role in sustaining the allergic status in the intestine.

Materials and methods

Patients

Thirty patients (men: 15, aged 18–58; women: 15, aged 18–61) with peptic ulcer with or without FA were recruited to this study at the Department of Gastroenterology, Zhengzhou University. The diagnosis of FA is described in Supporting Information. The patients did not use any antiallergy drugs at least 1 month before the study. Ten healthy control subjects were also recruited in which allergic disorders could be ruled out. The demographic data of patient are presented in Table S1.

Duodenal biopsy

Duodenal biopsies were taken from 30 patients with peptic ulcers with or without FA under endoscopy for the purpose of ruling out malignant disorders. Two pieces of biopsies were taken from the edge of ulcers of each patient. The procedures using human tissue in the study was approved by the Research Ethic Committee at Zhengzhou University. Informed written consent was obtained from each subject.

Mice

Balb/c mice, 6–8 weeks old, were purchased from Charles River Canada (St Constant, QC, Canada). Mice were maintained in a pathogen-free environment. The procedures of animal experiments in this study were approved by the Animal Care Committee at McMaster University.

Flow cytometry

Cells were collected and incubated with fluorescence-labeled primary antibodies on ice for 30 min. (For the intracellular staining, cells were fixed with 1% paraformaldehyde on ice for 30 min and incubated with permeabilization reagents for 30 min on ice) The stained cells were analyzed using a flow cytometer (BD FACSCanto; BD Bioscience, San Jose, CA, USA).

Intestinal epithelial cells and HMC1 cell culture

Intestinal epithelial cells (IEC) were seeded in inserts of transwell system and cultured with Dulbecco's modified eagle medium (DMEM) (HMC1 cells, a gift from Dr. Butterfield at Mayo Clinic, were cultured with RPMI1640 medium) at 37°C with 5% CO₂. After coculture with HMC1 cells (the coculture medium was DMEM), IEC were removed from the support filter with 0.5% trypsin–EDTA, smeared onto slides, and used for immunocytochemistry. For the purposed of activating HMC1 cells, compound 48/80 (C48/80 in short; 5 µg/ml; a nonspecific mast cell activator) was added to basal chambers; cells were collected at 48 h later for further experiments.

Statistics

All values were expressed as the means $\pm$ SD of at least three independent experiments. The values were analyzed using the two-tailed unpaired Student's *t*-test when data consisted of two groups or by ANOVA when three or more groups were compared. *P* < 0.05 was accepted as statistically significant.

Other experimental procedures were listed in Supporting Information.

Results

Expression of Gal-9 is increased in the gut of patients with FA

Previous studies indicate that human intestinal cell line Caco-2 cells and human intestinal M cells express Gal-9 (18). Whether Gal-9 is involved in the pathogenesis of intestinal immune disorders is unclear. Thus, we collected a batch of duodenal biopsies from patients with peptic ulcers with or without FA. The results showed that Gal-9-positive immune products were observed in all 20 intestinal biopsies from patients with FA and 10 non-FA intestinal samples (Fig. 1A–C); most epithelial cells were positively stained in FA group, much less frequency of positively stained cell was observed in non-FA group. The semiquantitative results of immune staining indicated that the Gal-9 staining score was 3.75 ± 0.23 in FA group, whereas for non-FA group, the score was 1.0 ± 0.1 (*P* < 0.01). Some positively stained cells were also observed in the lamina propria. The results of Gal-9 expression in the duodenum were confirmed by RT-PCR (Fig. 1D) and Western blotting (Fig. 1E). The serum Gal-9 levels (Fig. 1F) were significantly higher in FA group than in healthy group.

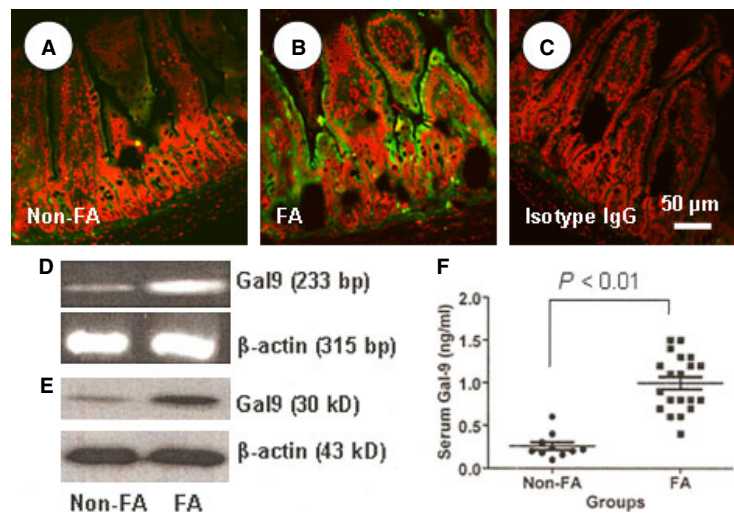


Figure 1 Expression of Gal-9 in IEC is increased in food allergy (FA). Duodenal biopsies were obtained from 30 patients with peptic ulcers including 20 FA patients and 10 non-FA patients. The samples were processed for immunohistochemistry, RT-PCR, and Western blotting to detect the expression of Gal-9. (A–C) Representative confocal images show Gal-9-positive immune products (in green) in duodenal samples of non-FA patient (A) and FA patient

(B). C is a negative control (isotype IgG staining). (D–E) Representative RT-PCR results (D) and immune blots (E) show the expression of Gal-9 in biopsies. The β -actin gels are used as an internal control. (F) The sera were collected from each subject and processed to determine the levels of Gal-9 (by ELISA). Scatter dot plots show the levels of Gal-9. Each dot represents a single data point from one patient.

