

Bcl2L12 plays a critical role in the development of intestinal allergy

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ARTICLE INFO

Keywords:

Intestine
Inflammation
Food allergy
T cell
Bcl2L12

ABSTRACT

The skewed T helper (Th) 2 response plays a central role in the pathogenesis of allergic diseases, while its initiating factors remain elusive. Recent studies indicate that Bcl2 like protein-12 (Bcl2L12) is associated with the Th2-biased inflammation. This study is designed to test a hypothesis that Bcl2L12 plays a critical role in the initiation of allergic response. In this study, peripheral CD4⁺ T cells were isolated from food allergy (FA) patients and healthy subjects; A mouse FA model was developed to test the role of Bcl2L12 in induction of allergic response in the intestine. The results showed that expression of Bcl2L12 by CD4⁺ T cells was higher in FA patients and FA mice and positively correlated with expression of Th2 cytokines. CD4⁺ T cells from FA patients showed a Bcl2L12-dependent tendency to differentiate into Th2 cells. Bcl2L12 played a crucial role in induction of allergic response in the intestine. Physical contact between Bcl2L12 and GATA3 facilitated GATA3 to bind *Il4* promoter to promote expression of IL-4. Adoptive transfer with Bcl2L12-deficient CD4⁺ T cells to Rag2^{-/-} mice did not reconstitute the efficient CD4⁺ T cell response as the mice could not be induced FA, while Rag2^{-/-} mice received WT CD4⁺ T cell transfer were induced FA. In conclusion, Bcl2L12 plays a crucial role in the induction of Th2 polarization and allergic response in the intestine. The Bcl2L12 in CD4⁺ T cells may be a potential target for the treatment of FA.

1. Introduction

Food allergy (FA) is an adverse reaction to food allergens mediated by immune response, characterized by the T helper-2 (Th2) polarization; the underlying mechanism is unclear. FA has two major categories, immunoglobulin (Ig)E-mediated FA and non-IgE-mediated FA. In the IgE-mediated FA, food allergens induce IgE production by plasma cells; the IgE binds the high affinity IgE receptors on the surface of mast cells to make the mast cells sensitized. Re-exposure to specific allergens activates sensitized mast cells and causes mast cells to release chemical mediators to evoke FA symptoms. In the non-IgE-mediated FA, adverse reaction is mainly mediated by immune cells, such as allergen-specific T cells and eosinophils [1]. The initiating factors of FA are not fully understood yet. It is accepted that the Th2-biased inflammation plays a critical role in the pathogenesis of FA [2]. Profound infiltration of CD4⁺

T cells in the intestinal mucosa and over production of T helper (Th) 2 cytokines [including interleukin (IL)-4, IL-5 and IL-13, etc.] in the intestinal tissue were observed in the mouse and human [3,4]. IL-4 further induces production of IL-5 and IL-13 by CD4⁺ T cells as well as induces production of IgE by plasma cells. However, the causative factors of Th2-biased responses are not fully elucidated yet.

Our recent studies revealed that the Bcl2 like protein 12 (Bcl2L12) was associated with the pathogenesis of inflammatory bowel disease related to Th2-biased inflammation [5] as well as involved in the pathogenesis of allergic rhinitis [6]. The Bcl2L12 is a member of the Bcl2 family. Bcl-2 family members act as pro- or anti-apoptotic regulators that are involved in a wide variety of cellular activities [7]. Bcl2L12 expression is upregulated in most human glioblastomas [8]. Bcl2L12 can induce resistance to apoptosis via directly neutralizing caspase-7 and indirectly neutralizing caspase-3 [8]. The activities of Bcl2L12 are

Abbreviations: Th, T helper; Bcl2L12, Bcl2 like protein-12; FA, food allergy; Ig, immunoglobulin; IL, interleukin; sIgE, specific IgE; PBMC, peripheral blood mononuclear cells; MACS, magnetic cell sorting; KO, knockout; HEK293 cells, human embryonic kidney cells 293; IP, immunoprecipitation; ChIP, chromatin IP; ANOVA, analysis of variance; RT-qPCR, real time quantitative RT-PCR

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<https://doi.org/10.1016/j.imlet.2018.09.001>

Received 13 April 2018; Received in revised form 1 August 2018; Accepted 3 September 2018

Available online 05 September 2018

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Table 1
Demographic data of human subjects.

Items	FA patients	Healthy subjects
Number of Subjects	20	20
Age (yr) (median)	31.5 ± 5.6	28.8 ± 5.8
Male	10 (50)	10 (50)
Female	10 (50)	10 (50)
SPT (diameter)*	0	20
< 3 mm	10 (50)	0
10–15 mm	10 (50)	0
> 15 mm	0	0
Serum sIgE#	4(20)	0
17.5–50 KU/L	12 (60)	0
50–100 KU/L	4 (20)	0
> 100 KU/L	0	0

The numbers in () indicate %.
* A wheal diameter greater than 3 mm of the negative saline control was considered SPT positive.
The serum sIgE to specific antigens was measured by the ImmunoCap test.

not only associated with the pathogenesis of tumors, others also found that blood vessel endothelial cells expressed Bcl2L12 [9] and associated with cirrhosis [10].
In this study, we found that Bcl2L12 played a critical role in the development of allergic response by promoting the expression of IL-4 in CD4⁺ T cells. The expression of Bcl2L12 was detected in peripheral

CD4⁺ T cells, which was markedly higher in FA patients and FA mice. CD4⁺ T cells from FA subjects showed a Bcl2L12-dependent tendency to differentiate into Th2 cells. The data suggest that to regulate the expression of Bcl2L12 in CD4⁺ T cells has therapeutic significance for the management of FA.

2. Materials and methods

2.1. Ethic statement

The using human tissue and animals in the present study was approved by the human ethics committee and the animal ethics committee at Zhengzhou University. An informed written consent was obtained from each human subject.

2.2. Patients

The FA patients were recruited at the affiliated hospitals of Zhengzhou University. The diagnosis and management of FA patients were carried out by the physicians of our hospitals following the established procedures [11]. The healthy subjects were selected from those with no food allergy history, negative results of skin test of the food allergen set, and the serum total IgE levels less than 0.35 kU/L. The demographic data of human subjects are presented in Table 1; the skin prick test results are presented in Table S1; the specific IgE (sIgE) results are presented in Table 1 and Table S2 and the clinical symptoms of FA are presented in Table S3. Patients with following conditions were

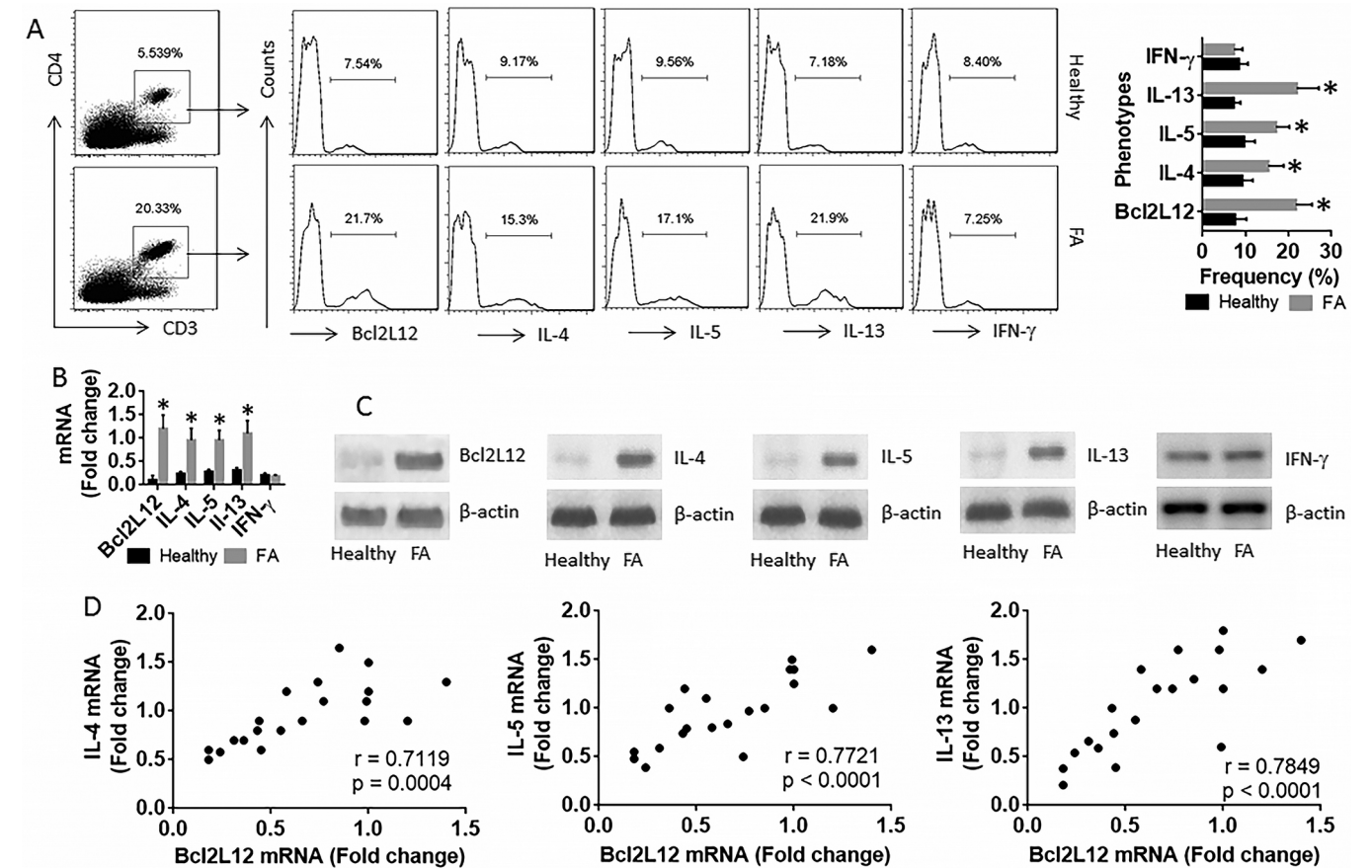


Fig. 1. Bcl2L12 is positively correlated with Th2 cytokines in peripheral CD4⁺ T cells of FA patients. Peripheral blood samples were collected from FA patients (n = 20) and healthy subjects (n = 20). A, PBMCs were prepared and analyzed by flow cytometry. The gated histograms show the frequency of Bcl2L12⁺ cells and phenotypes of Th1 and Th2 cells in the gated dot plots (the CD4⁺ T cells). The bars indicate the summarized data of the histograms. B–C, CD4⁺ T cells were isolated from PBMCs by MACS and analyzed by RT-qPCR and Western blotting. The bars show mRNA (B) and the immunoblots show protein (C) levels of Bcl2L12 and cytokines in the CD4⁺ T cells. D, the correlation between Bcl2L12 and Th2 cytokines in FA patients. The data of bars are presented as mean ± SD. *p < 0.01, compared with the healthy group.

