Bcl2-like protein 12 plays a critical role in development of airway allergy through inducing aberrant Th2 polarization

To the Editor:

Although it is well understood that skewed Th2 polarization plays a critical role in the pathogenesis of allergic asthma, the mechanism of Th2 polarization remains elusive. Our recent studies showed that Bcl2-like protein 12 (Bcl2L12) was associated with pathogenesis of Th2-biased inflammation in the intestine by facilitating Th2 polarization. Thus we hypothesize that Bcl2L12 might be also associated with the pathogenesis of allergic asthma.

In this study we developed a mouse strain with Bcl2L12 knockout (KO) CD4+ T cells (ie, KO mice; see Fig E1 in this article’s Online Repository at www.jacionline.org) KO mice and wild-type (WT) mice were sensitized to ovalbumin (OVA) to develop airway allergy according to published procedures. After sensitization, WT mice showed asthma-like inflammation in the airway, including profound infiltration of mononuclear cells in lung tissue, increase in thickness of the basal membrane of bronchial walls (Fig 1, A), antigen-specific IgE in sera, and high levels of Th2 cytokines and mouse mast cell protease 1 in both sera and bronchoalveolar lavage (BAL) fluid, which were much lower in KO mice. The signature Th1 cytokines IFN-γ, IL-17, and TNF were also detected in sera and BAL fluid of both WT and KO mice, although levels were lower in sensitized WT mice compared with those in control mice (Fig 1, B-E). In BAL fluid total cell, eosinophil, lymphocyte, neutrophil, and macrophage counts were significantly greater in sensitized WT mice than those in sensitized KO mice (Fig 1, F-J). Lung resistance was much greater in sensitized WT mice than in sensitized KO mice (Fig 1, K). The data suggest that Bcl2L12 plays a critical role in development of asthma-like inflammation in the airway.

BAL fluid was obtained from asthmatic patients and healthy subjects (see Table E1 for patient demographic data in this article’s Online Repository at www.jacionline.org). Cellular elements were prepared from BAL fluid samples. Compared with samples from healthy subjects, BAL fluid samples from asthmatic patients showed a Th2-dominant profile, as indicated by a higher frequency of Th2 cells and high levels of Th2 cytokines (Fig 2, A-C, and see Fig E2 in this article’s Online Repository at www.jacionline.org). Expression of Bcl2L12 in CD4+ T cells isolated from BAL fluid samples was greater in the asthma group than that in the healthy group (Fig 2, D and E; see Table E2 for primers used in RT-qPCR in this article’s Online Repository at www.jacionline.org). A positive correlation was identified between Bcl2L12 and IL-4 (r = 0.7172, P = .0196), IL-5 (r = 0.7545, P = .0117), and IL-13 (r = 0.8076, P = .0047) in BAL fluid CD4+ T cells collected from the asthmatic group (Fig 2, F-H). A negative correlation (r = 0.8953, P = .0005) was identified between Bcl2L12 expression in BAL fluid CD4+ T cells and FEV1 of asthmatic patients (Fig 2, I). Similar phenomena were also found in peripheral blood CD4+ T cells of the same group of asthmatic patients (see Fig E3 in this article’s Online Repository at www.jacionline.org). No correlation was detected between Bcl2L12 and IFN-γ, IL-17, or TNF (data not shown). These data imply that Bcl2L12 might be involved in the development of the skewed Th2 response in asthmatic patients.

CD4+ T cells were isolated from blood samples collected from asthmatic patients and healthy subjects to gain insight into the mechanism by which Bcl2L12 regulates Th2 response. After stimulation with activators in culture for 4 days, more IL-4+ T cells were induced in naïve CD4+ T cells collected from asthmatic patients than those from healthy subjects, which was abolished by knocking down Bcl2L12 expression.

