

B cell lymphoma-2-like protein-12 association with T-helper 2 inflammation in chronic rhinosinusitis with allergy

Yun-Fang An, MD, PhD¹, Yong-Jin Wu, MD, PhD^{2,3}, Xian-Hai Zeng, MD^{2,3}, Li-Juan Song, MD⁴, Fei Ma, MD, PhD⁴, Wen-Jing Liao, MD⁴, Zhi-Qiang Liu, MD, PhD^{2,3} , Gui Yang, MD^{2,3}, Xiao-Wen Zhang, MD, PhD⁴, Zhi-Gang Liu, MD, PhD², Chang-Qing Zhao, MD, PhD¹ and Ping-Chang Yang, MD, PhD² 

Background: T-helper 2 (Th2) polarization plays a critical role in the pathogenesis of chronic rhinosinusitis (CRS) with accompanying nasal allergy. Recent studies indicate that B cell lymphoma-2-like protein-12 (Bcl2L12) is associated with immune dysregulation. The purpose of this study was to elucidate the role of Bcl2L12 in the pathogenesis of Th2 polarization of CRS patients.

Methods: CRS patients with nasal allergy (CRSa) and without nasal allergy (CRSna) were recruited into this study. CD4⁺ T cells were isolated from the blood samples of human subjects. A variety of immunologic molecular strategies were used to assess Th2 polarization and Bcl2L12 expression.

Results: Twenty CRSa patients, 20 CRSna patients, and 20 healthy subjects were recruited into this study. High levels of immunoglobulin E (IgE), interleukin 4 (IL-4), IL-5, IL-13, and Bcl2L12 were detected in nasal extracts of CRSa patients, but not in CRSna patients. The levels of Bcl2L12 were

positively correlated with Th2 cytokines. CD4⁺ T cells from CRSa patients were prone to differentiate into Th2 cells, in which Bcl2L12 was required.

Conclusion: Bcl2L12 is positively correlated with Th2 cytokine levels in the nasal mucosa of CRSa patients. Bcl2L12 contributes to the Th2 polarization, which may be a novel therapeutic target in the treatment of CRSa. © 2018 ARS-AAOA, LLC.

Key Words:

rhinosinusitis; inflammation; Bcl2L12; immunity; lymphocyte

How to Cite this Article:

An YF, Wu YJ, Zeng XH, et al. B cell lymphoma-2-like protein-12 association with T-helper 2 inflammation in chronic rhinosinusitis with allergy. *Int Forum Allergy Rhinol.* 2018;00:1-8.

Chronic rhinosinusitis (CRS) is a chronic inflammation in the sinus mucosa and nasal mucosa.¹ CRS can be

categorized into 2 types, CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP).¹ In CRSwNP, the immune inflammatory type usually is T-helper 2 (Th2) polarization; as shown by high peripheral Th2/Th1 cell ratio, high frequency of myeloid dendritic cells (DCs),² and high frequency of group 2 innate lymphoid cells (ILC2s),³ while in CRSsNP, Th1 polarization is the major inflammatory feature.⁴ On the other hand, CRS may be categorized into 2 types: CRS with nasal allergy (CRSa) and CRS without nasal allergy (CRSna). CRSa is also characterized as Th2 polarization and accompanied with allergic rhinitis (AR).⁵ To date, the pathogenesis of CRSa still remains elusive; the treatment of CRS is refractory currently.⁶

The Th2 polarization is featured as aberrant infiltration of Th2 cells and Th2 cytokine production in the local tissue, which can be reflected in the peripheral circulation system.⁷ The signature Th2 cytokine is interleukin 4 (IL-4). IL-4 is mainly produced by Th2 cells and can promote the production of other Th2 cytokines, such as

¹Department of Otolaryngology, Head & Neck Surgery, the Second Hospital, Shanxi Medical University, Taiyuan, China; ²Ear-Nose-Throat (ENT) Institute and Research Center of Allergy & Immunology, Shenzhen University School of Medicine, Shenzhen, China; ³Longgang Ear-Nose-Throat (ENT) Hospital, Shenzhen, China; ⁴Department of Otolaryngology, Head & Neck Surgery, the First Hospital, Guangzhou Medical University, Guangzhou, China

Correspondence to: Ping-Chang Yang, Room 505 of Medical School Building, Shenzhen University, 1066 Xueyuan Blvd, Shenzhen 518055, China; e-mail: pcy2356@szu.edu.cn

Y.F.A., Y.J.W., and X.H.Z. contributed equally to this work.

Funding sources for the study: Innovation of Science and Technology Commission of Shenzhen Municipality (JCYJ20150403091931195, CXZZ20140902151802864, and JCYJ20160429091935720); National Natural Science Foundation of China (31570932, 31400856, 81571790, and 81501573).

Potential conflict of interest: None provided.

Received: 3 June 2018; Revised: 10 September 2018; Accepted: 12 September 2018

DOI: 10.1002/alr.22223

View this article online at wileyonlinelibrary.com.

IL-5 and IL-13.⁸ The regulation of Th2 cytokines and Th2 polarization has been investigated extensively. It is known that the aberrant response to innocent antigens can induce Th2 polarization and allergic diseases,⁹ including AR.¹⁰ The abnormally high activities of GATA3, the IL-4 gene transcription factor, induce IL-4 overproduction to contribute to the development of Th2 polarization.¹¹ Yet the underlying mechanism of Th2 polarization is still not fully understood.