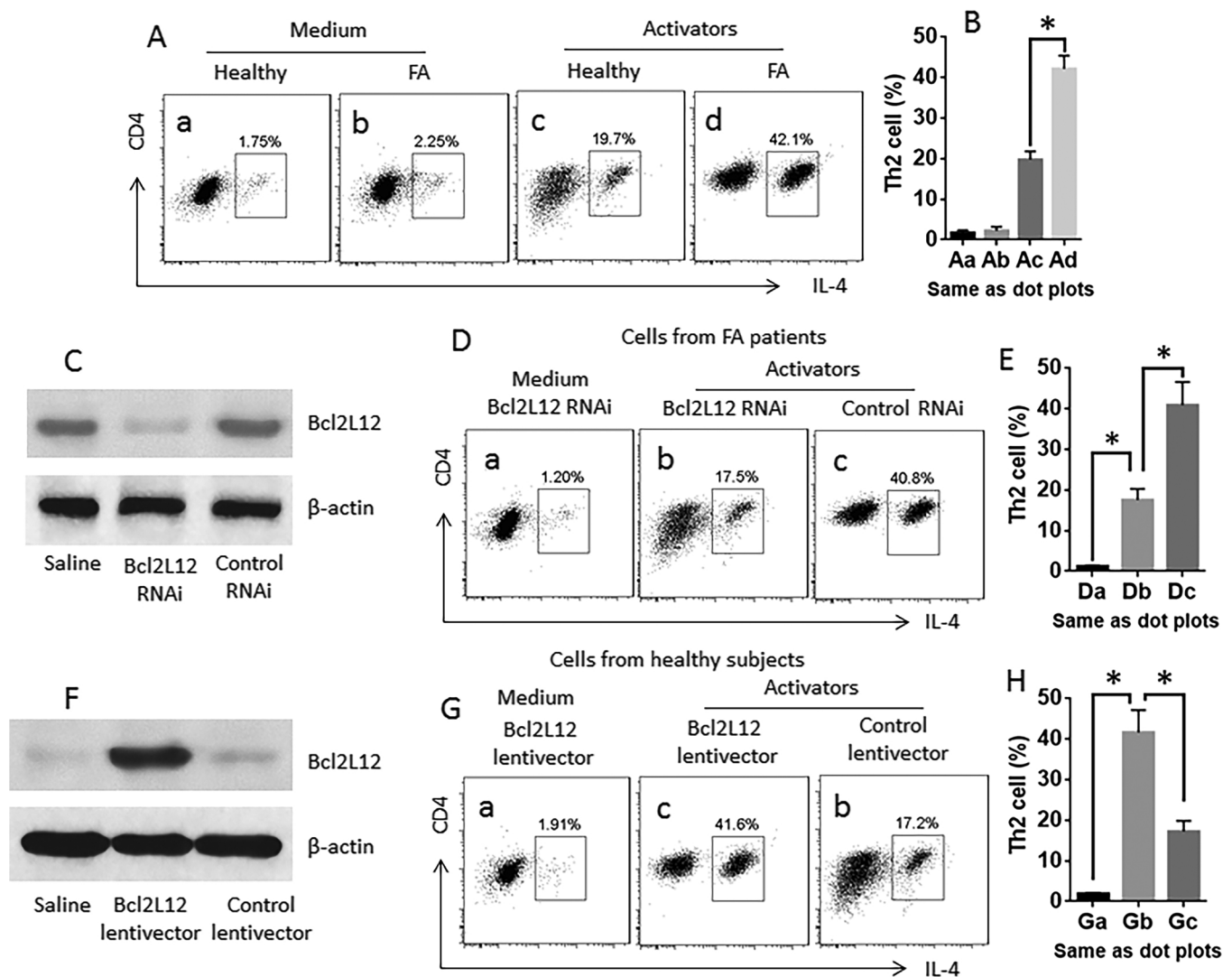


Fig. 2. Bcl2L12 facilitates Th2 cell differentiation. Naive CD4⁺ T cells are prepared from healthy subjects (n = 20) and FA patients (n = 20) and cultured for 4 days in the presence or absence of T cell activators (IL-2/PMA/ionomycin). The gated dot plots (A, D, G) indicate the frequency of Th2 cells differentiated from naive CD4⁺ T cells. The bar graphs (B, E, H) show the summarized data of the dot plots. The immune blots show the results of Bcl2L12 RNAi (C) and the Bcl2L12 overexpression (F) in CD4⁺ T cells. The data of bars are presented as mean $\pm$ SD. *p < 0.01. Each experiment was repeated 3 times.

excluded: Suffering from other autoimmune diseases, severe organ diseases and cancers.

2.3. Activation of CD4⁺ T cells in the culture

Naive CD4⁺ T cells (CD3⁺ CD4⁺ CD25⁻ CD62L⁺) were isolated from PBMCs by MACS with reagent kits following the manufacturer's instructions. The cells were cultured in the presence of activators, including IL-2 (20 ng/ml); PMA (Phorbol 12-myristate 13-acetate; 50 ng/ml) and ionomycin (100 ng/ml), for 4 days.

2.4. Mice

The Bcl2L12 knockout (KO) mice (BALB/c background; see more information in the supplemental materials for the protocol of the KO mouse development) were provided by the Institute of Bioplatrics at Chinese Academy of Agricultural Sciences (Beijing, China). Rag2^{-/-} mice (8–10 week old) were purchased from the Jackson Laboratory (Bar Harbor, ME). BALB/c mice and C57BL/6 were purchased from the Beijing Experimental Animal Center (Beijing, China). The mice were maintained in a specific pathogen free environment with accessing food and water freely. The expression of Bcl2L12 in the major organs of KO

mice and WT mice is presented in Fig. S1 in supplemental materials.

2.5. Bcl2L12 expressing plasmids

The full-length Bcl2L12 gene plasmids carried by lentivirus were constructed by the Sangon Biotech (Shanghai, China) and transduced into isolated CD4⁺ T cells following the manufacturer's instructions with the aid of electroporation using a Bio-Rad Gene Pulse set at 950 μ F and 280 V. The gene expressing effects were assessed 48 h later by Western blotting. The design of mutated Bcl2L12 plasmid was replaced the "c" with "t" at position 181 and replaced the "c" with "g" at position 323.

2.6. Test the formation of Bcl2L12/GATA3 complex with HEK293 cells

The lentivectors carrying Bcl2L12 gene (tagged by FLAG) or GATA3 gene (tagged by HIS) were constructed by the Sangon Biotech (Shanghai, China). The two vectors were transduced into HEK293 cells (Human embryonic kidney cells 293; because of their reliable growth and propensity for transfection, HEK293 cells are widely used in cell biology study) following the manufacturer's instructions. The complex of Bcl2L12/GATA3 in HEK293 cells was assessed by IP