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Overexpression of Bcl2L12 induced naive CD4+ T cells to differentiate into TH2 cells (Fig 2, J, and see Fig E4 in this article’s Online Repository at www.jacionline.org). Results of co-immunoprecipitation showed that Bcl2L12 formed a complex with GATA-3 in CD4+ T cells; the quantity of the complex was greater in samples collected from asthmatic patients than that from healthy subjects (Fig 2, K). Binding between Bcl2L12 and GATA-3 was verified in in vitro experiments. HEK293 cells were transfected with Bcl2L12-expressing and GATA-3–expressing plasmids. A complex of rBcl2L12 and rGATA-3 was detected in the HEK293 cells (Fig 2, L). By using the co-chromatin immunoprecipitation (co-ChIP) approach, the complex of Bcl2L12 and GATA-3 was detected in the Il4 promoter locus, which was much greater in CD4+ T cells collected from asthmatic patients than that from healthy subjects (Fig 2, M).

To corroborate these data, CD4+ T cells were isolated from the spleens of WT and KO mice treated with airway sensitization procedures. The cells were analyzed by using co-ChIP. The results showed that the Bcl2L12 and GATA-3 complex was detected in the Il4 promoter locus of CD4+ T cells isolated from sensitized WT mice. Inhibition of Bcl2L12 suppressed levels of GATA-3 but did not affect its baseline in the Il4 promoter locus of CD4+ T cells (Fig 2, N). Data demonstrate that Bcl2L12 plays an important role in TH2 polarization in asthmatic patients by modulating the activities of GATA-3. The data indicate that Bcl2L12 might be a new member of the molecular family in regulation of GATA-3 activities in CD4+ T cells. Because of the proline-rich feature Bcl2L12 confers on binding ability,5,6 it can contribute to formation of the Bcl2L12 and GATA-3 complex. Because GATA-3 is the major transcription factor of IL-4, these results...
FIG 2. Correlation between Bcl2L12 and FEV1 and T_{h}2 cytokines in BAL fluid CD4^{+} T cells of asthmatic patients. BAL and blood samples were collected from healthy subjects (n = 10) and asthmatic patients (n = 10). Single cells of BAL and PBMCs were prepared and analyzed by using flow cytometry. A, Summarized frequency data of CD4^{+} T-cell phenotypes. B, BAL cytokine levels. C, Levels of mRNA in BAL CD4^{+} T cells. D, Bcl2L12 mRNA levels of BAL CD4^{+} T cells. E, Bcl2L12 protein levels in BAL CD4^{+} T cells. F-H, Correlation between Bcl2L12 mRNA and mRNA of T_{h}2 cytokines in BAL CD4^{+} T cells. I, The negative correlation between Bcl2L12 mRNA in peripheral CD4^{+} T cells and FEV1 of asthma patients. J, Bars indicate percentage of IL-4^{+} T cell converted from naive CD4^{+} T cells. K, Complexes of Bcl2L12 and GATA3 in CD4^{+} T cells. L, A complex of rBcl2L12 and rGATA3 in HEK293 cells. M-N, Bars indicate levels of Bcl2L12 and GATA3 at the Il4 promoter locus in CD4^{+} T cells of human (Fig 2, M) and mice (Fig 2, N). Data in bars are presented as means ± SDs. *P < .01 compared with the healthy group. Samples from individual subjects were analyzed separately.
suggestion that physical contact between Bcl2L12 and GATA-3 enhances expression of IL-4 in CD4+ T cells.

Overproduction of IL-4 is one of the main features of Th2-biased inflammation, such as allergic asthma. GATA-3 is the essential transcription factor of IL-4. Thus it is conceivable that inhibition of GATA-3 might attenuate or inhibit the Th2-biased inflammation. However, IL-4 is also an important cytokine in antibody production, which plays a crucial role in the defensive functions of the body. The present data indicate that inhibition of Bcl2L12 can inhibit binding between GATA-3 and the Il4 promoter to suppress IL-4 expression but does not affect its baseline expression. Data also suggest that inhibiting Bcl2L12 can inhibit the unnecessary amount of IL-4 and not affect its physiologic functions.