Mastocytosis is observed in the duodenum of FA patients

Mast cells were observed in the duodenal samples of both FA group and non-FA group by immunohistochemistry (Fig. 2A–C). The frequency of mast cells in the duodenum

was significantly higher in FA group than in non-FA group (Fig. 2D). The serum levels of tryptase were also higher in FA group than in non-FA group (Fig. 2E). Correlation assays were performed with the data that showed positive correlation between (i) serum tryptase and Gal-9 ($r = 0.606$;

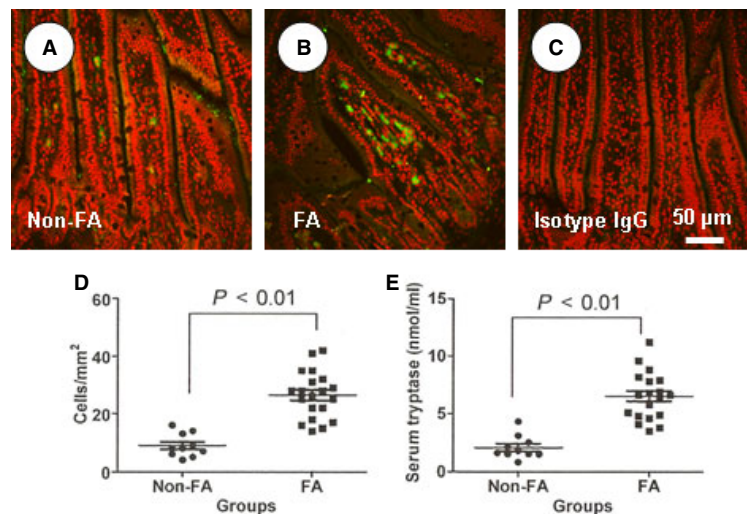


Figure 2 Mastocytosis is observed in FA duodenal mucosa. Cryosections of the duodenal biopsies were stained with antitryptase antibody for counting mast cells. (A–C) Representative confocal images show tryptase⁺ cells (mast cells; in green) in duodenal mucosa. C is a negative control (stained with isotype IgG). (D) The tryptase⁺ cells were counted on sections. The scatter dot plots

show frequency of tryptase⁺ cells in duodenal biopsies. Each dot represents an averaged number of tryptase⁺ cells from 20 fields per sample. (E) Serum levels of tryptase were also assessed by enzymatic approach. The scatter dot plots show the levels of serum tryptase. Each dot represents a single data point from each patient.

$P < 0.01$) and (ii) serum tryptase and mast cell number in the biopsies ($r = 0.556$; $P < 0.01$), but not between serum IgE and mast cell number ($r = 0.227$; $P > 0.05$).

Exposure to mast cells increases the expression of Gal-9 in human intestinal epithelial cells

Weak staining of Gal-9 could be seen in naïve IEC as shown by immunohistochemistry. The expression of Gal-9 was markedly increased in IEC after coculture with C48/80-activated HMC1 cells, which was abolished by pretreatment with mast cell stabilizer ketotifen (Fig. 3A). Similarly, we also observed Gal-9 expression in murine intestinal epithelial cell line IEC4.1 cells (Fig. S1). IEC exposed to C48/80 alone (without HMC1 cells) did not enhance the Gal-9 expression (Fig. 3A5). We also detected the enhancement of Gal-9 levels

in IEC culture supernatant after coculture with activated HMC1 cells (Fig. S2).

Tryptase plays a critical role in mast cell-induced Gal-9 expression in IEC

As tryptase plays an important role in the pathogenesis of allergic disorders (19), we further observed the role of tryptase in the production of Gal-9 in IEC. The results showed that exposure to tryptase markedly increased the expression of Gal-9 in IEC in a dose-dependent manner, which was abolished by knocking down the gene of protease-activated receptor (PAR)2 in IEC (tryptase activates IEC via cleaving PAR2 on IEC) (20) (Fig. 3B,C). Using human PAR2 agonist (peptide SLIGKV) (20) but not the control peptide (VKG-ILS), we also got similar results (Fig. 3A6–7).

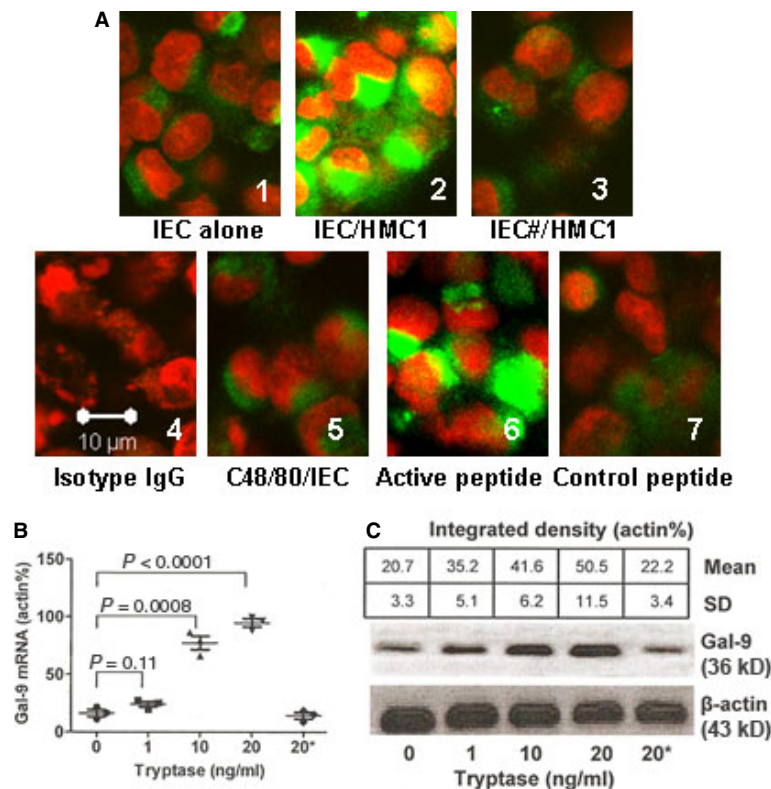


Figure 3 Mast cell increases Gal-9 expression in IEC. (A) IEC (T84 cells) were co-cultured with mast cells (HMC1 cell line) for 48 h in transwell system in the presence of C48/80 and processed to evaluate the expression of Gal-9 by immunocytochemistry, real-time RT-PCR, and Western blotting. (A) Representative confocal images show Gal-9 staining (in green) in IEC. The nuclei were stained with propidium iodide (in red). IEC alone: No HMC1 cells were added to IEC culture. IEC/HMC1: IEC were cocultured with HMC1 cells in the presence of C48/80. IEC#/HMC1: HMC1 cells were pretreated with mast cell stabilizer ketotifen (0.5 mg/ml) and then cocultured with IEC in the presence of C48/80. Isotype IgG: IEC cells were stained with matched isotype IgG used as a negative control.