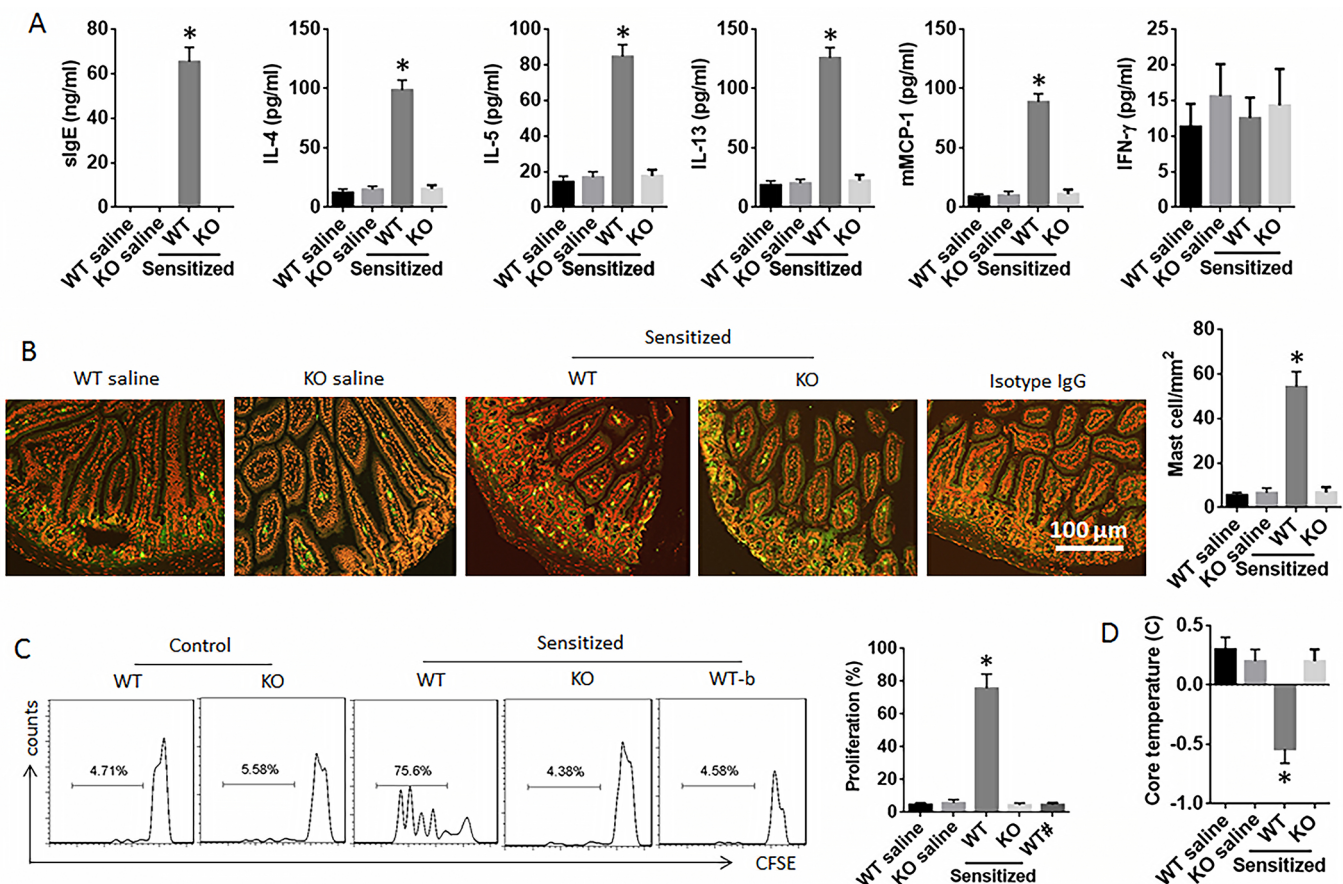


Fig. 3. Bcl2L12 promotes development of allergic response in the mouse intestine. WT mice and Bcl2L12 KO (KO, in short) mice were sensitized by feeding with OVA and CT or treated with saline used as controls. A, the bars indicate the serum levels of OVA-specific IgE (sIgE), IL-4, IL-5, IL-13, IFN-γ and mMCP-1 in WT and KO mice. B, the representative immunohistochemical images show mast cells (in green color; mMCP1 staining; $\times 200$) in the intestinal mucosa. The bars show the counts of mast cells (averaged from 20 fields per mouse). C, the histograms show the frequency of proliferating OVA-specific CD4⁺ T cells (KO-b: BSA was added to the culture used as an irrelevant antigen; others were added OVA in the culture). The bars show the summarized data of proliferating cells. D, the core temperature recorded from each mouse 30 min after specific antigen challenge. The data of bars are presented as mean $\pm$ SD. Each group consists of 6 mice. * $p < 0.01$, compared with the WT saline group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(immunoprecipitation). Briefly, samples were precleared by incubating with protein G agarose beads for 2 h. The supernatant was incubated with antibodies of FLAG, HIS or isotype IgG overnight. Immune complex in the sample was precipitated by incubating with protein G agarose beads for 2 h. The beads were collected and treated with an eluting buffer. The precipitated proteins were analyzed by Western blotting. The membranes were stained with anti-Bcl2L12 and anti-GATA3 antibodies, respectively.

2.7. Luciferase *Il4* promoter reporter assay

The luciferase *Il4* promoter reporter plasmids were constructed by the Sangon Biotech (Shanghai, China) and transfected into the EL4 cell line (10×10^6 EL4 cells per transfection). EL4 cells are a cell line from murine lymphoma; the cells are prone to produce high levels of IL-4). The plasmids were transfected into EL4 cells via electroporation by using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Inc., Berkeley, CA) set at 950 μ F and 270 V. The transfected cells were cultured overnight, followed by stimulating with T cell activators (IL-2: 20 ng/ml; PMA: 50 ng/ml; ionomycin: 100 ng/ml) for 4 h. Cells were then harvested and analyzed by luciferase reporter assay by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The *Il4* promoter reporter activities were presented as fold change against the saline group.

2.8. Adoptive transfer with Bcl2L12 KO CD4⁺ T cells to Rag2^{-/-} mice

Spleen cells were prepared from Bcl2L12 KO mice or WT mice. Naive CD4⁺ T cells (Tc) were further isolated from the spleen cells by MACS. The KO-Tc or WT-Tc were adoptively transferred into Rag2^{-/-} mice at 10^6 cells (in 0.1 ml saline)/mouse via tail vein injection (control mice received saline injection). Two days after the transfer, mice were received OVA (0.2 mg/mouse, mixed in 0.3 ml alum) sensitization; the sensitization was repeated one week later. Three days after the second sensitization, mice were challenged with OVA (50 mg/mouse) via gavage-feeding. The challenge was repeated two more times with one day apart from each challenge. One day after the last challenge, mice were sacrificed. The allergic response parameters were assessed following our established procedures [12].

2.9. Statistics

The data are presented as mean $\pm$ SD. The difference was determined by Student *t* test between two groups. ANOVA followed by Dunnett's *t*-test or SNK test was performed for multiple comparisons. When necessary, correlation coefficient between two groups was calculated using Prism GraphPad software package. $P < 0.05$ was considered statistical significant.

The reagent information, experimental procedures of PBMC preparation, immune cell isolation, cell culture; flow cytometry; real time

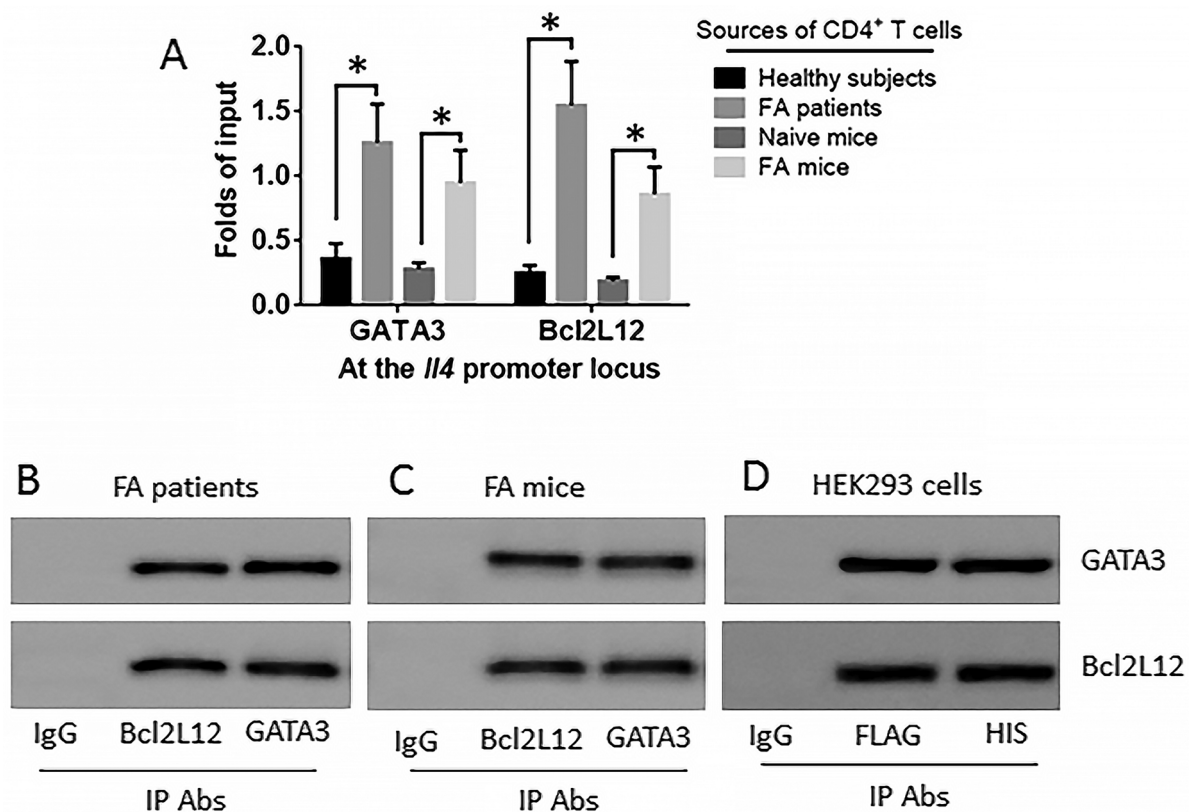


Fig. 4. Bcl2L12 promotes GATA3 to bind the *Il4* promoter in CD4⁺ T cells. A, nuclear extracts were prepared from CD4⁺ T cells isolated from PBMC (6 FA patients, 6 healthy subjects) and LPMC (6 naïve mice, 6 FA mice); the samples were analyzed by co-ChIP. The bars (mean $\pm$ SD; **p* < 0.01) indicate the levels of GATA3 and Bcl2L12 at the *Il4* promoter locus. B–C, the eluted proteins of co-ChIP were analyzed by Western blotting. The immunoblots show a complex of GATA3 and Bcl2L12 in CD4⁺ T cells. D, HEK293 cells were co-transfected with GATA3-HIS plasmids and Bcl2L12-FLAG plasmids. The cell extracts were analyzed by co-IP. The immunoblots show a complex of the recombinant GATA3 and Bcl2L12. The data of A–C represent 6 independent experiments. The data of D represent 3 experiments.

quantitative RT-PCR (RT-qPCR); preparation of cell protein extracts; Western blotting, co-chromatin immunoprecipitation (ChIP), RNAi, ELISA and development of an FA mouse model are presented in the supplemental materials.