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Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

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3. Li MG, Liu XY, Liu ZQ, Hong JY, Liu JQ, Zhou CJ, et al. Bcl2L12 contributes to food allergy being carried out at Severo Ochoa University Hospital (PREVALE [PREvalence of food allergies in Leganes pediatric population]) among the population of Leganes (Spain), FPIES characteristics and incidence have been analyzed.

The PREVALE study is a prospective longitudinal study that includes live births in the aforesaid hospital from April 2015 to April 2016. From the beginning of the study, 1142 patients (90.8% of total live births) are being closely monitored by telephone calls at 2, 4, 6, 12, 15, 18, 24, and 36 months of life. As of today, all the children involved are already, at least, 18 months old. In addition, and in collaboration with primary care pediatrics in the area, all children with symptoms suggestive of food allergy have been referred to the pediatric allergology specialist for their examination.

IgE-mediated food allergy (in the first year of life) in our cohort is 2.36%. Among these 1142 patients, 8 have been diagnosed with FPIES, a cumulative global incidence of 0.7%. The average age at onset was 7.62 months and 62% were males. Five out of the 8 patients (62.5%) were being breast-fed (not exclusively) at the time of diagnosis. The most frequently observed symptoms were persistent vomiting and lethargy (Table I).

Seven out of 8 patients (87.5%) had negative allergy test results (negative skin prick test result and undetectable food-specific serum IgE). One of the patients (12.5%) showed atypical FPIES (with a positive prick test result for casein and cow milk), which mainly affects infants and young children. It consists of gastrointestinal symptoms (persistent vomiting and, sometimes, diarrhea) and lethargy and/or pallor.

Despite growing scientific interest in recent years, this syndrome remains fairly unknown in terms of both its epidemiology and its pathophysiology, diagnosis, and natural history. This lack of knowledge hampers the development of studies aiming to determine the actual incidence of the disease.

Up to now, the study by Katz and Goldberg1 has been the sole prospective study published that refers to FPIES frequency, estimating a cumulative incidence of FPIES induced by cow’s milk protein of 0.34% live births in a hospital in Israel over a follow-up period of 2 years. A more recent study carried out in Australia7 determined the annual incidence of FPIES at 15.4 per every 100,000 children younger than 2 years (ie, 0.0154%).

In light of all the above, within the prevalence study on food allergies being carried out at Severo Ochoa University Hospital (PREVALE [PREvalence of food allergies in Leganes pediatric population]) among the population of Leganes (Spain), FPIES characteristics and incidence have been analyzed.

To the Editor:

Food protein–induced enterocolitis syndrome (FPIES) is a not well-understood type of non-IgE-mediated food hypersensitivity, which mainly affects infants and young children. It consists of gastrointestinal symptoms (persistent vomiting and, sometimes, diarrhea) and lethargy and/or pallor.

The most frequently implicated food was cow’s milk (4 patients [50%]), followed by fish (3 patients [37.5%]) and egg yolk (1 patient [12.5%]). Therefore, the cumulative incidence of FPIES was (1) 0.35% for cow’s milk protein, (2) 0.26% for fish, and (3) 0.09% for egg yolk. The fish involved in our 3 cases was hake. We have not observed cases of multiple FPIES and only 1 of the cases suggested chronic FPIES (Table I).