The Th2 polarization reflects a condition of immune dysregulation. Recent studies indicate that the abnormal expression of B cell lymphoma 2-like protein 12 (Bcl2L12) is associated with immune dysregulation.^{12,13} Bcl2L12 belongs to the Bcl2 family, is 1 of the antiapoptotic molecules.¹⁴ Early studies show that Bcl2L12 is associated with the pathogenesis of cancer by facilitating cancer cells to escape from apoptosis. Recent studies found that Bcl2L12 also associates with other cell activities, such as the vascular endothelial cell dysfunction,¹⁵ suppression of IL-10 expression in B cells,^{12,13} and Perthes disease.¹⁶ Therefore, we hypothesize that Bcl2L12 may be associated with the Th2 polarization status in CRS. To test the hypothesis, CD4⁺ T cells were collected from patients with CRS. The expression of Bcl2L12 in CD4⁺ T cells was analyzed; the relation between Bcl2L12 expression and Th2 response was investigated.

Materials and methods

Reagents

The antibodies of Bcl2L12 were purchased from Abcam (Cambridge, MA). The materials and reagents for Western blotting and real-time quantitative polymerase chain reaction (RT-qPCR) were purchased from Invitrogen (Carlsbad, CA). The enzyme-linked immunosorbent assay (ELISA) kits of total immunoglobulin E (IgE), mite-specific IgE, Bcl2L12, IL-4, IL-5, and IL-13 were purchased from BioMart (Beijing, China). The fluorescein isothiocyanate (FITC)-labeled anti-IL-4 antibody was purchased from BD Bioscience (Franklin Lakes, NJ). The chromatin immunoprecipitation (ChIP) kit and immunoprecipitation (IP) reagents were purchased from Sigma Aldrich (St. Louis, MO). The antibodies of GATA3, RNA polymerase II (Pol II), and IL-4 were purchased from Santa Cruz Biotech (Santa Cruz, CA).

Human subjects

Patients with CRS were recruited into this study. The diagnosis of CRS was carried out by our surgeons based on having compatible CRS clinical symptoms, with or without nasal polyposis and verified by computed tomography (CT) scan. CRS patients were categorized into CRSa and CRSna groups based on the presence or absence or perennial AR (including AR history, positive skin test, and serum antigen-specific IgE >0.35 IU/mL). The demographic data of human subjects are presented in Table 1. Patients

TABLE 1. Demographic data of human subjects

	CRSa	CRSna	Healthy
Male, n	10	10	10
Female, n	10	10	10
Age (years), mean $\pm$ SD	32.6 $\pm$ 6.5	33.6 $\pm$ 5.2	32.8 $\pm$ 6.8
Maxillary sinusitis, n	20	20	0
Ethmoidal sinusitis, n	12	11	0
Allergic rhinitis, n	20	0	0
DME SPT-positive, n	20	0	0
Polyposis, n	8	0	0
Serum total IgE (kU/L), mean $\pm$ SD	58.8 $\pm$ 8.8	<0.35 ^a	<0.35 ^a
Serum DME-specific IgE (kU/L), mean $\pm$ SD	74.5 $\pm$ 8.5	<0.35 ^a	<0.35 ^a
Blood eosinophils (%), mean $\pm$ SD	6.4 $\pm$ 2.1	2.5 $\pm$ 1.1 ^a	2.0 $\pm$ 1.2 ^a

^a $p < 0.01$, compared with the CRSa group.

CRSa = chronic rhinosinusitis with nasal allergy; CRSna = chronic rhinosinusitis without nasal allergy; DME = dust mite extract; IgE = immunoglobulin E; SD = standard deviation; SPT = skin-prick test.

with any of the following conditions were excluded from this study: Cancer, seasonal AR, autoimmune diseases, and severe organ diseases. Age- and gender-matched healthy subjects were also recruited (healthy subjects did not have histories of CRS or allergic disorders; serum IgE levels were less than 0.35 IU/mL; antigen skin test was negative). The using human tissue in the present study was approved by the Human Ethics Committee at Shanxi Medical University. An informed written consent was obtained from each human subject.

Nasal mucosa sample collection

All the CRS patients were treated with endoscopic rhinosinus surgery following published procedures. A piece of nasal mucosa around the rhinosinus orifice was sampled during surgery. The marginal normal tissue was collected from surgically removed nasal cancer (the histology was verified by pathologists). The samples were immediately processed for further analysis.

Preparation of protein extracts

Tissue was homogenized with a homogenizer in a lysing buffer. Cells were lysed with a lysing buffer. The lysates were centrifuged at 10,000 g for 10 minutes. The supernatant was collected and used as cytosolic extracts. The pellets were lysed with a nuclear lysing buffer. The lysates were centrifuged at 13,000 rpm for 10 minutes. The supernatant was collected and used as nuclear extracts. All the procedures were performed at 4°C.

ELISA

Cytokines in the serum or culture supernatant were assessed by ELISA with commercial reagent kits following the manufacturer's instructions.

Preparation of peripheral blood mononuclear cells and immune cell isolation

Blood samples were collected from human subjects by ulnar vein puncture. Peripheral blood mononuclear cells (PBMCs) were isolated from the samples by gradient density centrifugation. Immune cells for relevant experiments were isolated from the PBMCs by magnetic cell sorting (MACS) with commercial reagent kits following the manufacturer's instructions. The purity of the isolated cells was checked by flow cytometry. If the purity did not reach 95%, MACS was performed with the cells again.

Cell culture

Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI1640) culture medium supplemented with antibiotics (penicillin and streptomycin), glutamine, and fetal bovine serum (10%). The medium was changed in 2 to 3 days. The viability of the cells was greater than 99% as checked by Trypan blue exclusion assay.

Flow cytometry

In the surface staining, cells were stained with fluorescence-labeled antibodies of interest or isotype IgG for 1 hour at 4°C. In the intracellular staining, cells were treated with fixative and permeable reagents first, then stained with fluorescence-labeled antibodies of interest or isotype IgG for 1 hour at 4°C. After washing with phosphate buffered saline, the cells were analyzed with a flow cytometer (FACSCanto II; BD Biosciences, Franklin Lakes, NJ). The acquired data were analyzed with FlowJo software (FlowJo, LLC, Ashland, OR). The data generated by isotype IgG staining were used as gating references.