3. Results

3.1. Expression of Bcl2L12 is positively correlated with expression of Th2 cytokines in peripheral CD4⁺ T cells of FA patients

To understand the role of Bcl2L12 in the pathogenesis of Th2 polarization, we collected peripheral blood samples from FA patients. Peripheral blood mononuclear cells (PBMCs) were prepared with the blood samples and analyzed by flow cytometry. Bcl2L12⁺ CD4⁺ T cells were detected in the samples, which were much more in FA group than that in healthy control group (Fig. 1A). Bcl2L12 expression was also detected in other immune cells, including dendritic cells, B cells, CD8⁺ T cells and CD14⁺ cells; but no significant difference was found between healthy subjects and FA patients (Fig. S2). The results were supported by the data of RT-qPCR and Western blotting, which showed higher expression of Bcl2L12 in CD4⁺ T cells in FA group as compared to that in healthy group (Fig. 1B and C). With the same batch of samples, the expression of cytokines, including IL-4, IL-5, IL-13 and IFN- γ were also analyzed. The results showed that expression of Th2 cytokines in peripheral CD4⁺ T cells was higher in FA group than that of healthy group, while the signature cytokine of Th1, the IFN- γ , was not significantly different between healthy and FA groups (Fig. 1B and C). A correlation analysis was performed with data of Bcl2L12 and Th2 cytokines in CD4⁺ T cells collected from FA patients. The results showed a positive correlation between Bcl2L12 and either IL-4, or IL-5, or IL-13

(Fig. 1D), but not with IFN- γ (Fig. S3 in supplemental materials) in CD4⁺ T cells of FA patients. The data indicate that expression of Bcl2L12 is enhanced in peripheral CD4⁺ T cells of FA patients. Bcl2L12 may be involved in the promotion of Th2 cytokine expression in CD4⁺ T cells.

3.2. Bcl2L12 facilitates the differentiation of Th2 cells

Next, naïve CD4⁺ T cells were isolated from the peripheral blood of FA patients and healthy subjects. The cells were treated with non-specific T cell activators (IL-2/PMA/ionomycin) in the culture for 4 days. Significantly more CD4⁺ IL-4⁺ T cells were induced in CD4⁺ T cells collected from the FA group than that from healthy group (Fig. 2A and B). To elucidate the role of Bcl2L12 in Th2 cell differentiation, Bcl2L12 gene expression was knocked down by RNAi in naïve CD4⁺ T cells from FA patients (Fig. 2C). The cells were treated with IL-2/PMA/ionomycin for 4 days. Indeed, less Th2 cells were induced in cells treated by Bcl2L12 RNAi as compared to cells treated with control RNAi (Fig. 2D and E). On the other hand, naïve CD4⁺ T cells from healthy subjects were transduced with Bcl2L12 gene-carrying lentivectors, which resulted in overexpression of Bcl2L12 in the CD4⁺ T cells (Fig. 2F). The cells were treated with IL-2/PMA/ionomycin for 4 days. The results showed that overexpression of Bcl2L12 markedly increased the differentiation of Th2 cells (Fig. 2G and H). The results demonstrate that Bcl2L12 plays a critical role in differentiation of Th2 cells.

3.3. Mice with Bcl2L12-deficiency fail to induce allergic response in the intestine

Since skewed Th2 polarization plays very important role in the

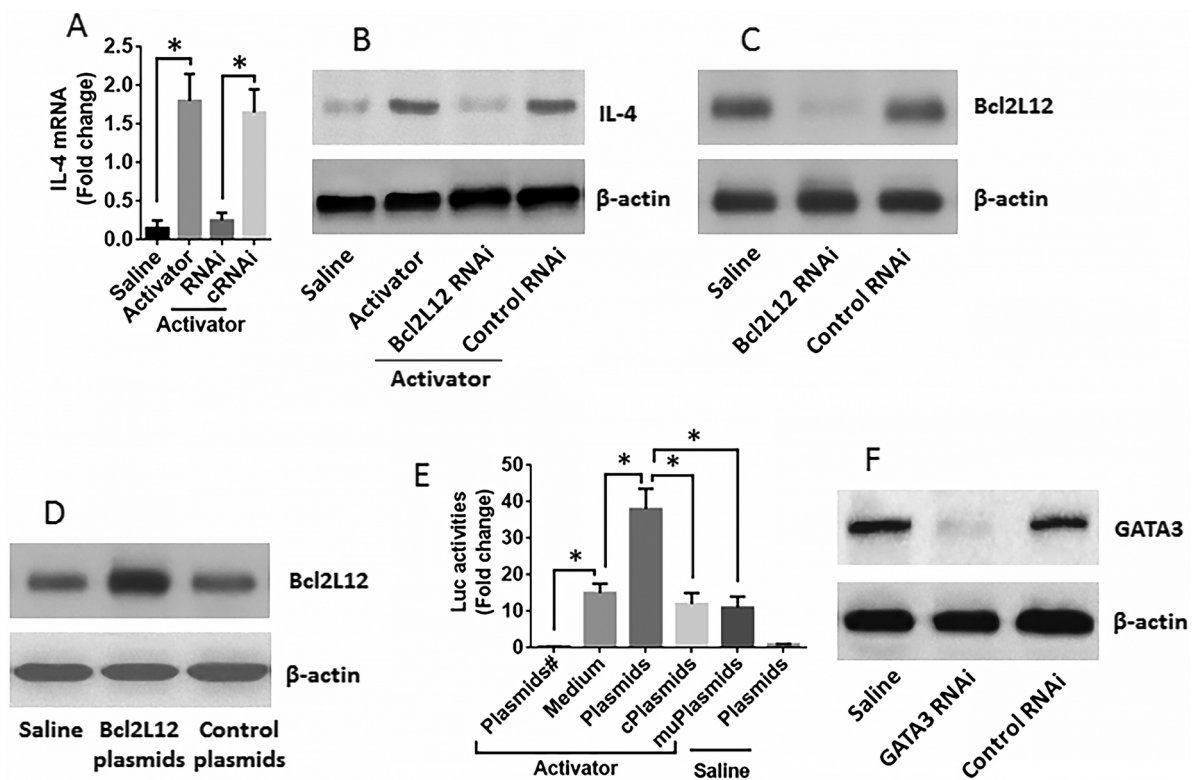


Fig. 5. Bcl2L12 overexpression promotes *Il4* promoter activation. A–B, Bcl2L12-deficient and sufficient EL4 cells were treated with activators (IL-2/PMA/ionomycin) or saline (control) for 3 days. The bars indicate the mRNA levels of IL-4 in the cells. The immunoblots show the IL-4 protein levels in EL4 cells. C, RNAi: EL4 cells were treated with Bcl2L12 RNAi. cRNAi: EL4 cells were treated with control RNAi. D, EL4 cells were transfected with Bcl2L12 plasmids or control plasmids. The immunoblots show the levels of Bcl2L12 in EL4 cells 3 days after the transfection. E, EL4 cells were co-transfected with an *Il4* promoter luciferase reporter and the procedures listed on the X axis of panel F. The bars show the luciferase (Luc) activities in the EL4 cells to represent the *Il4* promoter activation (normalized to the saline group). Plasmids: Bcl2L12 plasmids. cPlasmids: Control empty plasmids. muPlasmids: Mutated Bcl2L12 plasmids. The data of bars are presented as mean $\pm$ SD. * $p < 0.01$. The data represent 3 independent experiments. F, plasmid#: GATA3-deficient CD4⁺ T cells (made by RNAi) were transfected with Bcl2L12-expressing plasmids.

pathogenesis of allergic disorders [13], data reported above imply that Bcl2L12 may facilitate the development of allergy. To test this, Bcl2L12 knockout (KO) mice and wild type (WT; BALB/c) mice were sensitized to ovalbumin (OVA) following published procedures [12]. Higher levels of Th2 responses and allergic reaction, including higher levels of serum specific IgE, Th2 cytokines and mast cell protease-1 (Fig. 3A), profound mast cell infiltration in the intestinal mucosa (Fig. 3B), more antigen-specific CD4⁺ T cells in the intestine (Fig. 3C) and decrease in core temperature (Fig. 3D), in response to antigen challenge were induced in WT mice, which were not induced in Bcl2L12 KO mice (Fig. 3A–D). The data demonstrate that Bcl2L12 is a critical factor in the induction of this FA mouse model.

3.4. Bcl2L12 facilitates GATA3 to bind the *Il4* promoter in CD4⁺ T cells

Since IL-4 is the critical factor in the development of Th2 response, above data imply that Bcl2L12 may regulate expression of IL-4 in CD4⁺ T cells. By analyzing extracts of CD4⁺ T cells by chromatin IP (ChIP), higher levels of GATA3, the transcription factor of IL-4, were detected at the *Il4* promoter locus in FA group than that in healthy group. Similar results were obtained in CD4⁺ T cells isolated from mouse intestine. However, GATA3 was not detected at the *Il4* promoter locus of Bcl2L12 KO mice (Fig. S4 in supplemental materials). The results imply that Bcl2L12 may support GATA3 to bind the *Il4* promoter. To test this, CD4⁺ T cell extracts were analyzed by co-ChIP. Both GATA3 and Bcl2L12 were detected at *Il4* promoter locus, the quantity of which was much more in FA group than control group. The eluted proteins of co-ChIP were analyzed by Western blotting, a complex of GATA3 and Bcl2L12 was detected in both FA patients and FA mice, respectively

(Fig. 4B and C). The results demonstrate that Bcl2L12 can bind GATA3 to form a complex; the complex can be detected at *Il4* promoter locus. To corroborate the results, HEK293 cells were transfected with GATA3-HIS plasmids and Bcl2L12-FLAG plasmids. The recombinant GATA3 and Bcl2L12 also formed a complex in the cells (Fig. 4D).

3.5. Bcl2L12 is required for the activation of the *Il4* promoter

It is known that EL4 cells produce IL-4 upon activation. Such a function of EL4 cells was abrogated after knocking down the Bcl2L12 gene by RNAi. Overexpression of Bcl2L12 markedly increased *Il4* promoter activation in response to activators, which was not occurred in EL4 cells with the GATA3 gene knockdown (Fig. 5). The fact indicates that Bcl2L12 alone does not alter the activation of the *Il4* promoter; it facilitates the GATA3-induced *Il4* promoter activation (Fig. 6).

3.6. Reconstitution with Bcl2L12 KO CD4⁺ T cells in Rag2^{-/-} mice does not induce FA

To further test the role of Bcl2L12 in the induction of Th2-biased inflammation, Rag2^{-/-} mice were adoptively transferred with naive CD4⁺ T cells isolated from the spleen of Bcl2L12 KO mice or WT mice. The mice were then treated with procedures of sensitization. Upon challenging with specific antigens via garage-feeding, allergic response was observed in the intestine of mice received WT CD4⁺ T cells but not in those received Bcl2L12 KO CD4⁺ T cells. The results emphasize the importance of Bcl2L12 in CD4⁺ T cells in the induction of allergic response.