C48/80/IEC: C48/80 was added to IEC culture, and no HMC1 cells were added. Active peptide: IEC were stimulated by active PAR2 peptide (SLIGKV) in culture at a dose of 20 µg/ml for 24 h. Control peptide: IEC were stimulated by control PAR2 peptide (VKGILS) in culture at a dose of 20 µg/ml for 24 h. (B) Scatter dot plots show Gal-9 mRNA levels in RNA extracts from tryptase-stimulated IEC. The data were normalized to folds of β-actin (the internal control). Each dot represents a single data point. Data are from three separate experiments. *IEC were knocked down the gene of PAR2 (also see Fig. S3A). (C) A representative Western blotting gel shows Gal-9 immune blots (from three separate experiments) in IEC extracts. *IEC were knocked down the gene of PAR2.

Gal-9 regulates DC's functional status

We next generated bone marrow-derived dendritic cells (BmDC) to immature status and exposed to exogenous Gal-9 in culture. As shown by flow cytometry, marked increases in CD80 and CD86 but not in IL-12 were observed in BmDC. The effect of Gal-9 on BmDC could be

blocked by knockdown of TIM3 gene with RNA interference (RNAi) in BmDC (Fig. 4).

Interaction of Gal-9 with TIM3 induces DC to produce TIM4

It is reported that the interaction of Gal-9 with TIM3 induces DC activation (5, 6); it may drive DCs to produce

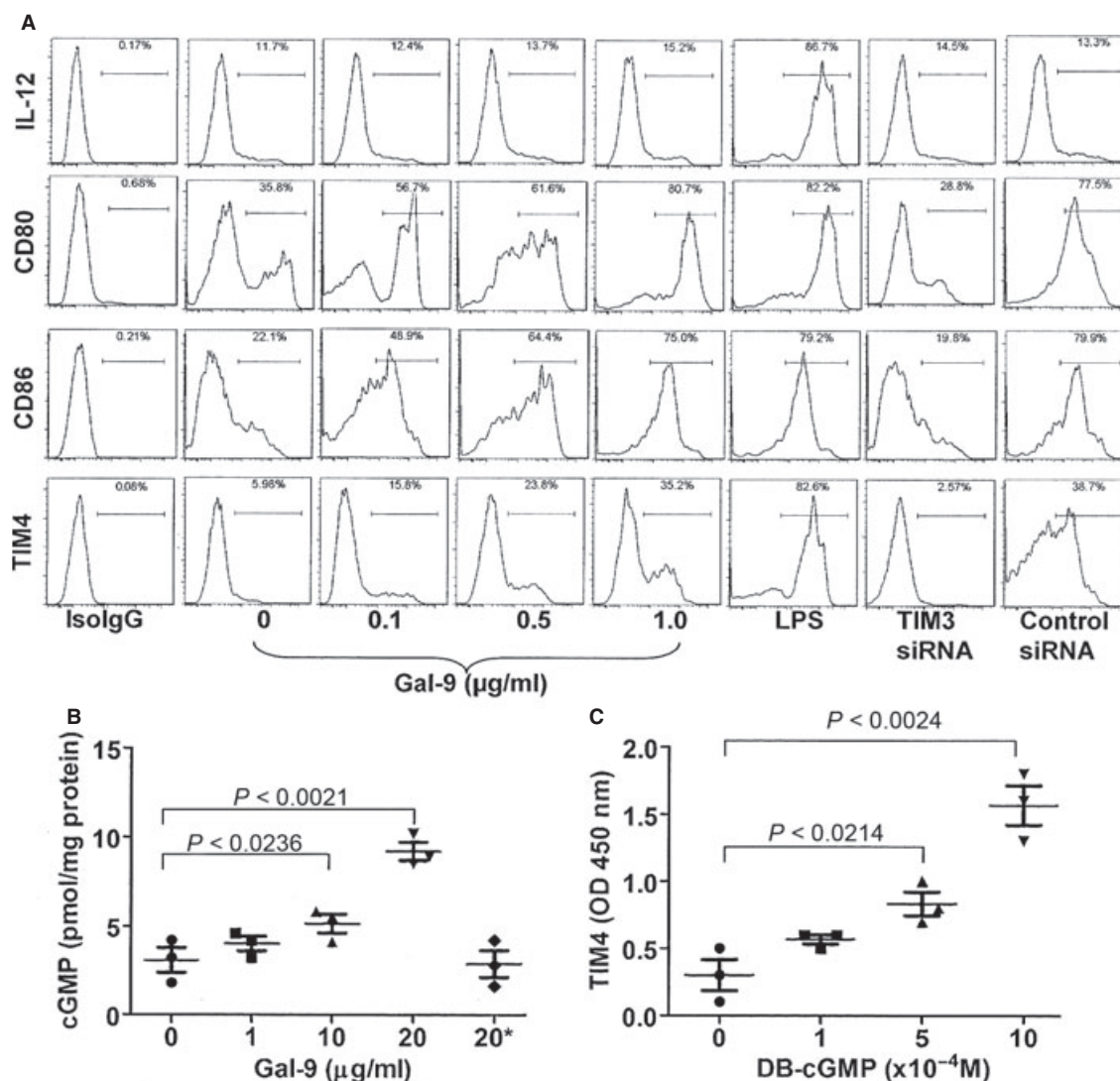


Figure 4 Gal-9 modulates bone marrow-derived dendritic cells (BmDC's) cytokine profile. Immature BmDCs were exposed to Gal-9 at indicated doses for 24 h. (A) The histograms show frequency of CD80⁺, CD86⁺, IL-12⁺, and TIM4⁺ BmDCs. Data represent three separate experiments. Blank: Cells that did not stain were used as negative staining control. Gal-9: Gal-9 was added to the culture at graded doses. LPS: LPS was added to the culture at a dose of 100 ng/ml using as positive control. TIM3 siRNA: TIM3-deficient BmDCs were prepared by knocking down the TIM3 gene from BmDCs by RNAi. The TIM3-deficient BmDCs were stimulated by adding Gal-9 at a dose of 1 μg/ml for 24 h (also

see Fig. S3B). Control siRNA: BmDCs were treated with control siRNA and then stimulated by Gal-9 at a dose of 1 μg/ml for 24 h. (B) BmDC were stimulated by Gal-9 for 24 h. Levels of cGMP in BmDC cellular extracts were evaluated by ELISA. Scatter dot plots indicate levels of cGMP in BmDC. Each dot represents a single data point. *BmDCs were pretreated with phosphodiesterase V (a cGMP inhibitor) at a dose of 1 μg/ml. (C) BmDC were stimulated by cGMP analog dibutyl-cGMP (DBcGMP) for 24 h. Cellular protein extracts were analyzed for TIM4 contents by ELISA. Scatter dot plots indicate TIM4 contents in DC as presented by OD value. Each dot represents a single data point.