Western blotting

The proteins were fractioned by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skim milk for 30 minutes, incubated with antibodies of interest overnight at 4°C, followed by incubating with the second antibodies (conjugated with peroxidase) for 1 hour. The membrane was washed with Tris-buffered saline Tween 20 for 3 times after each time of incubation. The immunoblots on the membrane were developed with the enhanced chemiluminescence and photographed with an imaging device.

RT-qPCR

Total RNA was extracted from cells collected from relevant experiments and reversely converted to complementary DNA (cDNA) with a reverse transcription kit following

the manufacturer's instructions. The samples were amplified in a qPCR device with the SYBR Green Master Mix and the presence of relevant primers, including IL-4 (aacgaggtcacaggagaagg and tctgcagctccatgagaaca) and Bcl2L12 (ttccgagttctatgccctgg and ccagttttacgatgcagagcc). The results are presented as fold change against the housekeeping gene beta actin.

IP

IP was performed with protein extracts with a commercial reagent kit following the manufacturer's instructions. Briefly, proteins (2 mg/mL) were precleared by incubating with protein G agarose beads for 2 hours. The beads were removed by centrifugation; the supernatant was incubated with antibodies (1:300 dilution) of interest overnight. The immune complexes were precipitated by incubating with protein G agarose beads for 2 hours. The beads were collected by centrifugation. The immune complexes on the beads were eluted with an eluting buffer and analyzed by Western blotting. All the procedures were performed at 4°C.

ChIP

ChIP was performed with a commercial reagent kit following the manufacturer's instructions. Briefly, cells were collected from relevant experiments and fixed with 1% formalin for 15 minutes. The cells were lysed with a lysing buffer. The lysates were sonicated to shear DNA into small pieces (300 to 500 base pairs [bp]). The samples were then treated with the procedures of IP (see IP section). Protein/DNA on agarose beads (from IP) were eluted with an eluting buffer. DNA was recovered from the eluted samples with a DNA extraction kit following the manufacturer's instructions. The DNA was analyzed by qPCR in the presence of primers of *Il4* promoter (ggcctctcccttctatgcaa and gattgtcagtcacttggggc). The results are presented as fold change against the input. All the procedures were performed at 4°C.

Bcl2L12 RNA interference

The Bcl2L12 gene in CD4⁺ T cells or EL-4 cells was knocked down by transducing with Bcl2L12 short hairpin RNA (shRNA)-carrying lentivectors by electroporation using a Bio-Rad Gene Pulse (Bio-Rad Laboratories, Hercules, CA) set at 950 μ F and 280 V. The effects of RNA interference (RNAi) were checked by Western blotting 48 hours after the transductions.

Bcl2L12 overexpression

The Bcl2L12-expressing plasmids and control plasmids (empty plasmids) were constructed by the Sangong Biotech (Shanghai, China). CD4⁺ T cells were transfected with the Bcl2L12-expressing plasmids or control plasmids by electroporation using a Bio-Rad Gene Pulse set at 950 μ F and 280 V. The effects of Bcl2L12