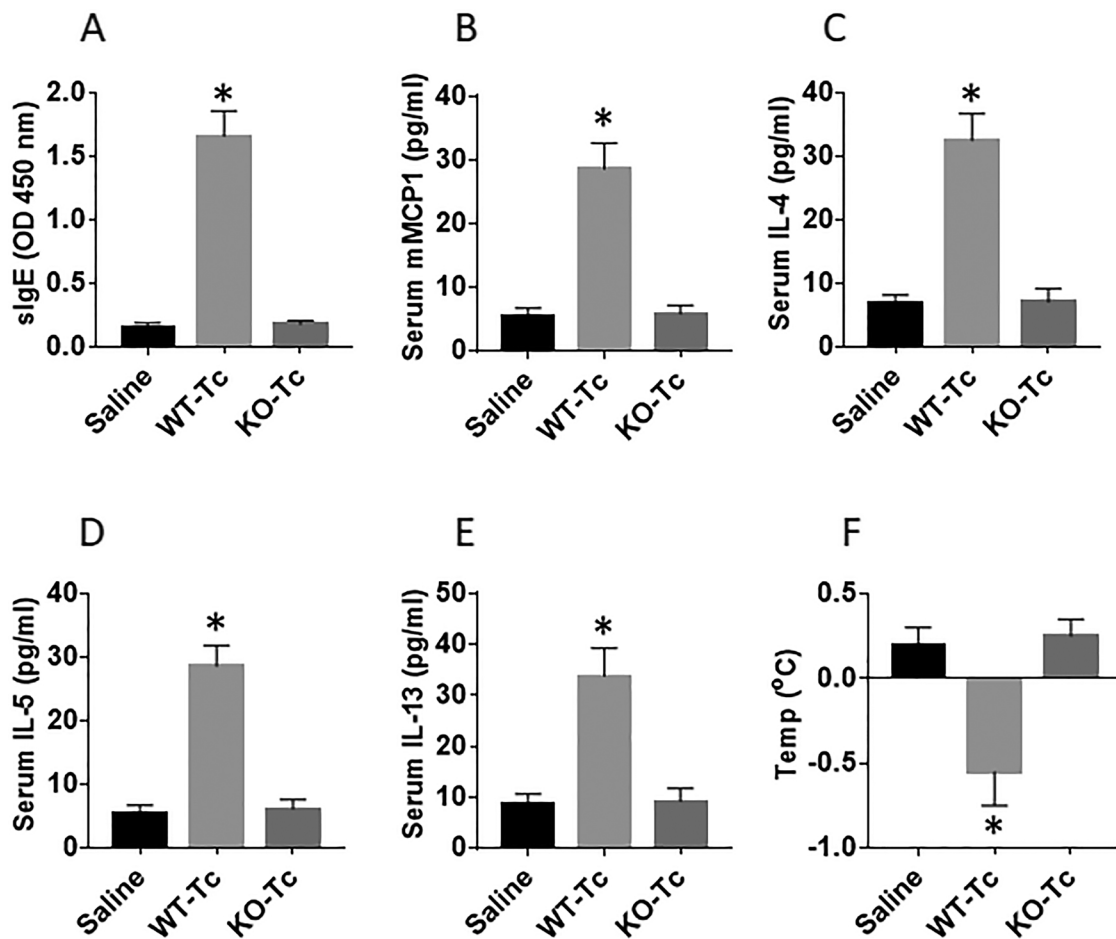


Fig. 6. Assessment of allergic response in $Rag^{-/-}$ mice adoptively transferred with Bcl2L12 KO or WT $CD4^{+}$ T cells. As denoted on the X axis, $Rag^{-/-}$ mice were adoptively transferred with Bcl2L12 KO $CD4^{+}$ T cells (Tc) (from Bcl2L12 KO mouse spleen) or WT $CD4^{+}$ T cells (from C57BL/6 mouse spleen) at 10^6 cells/mouse. The mice were sensitized to OVA. One day after challenging with OVA by gavage-feeding, the mice were sacrificed; allergic response was assessed. A–E, the bars indicate the serum levels of specific IgE (sIgE, A), mMCP1 (B) and Th2 cytokines (C–E). F, the bars indicate core temperature changes in mice recorded 30 min after antigen challenge. Each group consisted of 6 mice. Each sample was tested in triplicate. * $p < 0.01$, compared with saline group.

4. Discussion

The present study revealed a previously unknown regulator of Th2 responses. Bcl2L12 facilitated the development of Th2 cells. The $CD4^{+}$ T cells from healthy subjects expressed Bcl2L12 while it was significantly higher in FA patients. A positive correlation was detected between Bcl2L12 and Th2 cytokine expression, indicating that Bcl2L12 might promote the expression of Th2 cytokines. The inference was supported by further data that Bcl2L12 formed a complex with GATA3 to facilitate GATA3 to bind the *Il4* promoter in $CD4^{+}$ T cells, and thus enhanced the expression of IL-4.

It is known that the major immune pathological changes of FA are Th2-biased inflammation in the intestine [2]. However, the causes of the Th2 biased inflammation are less understood. Published data have revealed that several factors are associated with the initiation of allergic response. For example, TIM4 (T cell immunoglobulin domain 4) is a critical molecule for Th2 cell differentiation [14,15]. TIM4 binds to TIM1 in $CD4^{+}$ T cells to promote the expression of IL-4 and to drive the $CD4^{+}$ T cells to differentiate into Th2 cells [14]. Most studies on this topic focus on the mechanisms by which factors initiate or promote the expression of IL-4 to drive naive $CD4^{+}$ T cells to differentiate into Th2 cells [16]. Intestinal epithelial cells secrete TSLP (Thymic Stromal Lymphopoietin) and IL-33 to induce myeloid cells to secrete IL-4 to initiate the Th2 programme [17,18]. The present study revealed a novel factor, the Bcl2L12, inside $CD4^{+}$ T cells that promoted the expression of IL-4. The data suggest that such a function of Bcl2L12 contributes to

Th2-biased response as the $CD4^{+}$ T cells from FA subjects showed a Bcl2L12-dependent tendency to differentiate into Th2 cells. The data also rule out the possibility that the expression of Bcl2L12 by $CD4^{+}$ T cells is the results of Th2 polarization because using Bcl2L12 KO mice failed to induce Th2 polarization.

GATA3 is the major transcription factor for expression of IL-4. The present data show that Bcl2L12 regulates the expression of IL-4 in $CD4^{+}$ T cells via facilitating GATA3 to bind the *Il4* promoter. Early studies also showed that suppression of GATA3 activities could inhibit development of $CD4^{+}$ T cells. Such as administration with the oleanolic acid can inhibit experimental FA via suppression of GATA3 activities [19]. Mouse mast cell protease-6 increases expression of Bcl-6, the latter inhibits expression of GATA3, which converts Th2 cells to Foxp3⁺ regulatory T cells [20]. Our results showed a novel insight into the mechanism that Bcl2L12 formed a complex with GATA3, which is required for GATA3 to bind to *Il4* promoter in $CD4^{+}$ T cells. Thus, Bcl2L12 may be a novel target in the regulation of Th2 response.

Bcl2L12 is a member of the Bcl2 family. Early studies showed that Bcl2L12 facilitated cancer cells to survive via suppressing p53 [21] and inhibiting caspase 3/caspase 7 via physical contact [8]. The molecular structure of Bcl2L12 riches prolines and tends to bind to other proteins [22]. Our recent study also found that Bcl2L12 bound to the transcription factor of IL-10 to interfere with the function of IL-10 in B cells [5,6]. Although most studies about Bcl2L12 focus on the relation between Bcl2L12 and cancers, investigators have found that Bcl2L12 is also associated with other disorders, such as it is associated with

cirrhosis [10]. It is reported that human cord blood vessel endothelial cells express Bcl2L12 [9]. Whether the Bcl2L12 is involved in the pathogenesis of cardiovascular disorders needs to be further investigated.

Published data show that Bcl2L12 is an anti-apoptosis molecule by suppressing p53, caspase 3 and caspase 7 [7] in cancer cells to facilitate cancer cells to escape from the immune surveillance. The dysfunction of apoptosis is also noticed in CD4⁺ T cells of patients with allergic disorders [23]. The abnormal activities of both Fas ligand and p53 are involved in the defective activation-induced T cell death [23,24], which is one of the pathogenic aspects of the skewed Th2 polarization in allergic diseases. Whether the increase in Bcl2L12 in CD4⁺ T cells, as shown by the present data, also interferes with the apoptosis machinery in CD4⁺ T cells and plays a role in the skewed Th2 polarization is an interesting spot to be further investigated.

The core temperature drop is a sign of allergic reaction in food allergy mice. In this study, we also observed core temperature drop in sensitized mice after antigen challenge. Although dropped in a small scale, it was significantly lower than control mice. The core temperature drop after antigen challenge was also reported by other investigators; such as Manzano-Szalai et al. found a big drop of temperature in sensitized mice [25]. Similar phenomenon was also observed in our previous studies [26].

In the mouse model, mice with Bcl2L12 KO failed to develop FA. This mirrors the significance of Bcl2L12 in the development of Th2-biased inflammation in the intestine. On the other hand, since this mouse strain has global KO of Bcl2L12, other immune cells are also deficient of Bcl2L12. Whether other immune cells deficient of Bcl2L12 also contribute to the development of FA needs to be elucidated. The results from experiments of adoptive transfer with WT CD4⁺ T cells or Bcl2L12 KO CD4⁺ T cells into Rag2^{-/-} mice rule out this possibility. Only did those mice received WT CD4⁺ T cells show allergic response while those received Bcl2L12 KO CD4⁺ T cells did not.

In summary, the present study reveals that Bcl2L12 plays a critical role in IL-4 expression and Th2 response via facilitating GATA3 to bind the *IL4* promoter in the pathogenesis of FA. The results suggest that Bcl2L12 may be a novel therapeutic target in the management of FA.