TIM4 as well. To test the hypothesis, we measured TIM4 expression in DC after exposure to Gal-9. As shown by flow cytometry, only weak expression of TIM4 was observed on semi-mature BmDC (Fig. 4A). After exposure to Gal-9 for 24 h, the expression of TIM4 was markedly enhanced that could be abolished by knocking down the gene of TIM3 in BmDC (Fig. 4A). The data indicate that Gal-9 is capable of activating DC to produce TIM4 via ligation with TIM3.

Cyclic GMP is involved in Gal-9-induced TIM4 production by DC

To elucidate whether cGMP is involved in TIM4 production, in the first attempt, immature BmDCs were exposed to Gal-9 at graded doses for 24 h. We measured the levels of cGMP

in BmDC extracts by ELISA; the levels of cGMP in BmDCs were increased 2.5- to threefolds in a Gal-9 dose-dependent manner (Fig. 4B). We next added cGMP analog dibutyryl-cGMP (DBcGMP) to immature BmDC culture that resulted in the increase in TIM4 in DC in a DBcGMP dose-dependent manner (Fig. 4C). Finally, we pretreated BmDC with phosphodiesterase V (a cGMP inhibitor) that made Gal-9 have no effect on TIM4 production by BmDC (Fig. 4B).

Gal-9 contributes to sustaining the allergic status in the intestine of mice with FA

A mouse FA model was developed with the procedures in Fig. 5A. The results showed that the 'allergic hypersensitivity status' was established in the intestine (Figs 5B–E and

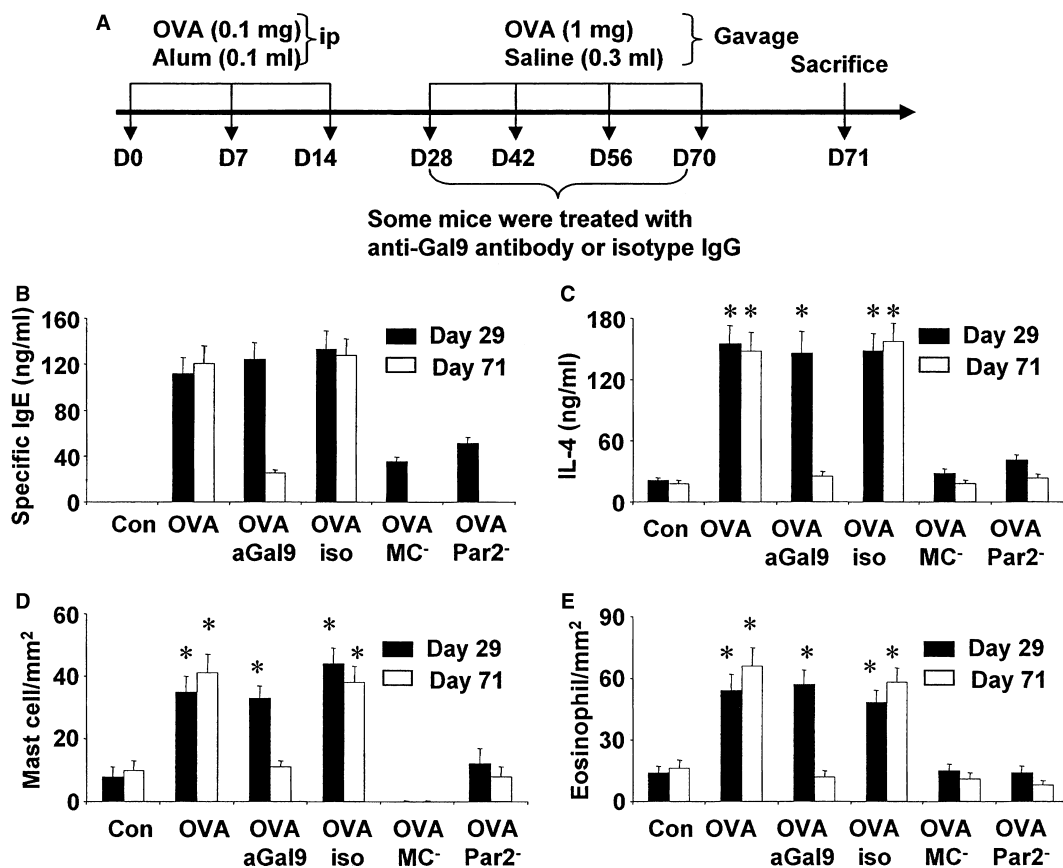


Figure 5 Gal-9 plays a critical role in maintaining the hypersensitivity status in food allergy. (A) A diagram of FA mouse model development. Balb/c mice (or mast cell-deficient mice or PAR2-deficient mice) were employed with 12 mice per group. The letter 'D' stands for 'day'. Six mice were killed on day 29; six mice were killed on day 71. (B) Bars indicate serum levels of OVA-specific IgE. The sera were collected during killing. OVA-specific IgE in the sera was measured by ELISA. (C) Bars indicate the levels of IL-4 in intestinal protein extracts that was determined by ELISA. D–E, Bars show mast cell (D) and eosinophil (E) Counts in jejunal tissue. Immune-positive cells were counted in 20 high power (×200) fields per mouse, six

mice a group. Con, control mice; naive mice were fed with saline. OVA, mice were treated with OVA and alum as depicted in A. OVA/aGal9: mice were treated with OVA and alum as well as with anti-Gal-9 antibody 30 min prior to each exposure to antigen OVA on day 28, 42, 56, and 70. OVA/iso: mice were treated with OVA and alum as well as with isotype IgG (matched to the anti-Gal-9 antibody) 30 min prior to each exposure to antigen OVA on day 28, 42, 56, and 70. OVA/MC^{-/-}: mast cell-deficient mice were treated as depicted in A. OVA/Par2^{-/-}: PAR2-deficient mice were treated as depicted in A.