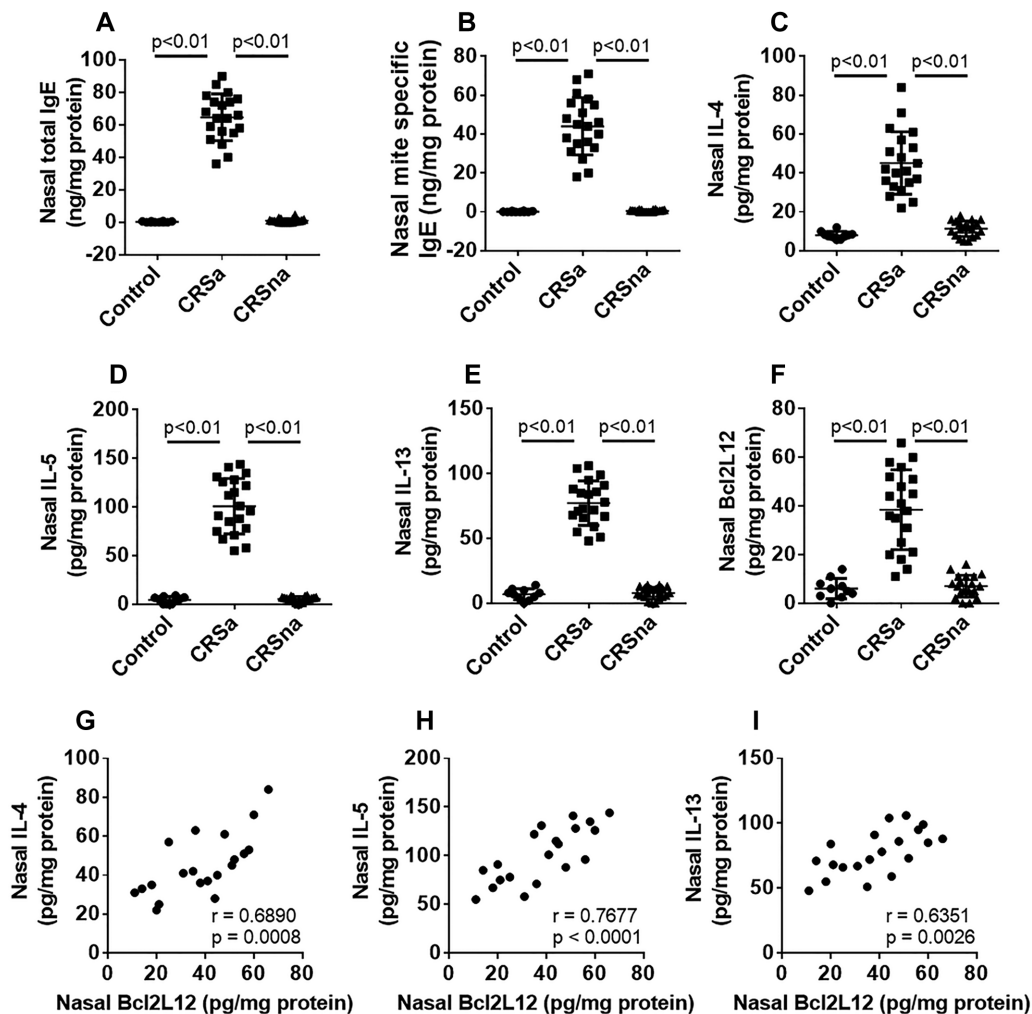


FIGURE 1. Bcl2L12 is positively correlated with Th2 polarization status in the nasal mucosa of CRSa patients. Surgically removed nasal mucosa samples were collected from CRSa ($n = 20$) and CRSna ($n = 20$) patients. The marginal normal tissue was collected from surgically removed nasal cancer ($n = 10$) used as control. Proteins were extracted from the tissue and analyzed by ELISA. The scatter dot plots show levels of total IgE (A), mite specific IgE (B), Th2 cytokines (C-E), and Bcl2L12 (F) in the nasal mucosa. (G, I) Scatter dot plots show the correlation between Bcl2L12 and Th2 cytokines. Each sample was analyzed in triplicate. Bcl2L12 = B cell lymphoma-2-like protein-12; CRSa = chronic rhinosinusitis with nasal allergy; CRSna = chronic rhinosinusitis without nasal allergy; ELISA = enzyme-linked immunosorbent assay; IgE = immunoglobulin E; Th2 = T helper 2.

overexpression were checked by Western blotting 48 hours later.

Statistics

The data are presented as mean $\pm$ standard deviation (SD). The difference between 2 groups was determined by Student *t* test. Correlation between groups was determined by Pearson correlation test. A value of $p < 0.05$ was set as a significant criterion.

Results

The IgE and Th2 cytokines are positively correlated with Bcl2L12 in the CRS nasal mucosa

With surgically removed nasal mucosa samples from CRS patients, we extracted proteins to be analyzed by ELISA. Based on the results of IgE analysis, antigen skin test, and

clinical symptoms, the CRS patients were categorized into the CRSa and CRSna groups. In the CRSa group, 8 patients also had nasal polyps. The levels of IgE, Th2 cytokines, and Bcl2L12 were much higher ($p < 0.01$) in nasal mucosa extracts of the CRSa group than the CRSna group (Fig. 1A-F). A correlation test was performed with the data of Th2 cytokines and Bcl2L12. The results showed a positive correlation between Th2 cytokines and Bcl2L12 in the CRSa group (Fig. 1G-I), but not in the CRSna group (not shown). On the other hand, 8 CRSa patients also had nasal polyps. The levels of IgE, Th2 cytokines, and Bcl2L12 expression in CRSa patients with or without nasal polyps were analyzed separately. The results showed that no significant difference was identified in these items between CRSa patients with or without nasal polyps (not shown). The results indicate a Th2 polarization status in the nasal mucosa of CRSa patients and suggest that Bcl2L12 may be associated with the Th2 polarization status in CRSa.

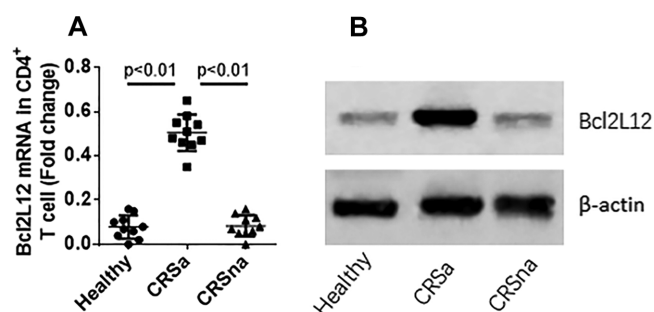


FIGURE 2. Peripheral CD4⁺ T cells in CRSa patients express high levels of Bcl2L12. Peripheral naive CD4⁺ T cells were collected from healthy subjects ($n = 10$), CRSa patients ($n = 10$), and CRSna patients ($n = 10$). (A) The dot plots indicate the mRNA levels of Bcl2L12 in CD4⁺ T cells. (B) The immunoblots from 1 experiment indicate the protein levels of Bcl2L12 in CD4⁺ T cells, which represent 10 independent experiments. Bcl2L12 = B cell lymphoma-2-like protein-12; CRSa = chronic rhinosinusitis with nasal allergy; CRSna = chronic rhinosinusitis without nasal allergy; mRNA = messenger RNA.

Peripheral CD4⁺ T cells of CRSa patients express high levels of Bcl2L12

Blood samples were collected from CRS patients and healthy subjects; naive CD4⁺ T cells were isolated from the blood samples by MACS and analyzed by RT-qPCR and Western blotting. The results showed that the expression of Bcl2L12 was detected in CD4⁺ T cells, which was much higher ($p < 0.01$) in the CRSa group than in the CRSna group and the healthy group (Fig. 2). On the other hand, no significant difference was identified in expression of Bcl2L12 in peripheral CD4⁺ T cells collected from CRSa

patients with or without nasal polyps because we analyzed the data separately (not shown).