The low incidence of FPIES induced by fish contrasts with that of other Spanish series3 where the rate is significantly higher. In

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METHODS

Reagents
Bcl2L12 antibody was purchased from Abcam (Cambridge, Mass). The reagent kit for Bcl2L12 RNA interference and GATA-3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). ELISA kits for specific IgE, mouse mast cell protease 1, IL-4, IL-5, IL-13, IFN-γ, IL-17, and TNF were purchased from Biomart (Beijing, China). Fluorescein-labeled antibodies (for flow cytometry) for CD3, CD4, IL-4, IL-5, IL-13, IFN-γ, IL-17, and TNF were purchased from BD Biosciences (Franklin Lakes, NJ). Immune cell isolation kits were purchased from Miltenyi Biotech (San Diego, Calif). Reagents for real-time quantitative RT-PCR, Western blotting, and gene transfection were purchased from Invitrogen (Carlsbad, Calif). Phorbol 12-myristate 13-acetate, ionomycin, reagents, and materials for immunoprecipitation and ChIP were purchased from Sigma-Aldrich (St Louis, Mo).

Human subjects
Patients with perennial asthma were recruited to this study at our affiliated hospitals. Diagnosis and management of asthma were carried out by the physicians of our hospitals, according to routine procedures. Demographic data of the patients are presented in Table E1. Patients with any of the following conditions were excluded from this study: severe organ diseases, cancer, and auto-immune diseases, as well as those using immune suppressors. Healthy subjects were also recruited to be used as control subjects. Use of human tissues in the present study was approved by the Human Ethics Committee at Shenzhen University. Informed written consent was obtained from each human subject.

Among the human subjects, BAL was performed in 10 asthmatic patients and 10 healthy subjects by using fiberoptic bronchoscopy (Olympus B2-10; Olympus, Tokyo, Japan), according to our routine procedures, which were also reported by others. Briefly, by using four 50-mL aliquots of warm saline instilled at the right-middle lobe of the lung, about 100 mL of fluid was recovered from each subject. Samples were centrifuged at 400g for 5 minutes at 4°C. Supernatants were collected and stored at −80°C until use. Cell pellets were resuspended in RPMI 1640 for cytologic analysis.

Mouse BAL fluid analysis
Lungs of each mouse were flushed with PBS (containing 0.5% FBS BSA in 1 mL of PBS). BAL fluid was obtained after lavage and centrifuged at 400g for 5 minutes at 4°C. Pellets were resuspended in 50 μL of PBS, and cytospin slides of BAL fluid cells were made with a cytospin device (Shandon Southern Instruments, Runcorn, United Kingdom). Slides were stained with May-Grunwald Giemsa. Cell counts were performed by using a light microscope. Absolute numbers and percentages of eosinophils, lymphocytes, neutrophils, and monocytes/macrophages were quantified. The supernatant was collected and stored at −80°C until use.

Induction of experimental asthma in mice
All animal experiments were approved by the Shenzhen University Institutional Animal Care and Use Committee. Male BALB/c mice (WT mice; provided by the Guangdong Experimental Animal Center, Guangzhou, China) and mice with Bcl2L12 KO CD4+T cells (provided by the Institute of Biophysics at Chinese Academy of Agricultural Sciences, Beijing, China; Fig E1) were injected intraperitoneally on days 0, 7, and 14 with OVA (100 μg mixed in 0.2 mL of alum) or PBS (control). The mice were challenged intranasally with 50 μL of 100 μg of OVA or PBS daily from days 21 to 27. Mice were killed on day 28.

Cytokine assessment in BAL fluid and serum
Cytokines in BAL fluid and serum were quantified by means of ELISA with relevant reagent kits, according to the manufacturer’s instructions.

Preparation of PBMCs
Blood samples were collected from each human subject by means of ulnar vein puncture. PBMCs were isolated from blood samples by means of density gradient centrifugation.

Assessment of respiratory mechanics of mice
Respiratory mechanics were evaluated by using the flexiVent system, according to the published procedures, to evaluate lung function in the mice. Mice were anaesthetized by means of intraperitoneal injection with xylazine and sodium pentobarbital. Mice were treated with increasing concentrations of aerosolized methacholine; measurements were performed with the flexiVent small-animal ventilator (SCIREQ, Montreal, Quebec, Canada).