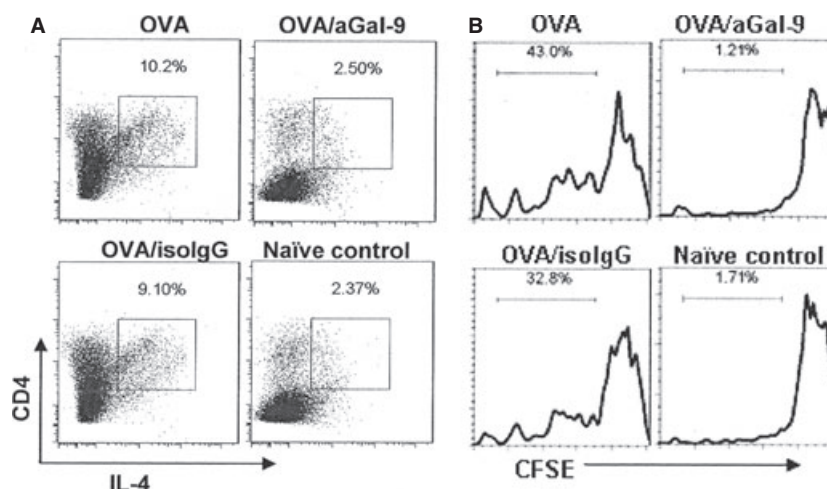


Figure 6 Gal-9 plays a critical role in sustaining antigen-specific Th2 responses. Mice and experimental procedures are the same as Fig. 5A. After killing, LPMCs were isolated for further experiments. (A) A part of the isolated LPMC was cultured for 3 days in the presence of specific antigen (OVA, 10 μ g/ml). Cells were collected at the end of culture, stained with fluorescence-labeled anti-CD4 and anti-IL-4 antibodies and analyzed by flow cytometry. The dot plots

show frequency of CD4⁺ IL-4⁺ T cells (Th2 cells; the gated cells). Data represent three separate experiments. (B) A part of the isolated LPMC was stained with CFSE and cultured for 3 days in the presence of specific antigen (OVA, 10 μ g/ml). Cells were collected at the end of culture and analyzed by flow cytometry. The histograms show the results of CFSE dilution. The gated portion is proliferated T cells.

Fig. 6), Fig. S4 manifesting a significantly increased (i) OVA-specific IgE and IL-4; (ii) infiltration of mast cells and eosinophils; (iii) diarrhea upon specific antigen challenge; (iv) frequency of intestinal CD4⁺ IL-4⁺ T cell; and (v) antigen-specific CD4⁺ T-cell proliferation. The allergic hypersensitivity status could be abolished by treatment with anti-Gal-9 antibody (Figs 5B–E and 6), Fig. S4. In addition, mast cell-deficient mice and PAR2-deficient mice were treated with the procedures in Fig. 5A. The results showed that these two strains had impaired capacity to be induced with allergic hypersensitivity status in the intestine.

Discussion

In this study, we have shown that IEC express Gal-9 that is significantly increased in patients with FA. Mast cell-derived tryptase has the ability to induce the expression of Gal-9 in intestinal epithelial cell line. Exogenous Gal-9 can bind TIM3 to activate DC to strengthen the expression of TIM4. Blocking Gal-9 suppresses the established food antigen-related hypersensitivity in the intestine.

Gal-9 has been identified in a number of cells, including endothelial cells (21), intestinal epithelial cell line Caco-2 cells (18), M cells (19), T cells (22), mast cells (23), and fibroblasts (24); the fact suggests that Gal-9 plays essential roles in basic cell function of these cells. Our data further demonstrate that human IEC also produce Gal-9. The novel aspect of this finding is that the expression of Gal-9 is very low in normal intestinal epithelia while it is markedly enhanced in patients with FA; the fact implies that Gal-9 may be involved in the pathogenesis of FA.

Mast cell is one of the major effector cells in allergic disorders; they distribute immediately under the epithelial layer to

defend pathogen invasion and release chemical mediators upon activation to induce inflammatory reaction. The location of mast cells makes it efficiently to influence the functional status of IEC via releasing mediators. Tryptase is one of the major mediators from mast cell that plays a critical role in mast cell-related allergic inflammation (25). Mastocytosis in the intestine is one of the pathologic features of FA that may be one of the sources of tryptase to stimulate IEC to produce Gal-9 in the intestine, probably via activating the PAR2 in IEC, which was supported by the present data; the production of Gal-9 by IEC was abolished by knocking down the gene of PAR2 in IEC. The finding is also supported by our previous work in which we show that mast cell-derived tryptase activates IEC via cleaving PAR2 (20). Others have shown evidence that mast cell-derived tryptase activates PAR2 to increase intestinal epithelial barrier permeability (26). On the other hand, mast cells also produce Gal-9 (27) that acts as an autocrine regulator of mast cell function to suppress excessive degranulation, which can be another source of Gal-9 in the intestine. The data indicate that apart from being an effector cell in allergic diseases, mast cell also promotes the production of Gal-9 in the intestine to modulate immune response.

Dendritic cells activation is the initiation point of immunity via initiating a series of subsequent immune responses. Published data indicate that Gal-9 can activate DCs and promote DC maturation (5, 6). In line with these pioneer studies, the present experiments also resulted in that Gal-9 activated DCs by showing the enhancement of CD80 and CD86 expression. It is noteworthy that Gal-9-activated DCs do not increase the production of IL-12. The fact implies that Gal-9-activated DCs may have the capability to induce Th2 pattern immune response. TIM4 is a critical molecule in the initiation of Th2 polarization (10, 13) that is produced by activated DCs (12).

Our previous study indicated that TIM4 was markedly increased in DCs in the intestine of mice with FA (13). The present data have expanded our previous experience on TIM4 study that Gal-9 can activate DCs to produce TIM4 via ligation with TIM3; the second messenger cGMP plays a critical role in Gal-9-induced TIM4 production by DCs.