Bcl2L12 facilitates naive CD4⁺ T cells of CRSa patients to develop into Th2 cells

We further investigated a possible role of Bcl2L12 in facilitating the development of Th2 cells. The peripheral naive CD4⁺ T cells were prepared and were stimulated with anti-CD3/CD28 antibodies in the culture for 72 hours and analyzed by flow cytometry. The results showed that much more IL-4⁺ T cells were induced in the CRSa group than in the CRSna group and healthy group (Fig. 3Aa-c, B), indicating that naive CD4⁺ T cells from the CRSa patients are prone to differentiate into Th2 cells. Considering that Bcl2L12 might play a role in the development of Th2 cells, the Bcl2L12 gene was knocked down by RNAi (Fig. 3C) in naive CD4⁺ T cells collected from CRSa patients. The cells were stimulated with anti-CD3/CD28 antibodies in the culture for 72 hours. As analyzed by flow cytometry, much fewer ($p < 0.01$) IL-4⁺ T cells were generated after the Bcl2L12 RNAi (Fig. 3Ad-e, B). The results suggest that Bcl2L12 contributes to the development of Th2 cells in CRSa patients. To corroborate the results, naive CD4⁺ T cells were collected from healthy subjects. The cells were transfected with Bcl2L12-expressing plasmids to make a Bcl2L12 overexpressing status in the cells (Fig. 3D). The cells were stimulated with anti-CD3/CD28 antibodies for 72 hours. Indeed, much more ($p < 0.01$) IL-4⁺ T cells were generated from the CD4⁺ T cells with Bcl2L12-overexpression (Fig. 3Af-g, B). The results demonstrate that Bcl2L12 plays a critical role in the Th2 cell differentiation.

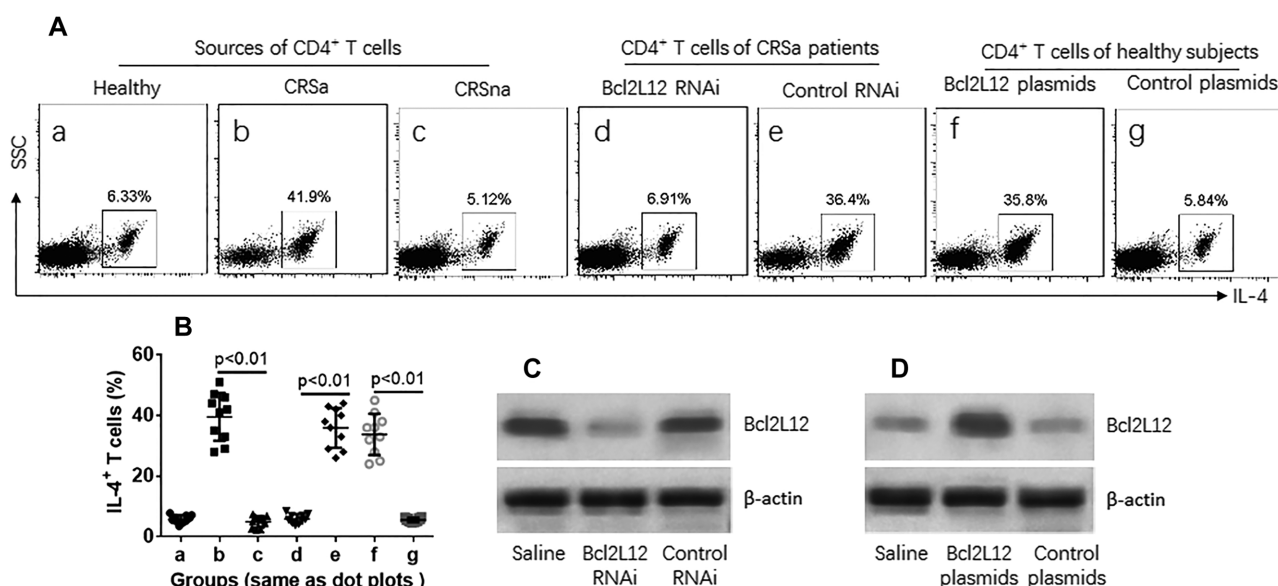


FIGURE 3. Bcl2L12 facilitates Th2 cell development in CRSa patients. Peripheral naive CD4⁺ T cells were collected from healthy subjects ($n = 10$), CRSa patients ($n = 10$), and CRSna patients ($n = 10$). (A) The gated cell population indicates the frequency of IL-4⁺ T cells generated from naive CD4⁺ T cells after the treatment denoted above each subpanel. (B) The dot plots show the summarized data of panel A. (C) The results of Bcl2L12 RNAi in CD4⁺ T cells after transfecting with Bcl2L12 shRNA lentivectors or control lentivectors. (D) The results of Bcl2L12 overexpression in CD4⁺ T cells induced by transfecting with Bcl2L12-expressing plasmids or control plasmids. Bcl2L12 = B cell lymphoma-2-like protein-12; CRSa = chronic rhinosinusitis with nasal allergy; CRSna = chronic rhinosinusitis without nasal allergy; IL-4 = interleukin 4; RNAi = RNA interference; shRNA = short hairpin RNA; SSC = Side scatter; Th2 = T helper 2.