Conflict of interest

None to declare.

Acknowledgement

This study was supported by grants from the innovation of science and Technology Commission of Shenzhen Municipality (JCYJ20160422101725667, JCYJ20140418095735611 and ZDSYS201506050935272, CXZZ20140902151802864), China Postdoctoral Science Foundation (2017M622792) and the Natural Science Foundation of China (31570932).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.imlet.2018.09.001>.

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Supplemental materials

Bcl2L12 plays a critical role in the development of intestinal allergy

Running title: Bcl2L12 promotes T helper 2 response

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* Equally contributed to this work

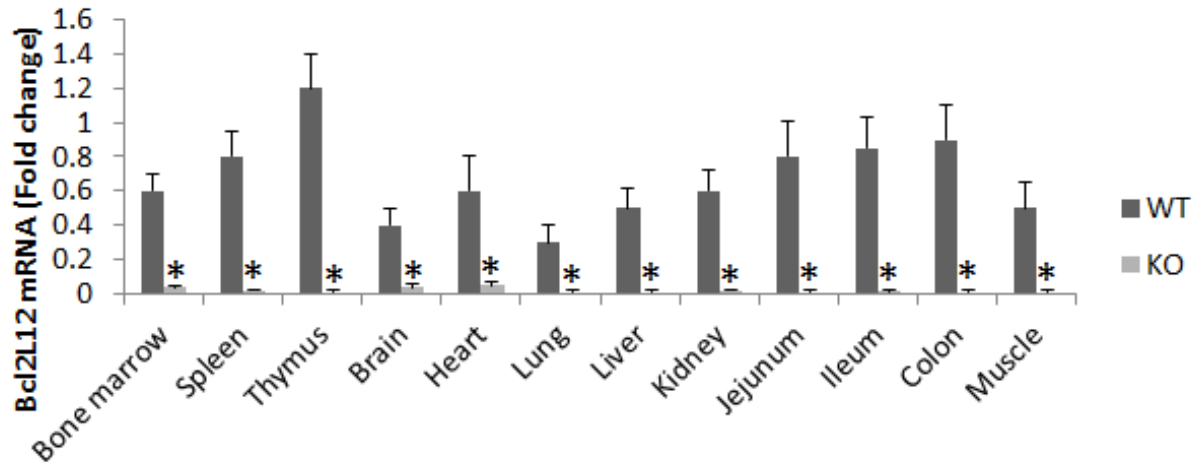


Fig. S1. Expression of Bcl2L12. Samples were taken from the major organs of Bcl2L12-deficient mice and WT mice as denoted on the X axis. The samples were analyzed by RT-qPCR. The bars indicate the mRNA levels. The data of bars are presented as mean $\pm$ SD. * $p < 0.01$, compared with the WT mice. Each group consists of 6 mice.

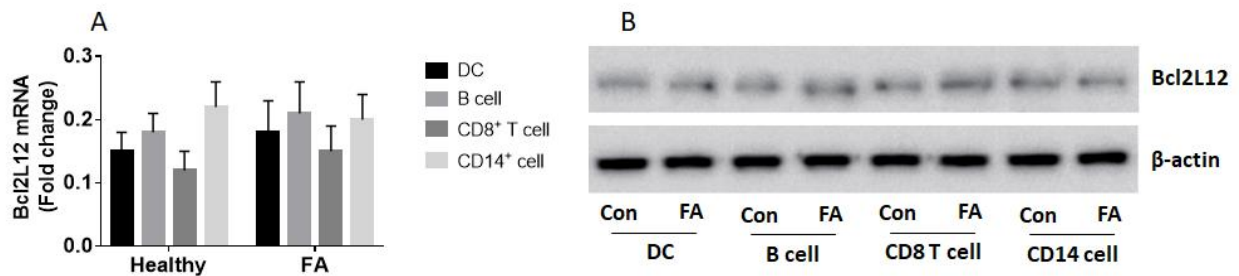


Fig. S2. Expression of Bcl2L12 in DC, B cell, CD8⁺ T cell and CD14⁺ cells. Peripheral blood samples were obtained from 6 healthy subjects (Con) and 6 FA patients. DC, B cells, CD8⁺ T cells and CD14⁺ cells were isolated from the blood samples. RNA and proteins were extracted from the isolated cells, pooled, respectively. The RNA was analyzed by RT-qPCR, the proteins were

analyzed by Western blotting, in triplicate. Data of bars are presented as mean $\pm$ SD. The data represent 3 independent experiments.

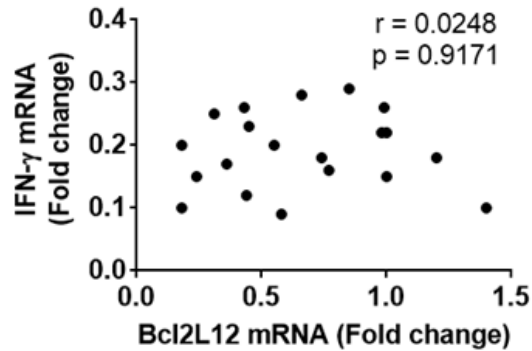


Figure S3. Correlation between Bcl2L12 and IFN- γ in CD4⁺ T cells of FA patients. Supplemental to Fig. 1D. The dot plots indicate the correlation between Bcl2L12 and IFN- γ in peripheral CD4⁺ T cells of FA patients. The data indicate no apparent correlation between the expression of Bcl2L12 and IFN- γ in CD4⁺ T cells.

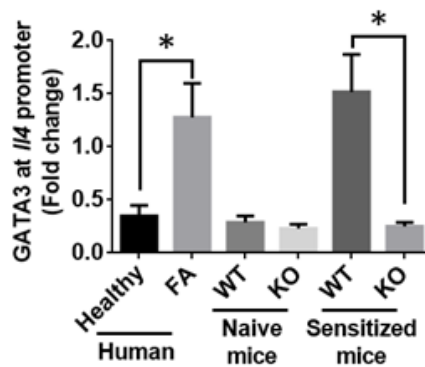


Figure S4. Assessment of GATA3 at the *Il4* promoter in CD4⁺ T cells. CD4⁺ T cells were isolated from PBMCs of healthy subjects (n=6) and FA patients (n=6), and LPMCs of WT mice (n=6) and Bcl2L12 KO mice (n=6). Nuclear extracts were prepared from the CD4⁺ T cells and analyzed by ChIP. The bars indicate the levels of GATA3 in the *Il4* promoter of CD4⁺ T cells. The data represent 6 independent experiments. *p<0.01.

Materials and methods

Reagents

The antibodies of IL-4 (HIL41, OX81), IL-5 (H-3, B-2), IL-13 (A-9, F-6), CD3 (PS1), GATA3 (HG3-31), Pol II (B6-1), IFN- γ (E-10, F3) and Bcl2L12 RNAi kits were purchased from Santa Cruz Biotech (Santa Cruz, CA). The antibody of Bcl2L12 (LS-C415684) was purchased from LSBio (Seattle, WA). The antibody of mMCP-1 (RF6.1) was purchased from eBioscience (San Diego, CA). The fluorescence-labeled antibodies of CD3 (145-2C11, UCHT1), CD4 (34930, GK1.5), IL-4 (34019, 3007) were purchased from BD Biosciences (Franklin Lakes, NJ). The lentivectors of Bcl2L12 were synthesized by the Sangon Biotech (Shanghai, China). The ELISA kits of IL-4, IL-5, IL-13, mMCP-1 and OVA-specific IgE were purchased from the Dakewe Biomart (Beijing, China). The reagents and materials for RT-qPCR, Western blotting and gene transfection were purchased from Invitrogen (Carlsbad, CA). The immune cell isolation kits were purchased from Miltenyi Biotech (San Diego, CA). The reagents/materials for IP and ChIP, IL-2, collagenase IV, EDTA,

PKH26-fluorescent dye, PMA and ionomycin were purchased from Sigma Aldrich (St. Louis., MO).

Peripheral blood mononuclear cell (PBMC) preparation

Peripheral blood samples were collected from FA patients and healthy subjects via ulnar vein puncture. PBMCs were isolated from the blood samples by gradient density centrifugation.

Immune cell isolation

Immune cells used in the present study were isolated from PBMCs or mouse tissues by the magnetic cell sorting (MACS) with purchased reagent kits following the manufacturer's instructions. The purity of isolated cells was checked by flow cytometry. If the purity did not reach 95%, the cells were re-purified with MACS.

Cell culture

The cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine. The medium was changed in 2-3 days. The cell viability was greater than 98% as checked by Trypan blue exclusion assay.

Flow cytometry

Cells were collected from relevant experiments. For the surface staining, the cells were stained with fluorescence-labeled antibodies of interest or isotype IgG for 30 min. For the intracellular

staining, the cells were fixed with 1% paraformaldehyde together with permeable reagents for 1 h, then stained with fluorescence-labeled antibodies of interest or isotype IgG for 30 min. The cells were analyzed with a flow cytometer (FACSCanto I; BD Bioscience). The data analyzed by the software package flowjo with the isotype IgG staining data as gating references. The dilutions of fluorochrome-labeled antibodies were 1:100.

Real time quantitative RT-PCR (RT-qPCR)

The total RNA was extracted from cells collected from relevant experiments. The cDNA was synthesized with the RNA and a reverse transcription kit following the manufacturer's instructions. The samples were amplified in a qPCR device (CFX96 Touch™ Real-Time PCR Detection System; Bio-Rad) with the SYBR Green Master Mix and relevant primers (Table S4). The results were normalized into fold change against the housekeeping gene β -actin.

Preparation of cell protein extracts

Cells were collected from relevant experiments and lysed with a cell lysing buffer (containing protease inhibitor) for 30 min at 4 °C. The samples were centrifuged for 15 min at 13,000 rpm. The supernatant was collected and used as the cytosolic extracts. The pellets were resuspended in a nuclear lysing buffer and incubated at 4 °C for 30 min. The samples were centrifuged for 15 min at 13,000 rpm. The supernatant was collected and used as the nuclear extracts.