To understand the mechanism by which FA sustains in the body may help to identify new effective remedies for the treatment of FA. The skewed Th2 polarization is proposed as one of the major pathologic changes in the intestine of individual with FA. However, our knowledge on sustaining the allergic hypersensitivity status in the intestine is still limited. Previous studies indicate that TIM4 plays a critical role in the initiation of Th2 polarization by ligating its receptor, TIM1, on CD4⁺ T cells (10, 13). TIM1 expression on CD4⁺ T cells has the tendency to decay in the absence of TIM4 (16). Thus, the endogenous steady supply of TIM4 is necessary to maintain the Th2 polarization and allergic hypersensitivity status. The present study has filled up this knowledge gap by providing evidence that mast cell-derived tryptase induces IEC to produce Gal-9, the latter activates DCs to produce TIM4 that can be a steady supplier of TIM4 in the body to ligate TIM1 on Th2 cells and thus sustains the allergic hypersensitivity status in the intestine of individuals with FA. Indeed, blocking of Gal-9 abolished the allergic hypersensitivity status in the intestine as we observed in the present study, which suggests that Gal-9 contributes to the sustaining of allergic hypersensitivity status in the intestine.

Author contribution

X.C., C.H.S., Z.Q.L., B.S.F., P.Y.Z., P.L., S.H.I., and S.G.T. were involved in performing experiments and revising the manuscript. P.C.Y. was the principle investigator, supervised

the project, and contributed to project design, data analysis, and paper writing.

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Conflict of interest

The authors do not have any financial conflict of interest to declare.

Supporting Information

Additional Supporting Information may be found in the online version of this article found at: www.wileyonlinelibrary.com.

Figure S1. Mouse small intestinal cells (IEC4.1 cell line) express Gal-9.

Figure S2. Gal-9 levels in IEC culture supernatant.

Figure S3. Gene knocking down for PAR2 in IEC and TIM3 in BmDC by RNAi.

Figure S4. Gal-9 regulates the expression of TIM1 and TIM4.

Table S1. Demographic data of FA patients (pt).

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Supplemental material

Intestinal epithelial cells express galectin-9 in patients with food allergy that plays a critical role in sustaining allergic status in mouse intestine

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Running title: Galectin-9 maintains allergic status

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Table S1. Demographic data of FA patients (pt)

Pt#	Age	Sex	Food allergens (determined by skin test)	Other allergies	Specific IgE (ng/ml)	Total IgE (ng/ml)
1	22	M	Milk	Asthma	56.3	88.3
2	18	M	Egg, milk	Asthma	81.2	112.3
3	51	M	Sea food, wheat		55.6	61.2
4	48	M	Milk, soy bean, wheat		48.3	81.5
5	33	M	Peanut, sea food, sesame		76.2	84.2
6	26	M	Egg, peanut, soy bean		28.5	58.3
7	21	M	Tree nuts, peanut	Rhinitis	61.2	91.3
8	48	M	Wheat, egg, sea food, milk		33.2	55.6
9	46	M	Peanut, tree nut, wheat		38.5	54.2
10	38	M	Milk		41.2	71.3
11	25	F	Sea food, sesame		18.6	62.3
12	61	F	Egg	Rhinitis	91.2	121.2
13	35	F	Milk		44.6	55.3
14	24	F	Milk		65.3	84.8
15	18	F	Peanut, tree nut, wheat	Dermatitis	41.2	61.2
16	21	F	Sea food, soy bean	asthma	44.6	55.8
17	23	F	Egg, milk		28.3	35.6
18	22	F	Egg, wheat		35.2	54.5
19	41	F	Milk, sea food, soy bean	Rhinitis	44.6	64.2
20	35	F	Milk, sea food, wheat		27.2	44.3

Reagents: Tryptase (Cat#G7061) was purchased from Promega (Madison, WI). nafamostat mesylate was purchased from Tocris Bioscience (Ellisville, Missouri). Antibodies of galectin-9 (H-80), TIM3 siRNA and PAR2 siRNA were purchased from Santa Cruz Biotech (Santa Cruz, CA). IL-4 ELISA kit (sensitivity = 2 pg), cGMP ELISA kit (sensitivity = 0.56 pMol/ml), Anti-TIM3 and TIM4 were purchased from R&D Systems (Burlington, ON, Canada). Fluorescence labeled antibodies against CD86, OX40L and IL-12p70 were purchased from BD Bioscience (Mississauga, ON, Canada). Ovalbumin (OVA) and tosyl-glycine-proline-arginine-pNitroanilide were purchased from Sigma Aldrich (Oakville, ON, Canada). IgE ELISA kit was purchased from AbD Serotec (Raleigh, NC; sensitivity = 8 ng/ml). Gal- 9 ELISA kit was purchased from Hölzel Diagnostika GmbH (Cologne, Germany). Gal-9 was synthesized by GL Biochem Ltd. (Shanghai, China). The lipopolysaccharide (LPS) levels in all reagents were detected using limulus assay (Limulus ameocyte lysate QCL 1000, Biowhittaker, Walkersville, MD). Less than 30 pg LPS/ml in all reagents was used in this study.

Real time reverse transcription polymerase chain reaction (qPCR): Total RNA was extracted from the duodenal biopsy using an RNeasy Mini kit. cDNA was synthesized using iScript™ cDNA Synthesis Kit. The resulted cDNA was subjected to qPCR that was performed with a LightCycler using a SuperScript III Platinum SYBR Green Two-Step qPCR Kit. The amplified product was detected by the presence of an SYBR green fluorescent signal. β -actin cDNA was used as the internal control. The primers of Gal-29 were: forward, ctggctctgcttctggctac and reverse, catggggcttcattcttact (NCBI, AB006782).

Western blotting: IEC lysates were prepared by resuspending cell pellet in lysis buffer. The homogenates were centrifuged at 15,000 rpm for 20 min at 4°C. A total of 50 µg denaturized protein were loaded onto 12.5% SDS-PAGE gel and transferred onto nitrocellulose membrane. Membranes were then incubated with anti-Gal-9 antibody (1:1000) or isotype IgG (using as negative control) overnight at 4°C. Reactions were developed using the Pierce ECL chemiluminescence substrate kit. Results were recorded with X ray film. Bands were semi-quantified by densitometry. The intensity of the specific band was normalized to the intensity of β -actin band.