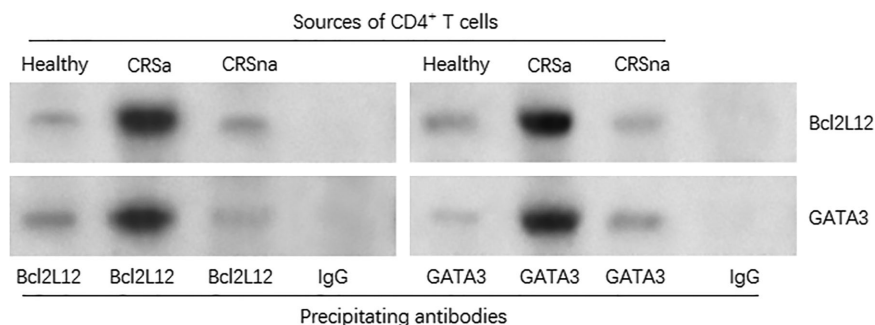


FIGURE 4. A complex of Bcl2L12 and GATA3 in CD4⁺ T cells. Peripheral CD4⁺ T cells were isolated from the blood samples collected from healthy subjects (n = 10), CRSa patients (n = 10), and CRSna patients (n = 10). Cell extracts were prepared with the cells and analyzed by co-IP. The immunoblots show the complex of Bcl2L12 and GATA3 in CD4⁺ T cells. The data represent 10 independent experiments. Bcl2L12 = B cell lymphoma-2-like protein-12; co-IP = co-immunoprecipitation; CRSa = chronic rhinosinusitis with nasal allergy; CRSna = chronic rhinosinusitis without nasal allergy.

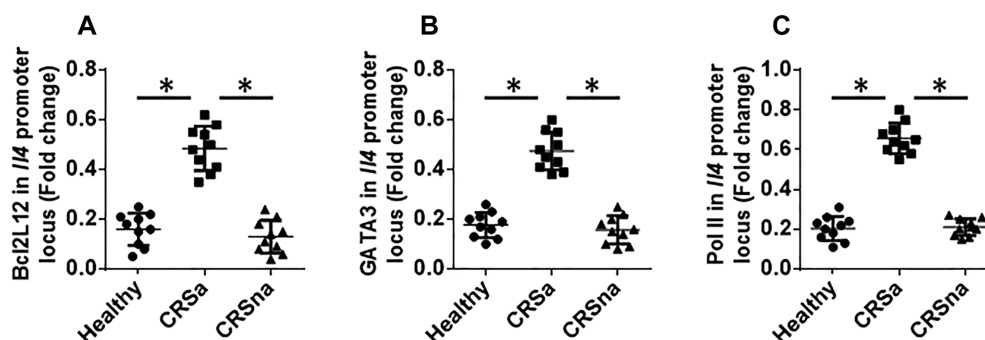


FIGURE 5. High levels of Bcl2L12, GATA3, and Pol II were detected at the *IL4* promoter locus of CD4⁺ T cells in CRSa patients. Peripheral CD4⁺ T cells were isolated from the blood samples collected from healthy subjects (n = 10), CRSa patients (n = 10), and CRSna patients (n = 10). Cell extracts were prepared with the cells and analyzed by ChIP. The dot plots show the levels of Bcl2L12 (A), GATA3 (B), and Pol II (C) at the *IL4* promoter locus. Bcl2L12 = B cell lymphoma-2-like protein-12; ChIP = chromatin immunoprecipitation; CRSa = chronic rhinosinusitis with nasal allergy; CRSna = chronic rhinosinusitis without nasal allergy; Pol II = RNA polymerase II.

Bcl2L12 facilitates IL-4 gene transcription in CD4⁺ T cells

Next, we looked into the mechanism by which Bcl2L12 regulated gene transcription of IL-4 in CD4⁺ T cells. By IP, we found a complex of Bcl2L12 and GATA3, the IL-4 gene transcription factor, in CD4⁺ T cells of CRSa patients, the amounts of which were much higher ($p < 0.01$) than that in the CRSna group and healthy group (Fig. 4). Both Bcl2L12 and GATA3 were detected at the *IL4* promoter locus (Fig. 5A, B), suggesting that the complex of Bcl2L12 and GATA3 moved to the *IL4* promoter locus. We also found that the levels of Pol II at the *IL4* promoter locus were higher ($p < 0.01$) in CD4⁺ T cells of the CRSa group than in the CRSna group and healthy group (Fig. 5C). Since levels of Pol II at promoter loci mirror gene transcription activity, the results suggest that Bcl2L12 may associate with the aberrantly higher expression of IL-4 in CD4⁺ T cells.

To elucidate if the physical contact between Bcl2L12 and GATA3 had any effects on IL-4 gene transcription, EL-4 cells (this cell line is prone to produce IL-4) were activated by exposure to phorbol myristate acetate (PMA) and ionomycin in the culture for 72 hours. The cells were harvested and analyzed by ChIP, RT-qPCR, and Western blotting. The results showed that, upon activation, the

levels of Bcl2L12, GATA3, and Pol II were increased at the *IL4* gene promoter locus (Fig. 6A-C), indicating that amounts of Bcl2L12 at the *IL4* promoter locus were in parallel to *IL4* gene transcription activities. The results imply that Bcl2L12 may be involved in the *IL4* gene transcription process. To test this, we knocked down the Bcl2L12 gene in EL-4 cells by RNAi (Fig. 6D). The Bcl2L12-insufficient EL-4 cells were stimulated with PMA/ionomycin. Indeed, the levels of Bcl2L12, GATA3, and Pol II at the *IL4* promoter locus were significantly lower ($p < 0.01$) in Bcl2L12-insufficient EL-4 cells as compared to wild-type EL-4 cells (Fig. 6A-C). The results were corroborated by analysis of Bcl2L12 mRNA and protein (Fig. 6E, F), which was in parallel to the *IL4* gene transcription activities (Fig. 6A-C). The results indicate that Bcl2L12 can enhance the binding of GATA3 to the *IL4* promoter and enhances *IL4* gene transcription. The absence of Bcl2L12 significantly reduced IL-4 expression, but not completely inhibited it, suggesting that Bcl2L12 is an enhancer of IL-4 expression.