Western blotting

Cells were collected from relevant experiments. The total proteins were extracted from the cells, fractioned by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), transferred onto a PVDF membrane. The membrane was blocked by incubating with 5% skim milk for 30 min, and then incubated with antibodies of interest (dilution at 1:500) overnight at 4 °C, washed with TBST (Tris-buffered saline Tween 20), incubated with the second antibodies (labeled with peroxidase) for 1 h at room temperature and washed with TBST. The immunoblots on the membrane were developed by the enhanced chemiluminescence and photographed with an imaging device (UVI, Cambridge, UK).

Co-chromatin immunoprecipitation (ChIP)

Cells were collected from relevant experiments and fixed with 1% formalin for 15 min. The cells were lysed with a lysing buffer and sonicated to shear the DNA into small pieces. The samples were precleared by incubating with protein G agarose beads for 2 h. The supernatant was incubated with antibodies of Bcl2L12, GATA3 (diluted at 1:200) or isotype IgG overnight. The immune complex in the sample was precipitated by incubating with protein G agarose beads for 2 h. The beads were collected and treated with an eluting buffer. DNA was recovered from the eluted samples with a DNA recovering kit following the manufacturer's instructions. The DNA was analyzed by qPCR with the presence of the primers of the *Il4* promoter (ggcctctcccttctatgcaa and gattgtcagtcacttggggc; -1000 to 100). The results were normalized into fold change against the input. The proteins were analyzed by Western blotting; the membranes were stained with anti-Bcl2L12 and anti-GATA3 antibodies, respectively.

RNA interference (RNAi)

CD4⁺ T cells were isolated from PBMC as described above. The CD4⁺ T cells and EL4 cells were treated with the Bcl2L12 shRNA lentivector or control lentivector following the manufacturer's instructions. The inhibitory effects of RNAi on Bcl2L12 expression were assessed by Western blotting 48 h after the transduction.

Enzyme-linked immunosorbent assay (ELISA)

Cytokines, specific IgE and mMCP-1 (mouse mast cell protease-1) were assessed by ELISA with purchased reagent kits following the manufacturer's instructions.

Development of an FA mouse model

Sensitization: The mice were gavage-fed with ovalbumin (OVA; 1 mg/mouse) and cholera toxin (CT; 20 µg/mouse) in 0.3 ml saline once a week for 4 consecutive weeks. Control mice were fed with saline.

Challenge: In the fifth week, the mice were gavage-fed with OVA (5 mg/mouse) in 0.3 ml saline.

Recording allergic response: The core temperature was measured in the rectum of each mouse with a digital thermometer 30-60 min after the oral challenge.

Assessment of systemic allergic response: The blood samples were collected at the sacrifice. The sera were isolated and analyzed by ELISA to determine the levels of OVA specific IgE, IL-4, IL-5, IL-13 and mMCP-1.

Counts of mast cells in the mouse intestinal mucosa: A segment of jejunum was excised at the sacrifice. Cryosections were prepared with the intestinal samples and stained with the anti-mMCP-1 antibody or isotype IgG. The sections were observed under a fluorescent microscope. Twenty fields ($\times 200$) were randomly selected from the samples of each mouse; the positively stained mast cells were counted and presented as mast cells/mm². The slides were observed in an observer-blinded fashion.

Assessment of antigen specific CD4⁺ T cells in the intestinal mucosa: Small intestinal tissue was collected at sacrifice, treated with 0.5% EDTA (ethylenediaminetetraacetic acid) in the culture for 30 min and cut into small pieces (2 $\times$ 2 $\times$ 2 mm). The samples were incubated with collagenase IV (1 mg/ml) for 2 h at 37 °C with mild agitation. The single cells were filtered through a cell strainer (100 μ m first, then 70 μ m) and collected by centrifugation (1000 rpm; 5 min). The LPMCs were isolated from the single cells by gradient density Percoll centrifugation. CD3⁺ CD4⁺ CD25⁻ T cells and dendritic cells (DC) were isolated from LPMCs by MACS as described above. The T cells (labeled with CFSE, carboxyfluorescein succinimidyl ester) and DCs were co-cultured at a ratio of 5 $\times$ 10⁴:1 $\times$ 10⁴/well in the presence of OVA (5 μ g/ml; a specific antigen) or bovine serum albumin (BSA; 5 μ g/ml; an irrelevant antigen). Three days later, the cells were analyzed by flow cytometry. The proliferating T cells were regarded as the antigen specific CD4⁺ T cells.

Mouse Bcl2112 Knockout Project (CRISPR/Cas9)

1. OBJECTIVE

To create a Bcl2112 knockout mouse model (C57BL/6) by CRISPR/Cas-mediated genome

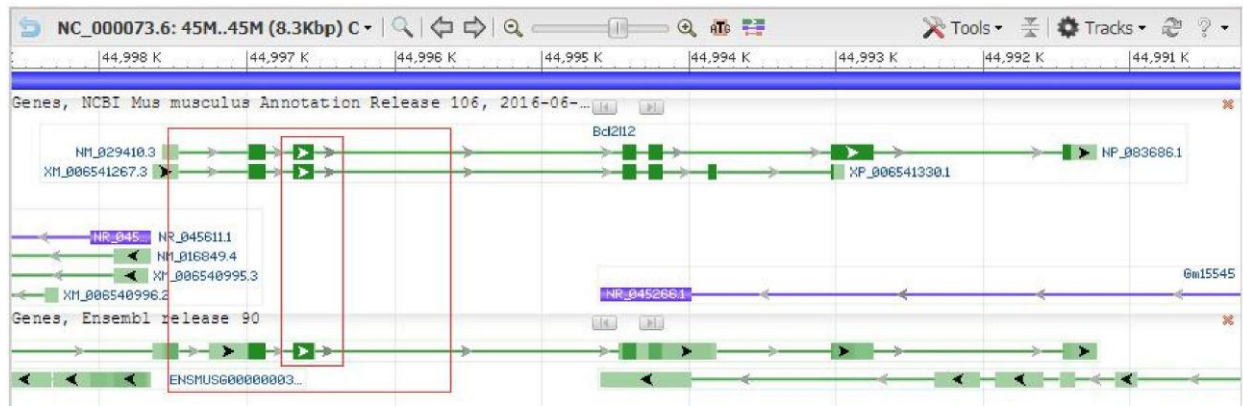
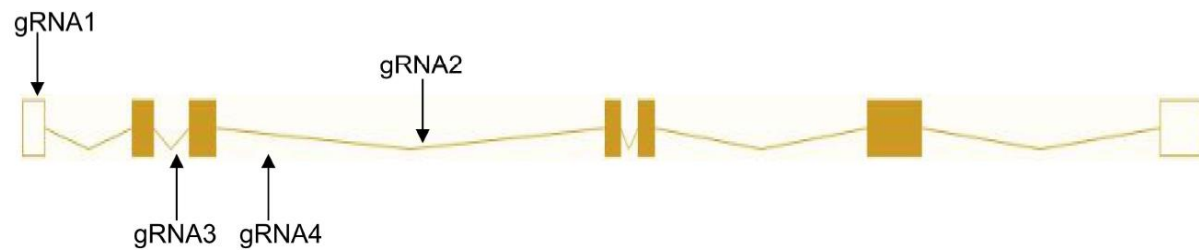
2. PROJECT SUMMARY

- The mouse Bcl2112 gene (GenBank accession number: NM_029410.3; Ensembl: ENSMUSG00000003190) is located on mouse chromosome 7.
- Seven exons have been identified, with the ATG start codon in exon 2 and TGA stop codon in exon 7.
- Exon 2 to exon 3 and exon 3 are selected as target sites (sequences shown below).
- Two pairs of gRNA targeting vectors are constructed and confirmed by sequencing.
- Cas9 mRNA and gRNA generated by in vitro transcription are co-injected into fertilized eggs for KO mouse production.
- The pups are genotyped by PCR followed by sequence analysis.

3. TARGETING STRATEGY

3a. Schematic depiction of targeting strategy

Genomic region of mouse Bcl2l12 locus is diagrammed below (gene is oriented from left to right; total size is 6.36 kb). Solid bars represent ORF; open bars present UTRs.



3b. gRNA target sequence Pair 1

gRNA1 (matches forward strand of gene): AGCGTG TACGCCAGTGCGAC

gRNA2 (matches forward strand of gene): TTCCGTGGTTGACC CGGGAGAGG

Pair 2

gRNA3 (matches reverse strand of gene): GGAGT TATCCCGAAGGGG TC1'..§

gRNA4 (matches forward strand of gene):GTGGGACGTGTCCCG ATGGAGGG

3c. The links of gRNA Vector Builder

gRNA1: https://www.vectorbuilder.com/vector/VB_171020-1011mns.html

gRNA2: https://www.vectorbuilder.com/vector/VB_171020-1012soh.html

gRNA3: <https://www.vectorbuilder.com/vector/VB171020-1013vdk.html>

gRNA4: https://www.vectorbuilder.com/vector/VB_171020-1014kmz.html

3d. Assay of CRISPR-induced mutation

The target region of mouse Bcl2l12 locus is amplified by PCR with specific primers.

PCR product is sequenced to confirm targeting.