RNA interference: siRNA Transfection was performed to knockdown the gene expressions of TIM3 on DC or PAR2 in IEC following the manufacturer instruction and our established procedures (Jacob C, Yang PC, Darmoul D, Amadesi S, Saito T, Cottrell GS, et al: Mast Cell Tryptase Controls Paracellular Permeability of the Intestine. Journal of Biological Chemistry 9-9-2005;280:31936-31948). The efficiency of siRNA transfection was determined by Western blotting. In both molecules, the peak inhibitory effect was reached 24-36 h post-transfection that sustained at least for another 72 h and declined thereafter; the expression of target molecules in the cells returned to normal levels 4 days after transfection. The control siRNAs did not affect the target molecule expression.

Counting eosinophil and mast cell in the intestinal mucosa: Numbers of eosinophil and mast cell in the intestine were counted with our established procedures [21]. Briefly,

intestinal segments were fixed with 4% paraformaldehyde or Carnoy solution (for mast cell counting) overnight and processed for paraffin sections. The sections were stained with hematoxylin or 0.5% toluidine blue (for mast cell counting) and observed under a light microscope.

Bone marrow-derived DC (BmDC) generation: Bone marrow cells were seeded in tissue culture flasks at 10^6 cells/ml in RPMI 1640-based complete medium with 20 ng of GM-CSF/ml. On day 5, loosely adherent cells were collected and purified with magnetic bead coupled anti-CD11c antibody. Isolated BmDCs (the purity was over 95% as determined by flow cytometry) were resuspended in fresh medium for further experiments.

Isolation of lamina propria mononuclear cells (LPMC): Mouse small intestine was washed with RPMI (no Peyer's patches) and cut into 2 mm pieces and then incubated in 0.5 mM EDTA in calcium and magnesium free Hanks' solution for 20 min at 37°C with shaking to remove mucin. Tissue then was incubated 20 min at 37°C in 20 ml RPMI containing 1 mg/ml collagenase. The tissue was filtered with a 0.1 mm cell strainer first, then 0.04 mm cell strainer. After washing, cells (up to 2×10^7) were layered onto a column of Percoll with a 30:70% gradient. Cells were spun at $2200 \times g$ at room temperature for 20 min. Subsequent cell viability was >90% as determined by trypan blue exclusion.

Immunohistochemistry: Duodenal biopsies were snap frozen and stored at -80°C until use. Cryosections were prepared and fixed with cold acetone for 20 min. Sections were

blocked by 1% bovine serum albumin (BSA) for 30 min and incubated with rabbit anti-human Gal-9 antibody (1:200), or anti-tryptase antibody (1:300), or isotype IgG (using as negative control) overnight at 4°C. After washing, sections were incubated with FITC-labeled anti-rabbit second antibody (1:300) for 1 h at room temperature. After washing, sections were mounted with cover slips and observed under a confocal microscope. The positive staining of Gal-9 was semi-quantified from 0-4; 0 indicates no staining; 4 indicates strong staining. The tryptase positive cells were regarded as mast cells and counted in 20 fields ($\times 200$) per samples. For cell staining, cell suspension was smeared onto glass slides, dried overnight. The rest procedures were the same as above. To avoid the observer bias, the sections were coded; the observer was not aware of the codes.

Tryptase activity assay

The sera were collected from patients with food allergy and healthy subjects. Ten microliters were added to 200 μ L of buffer (50 mmol/L Tris-HCl, pH 7.6, 120 mmol/L NaCl, 20 μ g/L heparin) containing 0.5 mmol/L substrate of mast cell tryptase (tosyl-glycine-proline-arginine-pNitroanilide) and incubated overnight at room temperature. The substrate cleavage was assessed using a microplate reader at 405 nm. The results were normalized to the serum protein content. Concentrations were calculated in comparison with a standard curve. Mast cell tryptase inhibitor (nafamostat mesylate) was used to confirm assay specificity.

Enzyme-linked immunoassay (ELISA): Levels of serum specific IgE, intestinal IL-4 and Gal-9 level in culture supernatant were measured by ELISA with commercial reagent

kits following the manufacturer's instruction. The evaluation of TIM4 was done by an in-house ELISA with similar procedure we did previously (Yang PC, et al: TIM-4 Expressed by Mucosal Dendritic Cells Plays a Critical Role in Food Antigen-Specific Th2 Differentiation and Intestinal Allergy. *Gastroenterology* 2007;133:1522-1533).

Diagnosis of food allergy:

Case history: The case history of food allergy was collected from each patient. Symptoms typically appear within minutes to two hours after the ingestion of the food to which he or she is allergic, which included a tingling sensation in the mouth, swelling of the tongue and the throat, difficulty breathing, hives, vomiting, abdominal cramps, diarrhea, drop in blood pressure, loss of consciousness, etc.

Skin prick test: One drop of extract of each fresh food (purchased from Beijing Xiehe Hospital, Beijing, China) was applied to the patients' forearm. The skin covered by the drop of food antigen was pricked with 1 mm single-peak lancets. Positive control was performed using 10 mg/ml histamine dihydrochloride; the negative control using saline solution. The results were read after 15 min. The outline of each wheal was documented using a piece of transparent paper to copy the wheal. The skin wheal areas were determined with scanned digital files using the software ImageJ. Skin prick tests were regarded positive when the area was larger than 7 mm². Subjects with a wheal area below 3 mm² in positive controls were excluded from this study.

Oral challenge with food antigens: Oral challenges were performed in all the subjects in this study using one of the positive food allergens (revealed by skin test; usually chose the one had the largest wheal area). The food allergens in the skin prick test

to screen the offending allergens in these patients are listed in Table S1 although some patients might also sensitize to other food antigens. The oral challenge was performed in double blind manner. The antigen extracts or placebos were contained in coded capsules. The patients took 3 capsules of antigen or placebo; they were asked to take another 3 capsules (placebo or antigens) one week later. The study subjects were closely monitored for 4 hours following oral challenge in our department where treatment of anaphylaxis was readily available. The healthy controls were also oral challenged with a mixture of the 10 antigens.