Immune cell isolation
Immune cells were isolated from PBMCs by means of magnetic cell sorting (MACS) with relevant reagent kits, according to the manufacturer’s instructions. Purity of the isolated cells was checked by using flow cytometry. If the purity was less than 95%, MACS was performed again with the cells.

Flow cytometry
On surface staining, cells were stained with antibodies (labeled with fluorochromes) of interest or isotype IgG for 30 minutes at 4°C. If intracellular staining was required, cells were treated with fixative/permeable reagents and stained with antibodies (labeled with fluorochromes) of interest or isotype IgG for 30 minutes at 4°C. Cells were analyzed with a flow cytometer (FACSCanto II; BD Biosciences). Data were processed with the software package FlowJo (Treestar, Ashland, Ore). Isotype IgG staining data were used as gating references.

Real-time quantitative RT-PCR
Cells were collected from relevant experiments. Total RNA was extracted from cells with TRIzol reagents (Thermo Fisher, Waltham, Mass). cDNA was converted with RNA samples with a reverse transcription kit, according to the manufacturer’s instructions. Samples were amplified in a quantitative PCR device (CFX96 Touch; Bio-Rad Laboratories, Hercules, Calif) with SYBR Green Master Mix in the presence of relevant primers (Table E2, see below). Results were normalized to fold change against the housekeeping gene β-actin.

Preparation of cytosolic and nuclear proteins
Cells were collected from relevant experiments and lysed with lysis buffer. Samples were centrifuged for 10 minutes at 13,000 rpm. Supernatants were collected and used as cytosolic extracts. Pellets were resuspended in nuclear lysing buffer for 30 minutes. Samples were centrifuged for 10 minutes at 13,000 rpm. Supernatants were collected and used as nuclear extracts. All procedures were performed at 4°C.

Western blotting
Proteins were fractionated by using SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. After blocking with 5% skin milk solution for 30 minutes, the membrane was incubated with the first antibodies of interest or isotype IgG overnight at 4°C, followed by incubating with the second antibodies (labeled with peroxidase) for 2 hours at room temperature. The membrane was washed with Tris-buffered saline–Tween 20 after each incubation. Immunoblots on the membrane were developed with enhanced chemiluminescence and photographed with an image device (UVI, Cambridge, United Kingdom).
Bcl2L12 RNA interference

CD4⁺ T cells were isolated from PBMCs of asthmatic patients by using MACS. Cells were treated with a Bcl2L12 short hairpin RNA reagent kit (Santa Cruz Biotech, Santa Cruz, Calif), according to the manufacturer’s instructions. The effects of RNA interference were assessed 48 hours later by using Western blotting.

Bcl2L12 RNA overexpression

Naive CD4⁺CD25⁻ T cells were isolated from PBMCs of healthy subjects by using MACS. Cells were transfected with the full-length Bcl2L12 gene-carrying plasmids or control plasmids (provided by the Sangon Biotech, Shanghai, China) by means of electroporation with a Bio-Rad Gene Pulse set at 950 μF and 280 V (Bio-Rad Laboratories). Bcl2L12 expression was assessed in cells 48 hours later by using Western blotting.

Immunoprecipitation

Protein samples were precleared by means of incubation of protein G agarose beads for 2 hours. The supernatant was incubated with antibodies of interest or isotype IgG overnight. Agarose beads were collected by means of centrifugation. Protein complexes on the beads were eluted with an eluting buffer and analyzed by means of Western blotting. All procedures were performed at 4°C.

ChIP

Cells were collected from relevant experiments and fixed with 1% formalin for 15 minutes. Cells were lysed with a lysis buffer, followed by sonication to shear DNA into small pieces. Samples were then processed with immunoprecipitation procedures. After eluting from the agarose beads, DNA was recovered from samples with a DNA recovering reagent kit (QIAGEN Sciences, Germantown, Md), according to the manufacturer’s instructions. DNA was analyzed by using qPCR in the presence of primers of the Il4 promoter (ggcctccctctatgcaa and gattgtcagtcacttggggc). Results were normalized to fold change against the input.