Discussion

This study revealed that Bcl2L12 was detected in the CRSa nasal mucosa, which was positively correlated with the

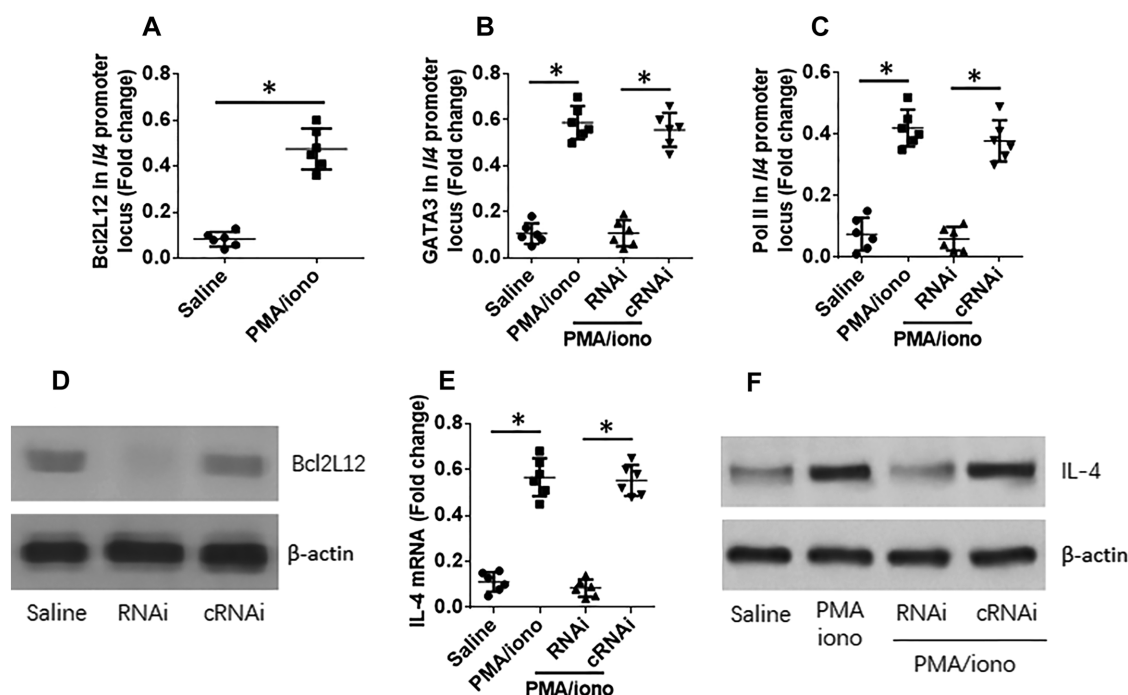


FIGURE 6. Bcl2L12 promotes the *Il4* gene transcription in EL-4 cells. The levels of Bcl2L12 (A), GATA3 (B), and Pol II (C) at the *Il4* promoter locus in EL-4 cells after the treatment denoted on the x-axis. (D) The results of Bcl2L12 knockdown in EL-4 cells. mRNA levels (E) and protein levels (F) of IL-4 in EL-4 cells after the treatment denoted on the x-axis. PMA/iono: EL-4 cells were stimulated with PMA (50 ng/mL) and ionomycin (100 ng/mL) in the culture for 3 days. The data represent 3 independent experiments. Bcl2L12 = B cell lymphoma-2-like protein-12; IL-4 = interleukin 4; mRNA = messenger RNA; PMA = phorbol myristate acetate; Pol II = RNA polymerase II.

Th2 polarization status in local tissue. Naive CD4⁺ T cells collected from CRSa patients were prone to differentiate into Th2 cells, which were Bcl2L12-dependent. The results demonstrate that Bcl2L12 acts as an enhancer in the development of Th2 polarization.

Bcl2L12 is 1 member of the Bcl2 family; it acts as an antiapoptosis protein. Previous studies indicate that Bcl2L12 is highly expressed in glioma cells and confers glioma cells the antiapoptotic property. The subsequent reports indicate that Bcl2L12 is also expressed by other cancer cells, such as leukemia,¹⁷ bladder cancer,¹⁸ and breast cancer.¹⁹ The Bcl2L12 is not only expressed by cancer cells, others found that Bcl2L12 was also expressed in vascular endothelial cells,¹⁵ is associated with cirrhosis,²⁰ and is involved in immune dysregulation.^{12,13} The present study adds novel information to this area that Bcl2L12 contributes to the pathogenesis of Th2 polarization in CRSa.

Our data show a Th2 polarization status in both the peripheral blood system and nasal mucosa of CRSa patients. This phenomenon was also observed by others. Pilette et al.¹⁰ reported that peripheral DCs from AR patients showed low Th1 signals (IL-12 and interferon α [IFN- α]) and IL-10 production and promoted Th2 and Th17 response. Ciprandi et al.²¹ reported high Th2 response in AR patients and suggested that the levels of IL-4 could be a marker in sublingual immunotherapy. Our previous work also showed Th2 polarization in AR patients, which was maintained by T cell immunoglobulin mucin

domain molecule (TIM)1/TIM4 interaction.²² The present data show that the Th2 polarization in CRSa patients, but not in CRSna patients; suggesting that Th2 polarization may not be the major causative factor in the pathogenesis of CRSna, which needs to be further investigated.

The data further show that Bcl2L12 has immune regulation function; it is involved in regulating CD4⁺ T cell activities by enhancing the development of Th2 cells from naive CD4⁺ T cells collected from CRSa patients. Our previous studies also found the immune regulatory function of Bcl2L12. We found that Bcl2L12 interfered with the IL-10 expression by B cells in patients with AR¹² and patients with inflammatory bowel disease.¹³

An immune complex of Bcl2L12 and GATA3 was found in CD4⁺ T cells collected from CRSa patients. Such physical contact between Bcl2L12 and GATA3 resulted in increasing in the binding of GATA3 and the *Il4* promoter in CD4⁺ T cells, and thus enhanced the *Il4* gene transcription and expression. The data demonstrate that Bcl2L12 acts as an enhancer of IL-4 expression in CD4⁺ T cells of patients with CRSa. It is noteworthy that inhibition of Bcl2L12 suppresses the development of Th2 cells with CD4⁺ T cells collected from CRSa patients. The fact suggests that to inhibit Bcl2L12 may have a role to regulate the Th2 polarization in CRSa patients, which needs to be further investigated.