Serum levels of cytokine and allergen-specific IgE: Blood samples were collected from each patient and healthy controls. The sera were separated immediately after withdrawn and stored at -70°C until use. The levels of total IgE, antigen specific IgE, IL-4, IL-13 and IFN- γ in the sera were measured with ELISA following the manufacturer's instructions. Negative controls included omitting the primary antibodies and using isotype IgG instead of the primary antibodies for each parameter.

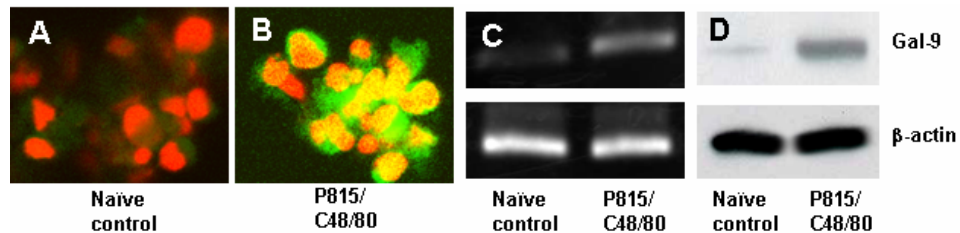


Figure S1. Mouse small intestinal cells (IEC4.1 cell line) express Gal-9. Mouse small intestinal cell line IEC4.1 cells were cocultured with mouse mast cell line p815 cells. The expression of Gal-9 in IEC4.1 cells was examined by confocal microscopy, RT-PCR and

Western blotting in the same procedures of figure 2. Naïve control: IEC4.1 cells were cultured with culture media alone. No other reagents were added. They were used as control. P815/C48/80: IEC4.1 cells were cocultured with p815 cells in the presence of C48/80 (5 µg/ml). B, representative confocal images show Gal-9 positive staining (in green) in IEC4.1 cells. The nuclei were stained with propidium iodide (in red) for morphological view. C, representative agarose gels that show RT-PCR results of Gal-9 expression in IEC4.1 cells. D, representative immune blots show Western blotting results of Gal-9 protein that was extracted from IEC4.1 cells. β-actin was used as an internal control.

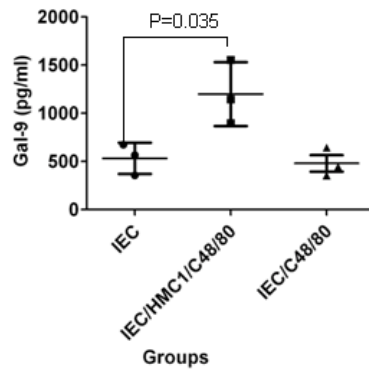


Figure S2. Gal-9 levels in IEC culture supernatant. IECs were cultured (10^5 cells/ml) in the presence or absence of C48/80-activated HMC1 cells (10^4 cells/ml) for 24h. Supernatant was collected and analyzed by ELISA. The scatter dot plots show Gal-9 levels. Each dot represents an individual datum. IEC: IECs were cultured with medium alone; no other reagents were added to culture. IEC/HMC1/C48/80: IECs were cocultured with HMC1 cells in the presence of C48/80 at a dose of 5 µg/ml. IEC/C48/80: IECs were cultured in the presence of C48/80 at a dose of 5 µg/ml (no HMC1 cells were added).

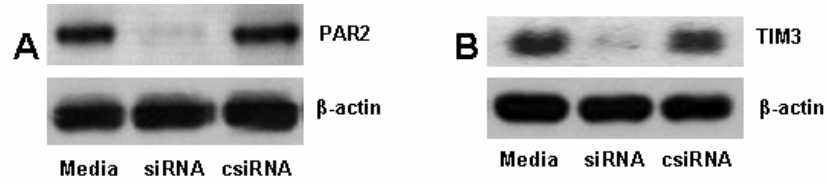


Figure S3. Gene knocking down for PAR2 in IEC and TIM3 in BmDC by RNAi.

RNAi was performed on IEC to knock down the PAR2 gene and on BmDC to knock down the TIM3 gene. IECs were cultured in the presence of C48/80-activated HMC1 cells for 24h. BmDCs were cultured in the presence of LPS for 24h. The cells were collected at the end of experiments; the cellular protein extracts were analyzed by Western blotting. The immune blots show the target proteins of PAR2 (A) and TIM3 (B). Media: Cells (IEC or BmDC) were cultured in medium. siRNA: Cells were treated with siRNA of PAR2 or TIM3. csRNA: Cells were treated with control siRNA that did not target on any genes.

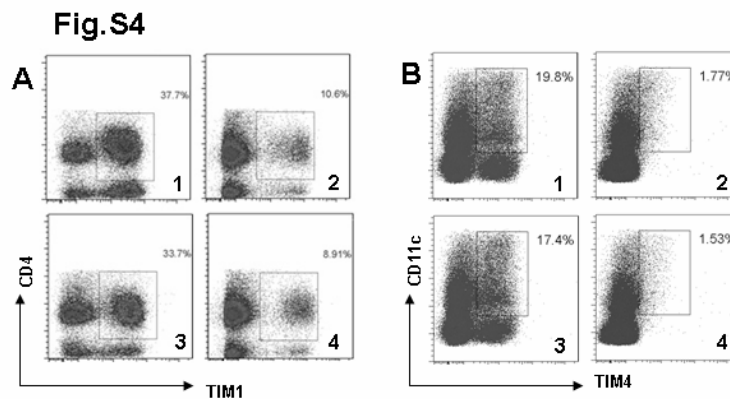


Figure S4. Gal-9 regulates the expression of TIM1 and TIM4. Sensitized mice (Sens) were treated with anti-Gal-9 antibody (or isotype IgG using as control) as described in text. After sacrifice, LPMCs were isolated by MACS. The LPMCs were stained with

anti-CD4, anti-TIM1 (or anti-TIM4) antibodies. The cells were analyzed by flow cytometry. A, dot plots show CD4⁺TIM1⁺ cells (the gated cell population). B, dot plots show CD11c⁺ TIM4⁺ cells (the gated cell population). The data represent three separate experiments. The cells in both panel A and B were isolated from mice with following treatments: 1, FA mice. 2, FA mice treated with anti-Gal-9 antibody. 3, FA mice treated with isotype IgG. 4, naïve control mice.