Statistics

Data are presented as means ± SDs. The difference between 2 groups was determined by using the Student t test or ANOVA, followed by the Dunnett t test or SNK test for multiple comparisons. Correlation between 2 groups was tested with the Pearson correlation assay. A P value of less than .05 was set as a significance criterion.

REFERENCES

FIG E1. Expression of Bcl2L12 in CD4^+ T cells with Bcl2L12 KO and WT mice. CD4^+ T cells were isolated from lung tissue and spleens of WT mice (BALB/c mice) and mice with Bcl2L12 KO CD4^+ T cells. Total RNA was extracted from CD4^+ T cells by using MACS. Cells were analyzed by using real-time quantitative RT-PCR and Western blotting. A, Bars show mRNA levels of Bcl2L12. B, Immunoblots show protein levels of Bcl2L12. Data represent 3 independent experiments.
FIG E2. BAL fluid CD4+ T-cell phenotypes. BAL fluid samples were collected from asthmatic patients (n = 10) and healthy subjects (n = 10). BAL fluid cells were prepared and analyzed by using flow cytometry. A, CD3+CD4+ T cells were gated. B, Gated histograms indicate the frequency of CD4+ T-cell phenotypes in BAL fluid cells.
FIG E3. Correlation between Bcl2L12 and FEV₁ and Th2 cytokines in peripheral blood CD4⁺ T cells of asthmatic patients. Blood samples were collected from healthy subjects (n = 30) and asthmatic patients (n = 30). PBMCs were prepared and analyzed by using flow cytometry. A-C, Gated dot plots show CD4⁺ T cells in PBMCs. Gated histograms show the frequency of CD4⁺ T-cell phenotypes. Bars show summarized frequency data of CD4⁺ T-cell phenotypes. D and E, Levels of mRNA (Fig E3, D) and protein (Fig E3, E) in peripheral CD4⁺ T cells. F, Integrated density of the immunoblots from Fig E3, E. G, Negative correlation between Bcl2L12 mRNA in peripheral CD4⁺ T cells and FEV₁ of asthmatic patients. H, mRNA levels of cytokines in CD4⁺ T cells (isolated from PBMCs). I, Correlation between Bcl2L12 mRNA and mRNA of Th2 cytokines in peripheral CD4⁺ T cells. Data in bars are presented as means ± SDs. *P < .01 compared with the healthy group. Samples from individual subjects were analyzed separately.
FIG E4. Assessment of the role of Bcl212 in Th2 cell differentiation. Naive CD4⁺ T cells (CD3⁺CD4⁺CD25⁻CD62L⁻) were prepared from PBMCs isolated from healthy subjects (n = 30) and asthmatic patients (n = 30). Cells were treated with procedures as denoted above each subpanel and exposed to activators (phorbol 12-myristate 13-acetate, 50 ng/mL; ionomycin, 0.5 μg/mL) in culture for 4 days. A, Gated flow cytometric histograms show representative data of the frequency of Th2 cells generated from naive CD4⁺ T cells from asthmatic patients. B, Gated flow cytometric histograms show representative data of the generated Th2 cells. C, Immunoblots show the results of Bcl2L12 RNA interference. D, Gated flow cytometric histograms show representative data of the frequency of generated Th2 cells. E, Results of Bcl2L12 overexpression in CD4⁺ T cells by transfecting with Bcl2L12-expressing plasmids.
TABLE E1. Clinical characteristics of study groups

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Values shown are means ± SDs.
DME, Dust mite extract; SPT, skin prick test; UD, undetectable.
*P < .05 compared with the healthy group (t test).
†Median (lower quartile–upper quartile).
### TABLE E2. Primers used in real-time quantitative RT-PCR

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