Primer sequence:

Mouse Bcl2l12-pair1-F: CTGAGACTCGCAGCCACGCAA

Mouse Bcl2l12-pair1-R: CCTTTCCC TAATCCCTAATGTGTGCTT

Mouse Bcl2l12-pair2-F: CGCCCTC TTCATTCAGTGATTCAG

Mouse Bcl2l12-pair2-R: CTGCAACCAAGGAGGACTGAC TTCTC

Expected PCR product size: Mouse Bcl2l12-pair1 · Wildtype allele: 2808 bp

Mutant allele: 810 bp, delete 2030 bp

Mouse Bcl2l12-pair2: Wildtype allele: 1245 bp

Mutant allele: 770 bp, delete 480 bp

3e. Off-target analysis

Off-target analysis for gRNA1:

Chrom	Strand	Start	End	Given query	Actual genomic hit	Number of mismatches	GC content	External links
7	-	44997481	44997503	AGCGTGTACGCCAGTGCAC	AGCGTGTACGCCAGTGCAC-GGG	0	65.0%	UCSC Genome Browser
10	-	80067276	80067298	AGCGTGTACGCCAGTGCAC	gtgGTGTACGCCAGTGCAC-AGG	4	65.0%	UCSC Genome Browser
13	-	107627820	107627842	AGCGTGTACGCCAGTGCAC	tGtGTGTaatCCAGTGCAC-AGG	4	50.0%	UCSC Genome Browser
13	-	73743668	73743690	AGCGTGTACGCCAGTGCAC	tGcGTGTaagCCAGTgtGAC-AGG	4	55.0%	UCSC Genome Browser
17	-	83315867	83315889	AGCGTGTACGCCAGTGCAC	tGCaGTACGCCAGTGccAg-AGG	4	60.0%	UCSC Genome Browser
2	+	153572015	153572037	AGCGTGTACGCCAGTGCAC	AtCGTgaACGCCaGctAC-AGG	4	55.0%	UCSC Genome Browser
17	-	66343002	66343024	AGCGTGTACGCCAGTGCAC	AGaGacACGCCAGTgaGAC-AGG	4	60.0%	UCSC Genome Browser
5	-	100712826	100712848	AGCGTGTACGCCAGTGCAC	AGtGTtTACTCCAGTgtGAC-AGG	4	45.0%	UCSC Genome Browser
11	+	96345346	96345368	AGCGTGTACGCCAGTGCAC	AGaGTGTccGctAaTGCGAC-GGG	4	55.0%	UCSC Genome Browser
17	-	25701185	25701207	AGCGTGTACGCCAGTGCAC	AGaGTGTccGCaAGTgctAC-AGG	4	55.0%	UCSC Genome Browser

Off-target analysis for gRNA2

Chrom	Strand	Start	End	Given query	Actual genomic hit	Number of mismatches	GC content	External links
7	-	44995453	44995475	TTCcGTGgTTGACCCGGGAG	TTCcGTGgTTGACCCGGGAG-AGG	0	65.0%	UCSC Genome Browser
11	+	108223122	108223144	TTCcGTGgTTGACCCGGGAG	TTCtGTGgcTGACctGGGAG-TGG	3	60.0%	UCSC Genome Browser
2	-	105017073	105017095	TTCcGTGgTTGACCCGGGAG	cTctGTGgcTGACCCGgaAG-CGG	4	65.0%	UCSC Genome Browser
11	-	86580725	86580747	TTCcGTGgTTGACCCGGGAG	aTCCGTGgTTGACagGGGtG-TGG	4	60.0%	UCSC Genome Browser
9	-	60089914	60089936	TTCcGTGgTTGACCCGGGAG	TgaCGTgTTGACcttGGAG-AGG	4	55.0%	UCSC Genome Browser
10	-	86795009	86795031	TTCcGTGgTTGACCCGGGAG	TgCaCaGgTaGACCCGGGAG-TGG	4	65.0%	UCSC Genome Browser
7	-	80328458	80328480	TTCcGTGgTTGACCCGGGAG	TaCtGTGgTTGcCCaGGGAG-GGG	4	60.0%	UCSC Genome Browser
19	-	10782645	10782667	TTCcGTGgTTGACCCGGGAG	TgCCcTGgTTGACacGGAG-TGG	4	65.0%	UCSC Genome Browser
15	+	87200138	87200160	TTCcGTGgTTGACCCGGGAG	TaCCGTtCaTgCCCCGGGAG-AGG	4	65.0%	UCSC Genome Browser
11	-	89979230	89979252	TTCcGTGgTTGACCCGGGAG	TtTacTgaTTGACCCGGGAG-AGG	4	50.0%	UCSC Genome Browser

Off-target analysis for gRNA3:

Chrom	Strand	Start	End	Given query	Actual genomic hit	Number of mismatches	GC content	External links
7	+	44996704	44996726	GGAGTTAICCCGAAGGGGTC	GGAGTTAICCCGAAGGGGTC-TGG	0	60.0%	UCSC Genome Browser
15	-	19943400	19943422	GGAGTTAICCCGAAGGGGTC	aGAGTATCtCGAAGGGaTC-TGG	4	45.0%	UCSC Genome Browser
10	+	27528999	27529021	GGAGTTAICCCGAAGGGGTC	cGAGITATgCCaAAGGGGIt-GGG	4	50.0%	UCSC Genome Browser
14	-	73325286	73325308	GGAGTTAICCCGAAGGGGTC	GagGgcATCCCGAAGGGGTC-TGG	4	70.0%	UCSC Genome Browser
11	-	109559592	109559614	GGAGTTAICCCGAAGGGGTC	GtAGgTtTCCCGAAGGtGTC-AGG	4	55.0%	UCSC Genome Browser
11	+	65847854	65847876	GGAGTTAICCCGAAGGGGTC	GtAGcTATCCaGATGGGGTC-TGG	4	55.0%	UCSC Genome Browser
8	+	94752891	94752913	GGAGTTAICCCGAAGGGGTC	GcAGTcATCCTGAAGGGcTC-TGG	4	60.0%	UCSC Genome Browser
18	-	35860509	35860531	GGAGTTAICCCGAAGGGGTC	GcAGTTAcCCCGAAGaGtTC-CGG	4	55.0%	UCSC Genome Browser
2	+	144839095	144839117	GGAGTTAICCCGAAGGGGTC	GcAGITATCCTcAAGtGGTC-TGG	4	50.0%	UCSC Genome Browser
15	+	86072376	86072398	GGAGTTAICCCGAAGGGGTC	GtAGITATCCCTaATGGGaC-AGG	4	45.0%	UCSC Genome Browser

Off-target analysis for gRNA4

Chrom	Strand	Start	End	Given query	Actual genomic hit	Number of mismatches	GC content	External links
7	-	44996231	44996253	GIGGGACGTGTCCGATGGA	GIGGGACGTGTCCGATGGA-GGG	0	65.0%	UCSC Genome Browser
17	+	5220997	5221019	GIGGGACGTGTCCGATGGA	cTGGGAtGTGTCCaGATGGA-CGG	3	55.0%	UCSC Genome Browser
1	+	184488778	184488800	GIGGGACGTGTCCGATGGA	GIGGGAgGaGTCCCTaTGA-GGG	3	60.0%	UCSC Genome Browser
1	-	23331174	23331196	GIGGGACGTGTCCGATGGA	GIGGGAtGTGTCCAgTGA-AGG	3	60.0%	UCSC Genome Browser
18	+	40181853	40181875	GIGGGACGTGTCCGATGGA	GIGGGActItTCCCGTGA-AGG	3	60.0%	UCSC Genome Browser
8	-	37462651	37462673	GIGGGACGTGTCCGATGGA	tTgctAgGTGTCCGATGGA-TGG	4	55.0%	UCSC Genome Browser
2	+	74354755	74354777	GIGGGACGTGTCCGATGGA	aTGGGAgcTGTGCCGATGGA-AGG	4	60.0%	UCSC Genome Browser
19	+	34252432	34252454	GIGGGACGTGTCCGATGGA	cTGGGACGTccCaCGATGGA-TGG	4	65.0%	UCSC Genome Browser
8	-	64596671	64596693	GIGGGACGTGTCCGATGGA	GaGaGaaGTGTCCCAATGA-AGG	4	55.0%	UCSC Genome Browser
18	+	11167052	11167074	GIGGGACGTGTCCGATGGA	CaGtGACaTGTCCCGaGGA-TGG	4	60.0%	UCSC Genome Browser

Table S1. Positive results (+)* of skin prick test of FA patients

Patient#	Fish	Egg	Soy	Hazelnut	Shrimp	Walnut	Milk	Peanut
1					+			
2	+							
3			+				+	
4	+							
5					+			
6		+						
7	+							
8			+				+	
9		+						
10					+			
11					+			
12		+						
13				+				
14				+				+
15						+		
16					+			
17					+		+	
18							+	
19						+		

20							+	+
Positive	3	3	2	2	5	2	5	2
%	15	15	10	10	25	10	25	20

* A wheal diameter greater than 3 mm of the negative saline control was considered SPT positive.

Table S2. Positive results of serum sIgE*

Patient#	Fish	Egg	Soy	Hazelnut	Shrimp	Walnut	Milk	Peanut
1					3.52			
2	3.26							
3			2.52				4.12	
4	1.96							
5					1.88			
6		4.55						
7	6.33							
8			4.23				3.96	
9		2.96						
10					1.65			
11					5.11			
12		4.22						
13				3.85				
14				5.28				4.23
15						3.87		
16					5.21			
17					3.55		3.65	
18							4.52	
19						4.25		

20							5.28	6.88
Positive	3	3	2	2	5	2	5	2
%	15	15	10	10	25	10	25	20

* Specific IgE titers were measured using the Immuno-CAP assay system (Thermo Fisher Scientific, Uppsala, Sweden). Specific IgE titers >0.35 kUA/L were considered positive.

Table S3. Symptoms of FA

Patient#	Nausea	Vomiting	Abdominal pain	Diarrhea	Oral allergy	Skin allergy	Airway allergy
1		+		+		+	
2	+		+	+	+		
3	+	+					+
4				+		+	
5	+	+					
6		+					+
7	+	+					
8			+				
9	+				+		
10	+			+		+	+
11	+	+					
12	+						
13			+				

14			+				
15		+					
16			+				+
17		+					
18	+			+		+	
19			+				
20				+			
Total	9	8	6	6	2	4	4
%	45	40	30	30	10	20	20

Table S4. Primers used in RT-qPCR

Molecules	Forward	Reverse
Bcl2L12	cttctatgctttggtggccc	tacagaacagctccaccagg
IL-4	gcagttctacagccaccatg	actctggttggttccttca
IL-5	gccatccccacagaaattcc	ccccttgcacagtttgactc
IL-13	catccgctcctcaatcctct	gatgctccataccatgctgc
β -actin	catggaatcctgtggcatcc	cacacagagtacttgcgctc