On the other hand, the data show that the expression of Bcl2L12 is significantly higher in CD4⁺ T cells

from CRSa patients than that in CRSn patients. The data imply that Bcl2L12 plays an important role in development of Th2-biased inflammation in CRS; however, the underlying mechanism still needs to be further investigated.

Conclusion

The present data show that Bcl2L12 plays a critical role in the pathogenesis of Th2 polarization in CRSa patients. Bcl2L12 may have the therapeutic potential in regulating the biased Th2 polarization in CRSa. 

References

- Sejima T, Holtappels G, Kikuchi H, Imayoshi S, Ichimura K, Bachert C. Cytokine profiles in Japanese patients with chronic rhinosinusitis. *Allergol Int*. 2012;61:115–122.
- O'Connell BP, Schlosser RJ, Wentzel JL, Nagel W, Mulligan JK. Systemic monocyte-derived dendritic cells and associated Th2 skewing in chronic rhinosinusitis. *Otolaryngol Head Neck Surg*. 2014;150:312–320.
- Miljkovic D, Bassiouni A, Cooksley C, et al. Association between group 2 innate lymphoid cells enrichment, nasal polyps and allergy in chronic rhinosinusitis. *Allergy*. 2014;69:1154–1161.
- Luo B, Feng L, Jintao D, et al. Immunopathology features of chronic rhinosinusitis in high-altitude dwelling Tibetans. *Allergy Rhinol (Providence)*. 2013;4:e69–e76.
- Han JK. Subclassification of chronic rhinosinusitis. *Laryngoscope*. 2013;123(Suppl 2):S15–S27.
- Bachert C, Zhang L, Gevaert P. Current and future treatment options for adult chronic rhinosinusitis: focus on nasal polyposis. *J Allergy Clin Immunol*. 2015;136:1431–1440.
- Henry EK, Inclan-Rico JM, Siracusa MC. Type 2 cytokine responses: regulating immunity to helminth parasites and allergic inflammation. *Curr Pharmacol Rep*. 2017;3:346–359.
- Mazzarella G, Bianco A, Catena E, De Palma R, Abbate GF. Th1/Th2 lymphocyte polarization in asthma. *Allergy*. 2000;55(Suppl 61):6–9.
- Neurath MF, Finotto S, Glimcher LH. The role of Th1/Th2 polarization in mucosal immunity. *Nat Med*. 2002;8:567–573.
- Pilette C, Jacobson MR, Ratajczak C, et al. Aberrant dendritic cell function conditions Th2-cell polarization in allergic rhinitis. *Allergy*. 2013;68:312–321.
- Barnes PJ. Role of GATA-3 in allergic diseases. *Curr Mol Med*. 2008;8:330–334.
- Xue JM, Yang LT, Yang G, et al. Protease-activated receptor-2 suppresses interleukin (IL)-10 expression in B cells via upregulating Bcl2L12 in patients with allergic rhinitis. *Allergy*. 2017;72:1704–1712.
- Guo X, Li MG, Li SS, Liu FH, Liu ZJ, Yang PC. Tumor necrosis factor suppresses interleukin 10 in peripheral B cells via upregulating Bcl2-like protein 12 in patients with inflammatory bowel disease. *Cell Biochem Funct*. 2017;35:77–82.
- Stegh AH, DePinho RA. Beyond effector caspase inhibition: Bcl2L12 neutralizes p53 signaling in glioblastoma. *Cell Cycle*. 2011;10:33–38.
- Teng W, Qiu C, He Z, Wang G, Xue Y, Hui X. Linc00152 suppresses apoptosis and promotes migration by sponging miR-4767 in vascular endothelial cells. *Oncotarget*. 2017;8:85014–85023.
- Szrentec S, Nikcevic G, Spasovski D, et al. Predictive genetic markers of coagulation, inflammation and apoptosis in Perthes disease-Serbian experience. *Eur J Pediatr*. 2015;174:1085–1092.
- Nishioka C, Ikezoe T, Takeuchi A, Nobumoto A, Tsuda M, Yokoyama A. The novel function of CD82 and its impact on BCL2L12 via AKT/STAT5 signal pathway in acute myelogenous leukemia cells. *Leukemia*. 2015;29:2296–2306.
- Foutadakis S, Aygeris M, Tokas T, Stravodimos K, Scorilas A. Increased BCL2L12 expression predicts the short-term relapse of patients with TaT1 bladder cancer following transurethral resection of bladder tumors. *Urol Oncol*. 2014;32:39.e29–39.e36.
- Tzouvaras A, Kladi-Skandali A, Michaelidou K, et al. BCL2L12: a promising molecular prognostic biomarker in breast cancer. *Clin Biochem*. 2014;47:257–262.
- Real LM, Caruz A, Rivero-Juarez A, et al. A polymorphism linked to RRAS, SCAF1, IRF3 and BCL2L12 genes is associated with cirrhosis in hepatitis C virus carriers. *Liver Int*. 2014;34:558–566.
- Ciprandi G, De Amici M, Murdaca G, et al. Serum IL-4 as a marker of immunological response to sublingual immunotherapy. *J Biol Regul Homeost Agents*. 2008;22:117–123.
- Zhao CQ, Li TL, He SH, et al. Specific immunotherapy suppresses Th2 responses via modulating TIM1/TIM4 interaction on dendritic cells. *Allergy*. 2010;65:986–995.