

# B Cell Immunity in Allergic Nasal Mucosa Induces T helper 2 Cell Differentiation

Shuqi Qiu · Yun Du · Xiaobei Duan · Xiaorui Geng ·  
Jianxiong Xie · Han Gao · Ping-Chang Yang

Received: 5 December 2011 / Accepted: 17 February 2012  
© Springer Science+Business Media, LLC 2012

## Abstract

**Background** The pathogenesis of allergic diseases is to be further understood. Recent studies indicate that B cells are involved in the immune regulation. The present study aimed to investigate the role of B cells in the initiation of skewed T helper (Th)2 polarization.

**Methods** The surgically removed nasal mucosal specimens from 24 patients with allergic rhinitis (AR) and 22 patients with non-AR (nAR) were collected. B cells isolated from the AR nasal mucosa were characterized. The effect of B cells on inducing naïve CD4<sup>+</sup> T cells to

differentiate into Th2 cells was evaluated with a cell culture model.

**Results** Abundant B cells were detected in the nasal mucosa of patients with AR, which also expressed high levels of T cell immunoglobulin mucin domain (TIM)4 and costimulatory molecules. High levels of Staphylococcal enterotoxin B (SEB) were detected in the AR nasal mucosa. Expression of TIM4 could be induced in naïve B cells in the presence of SEB in culture. TIM4<sup>+</sup> B cells could induce naïve CD4<sup>+</sup> T cells to differentiate into Th2 cells.

**Conclusions** TIM4<sup>+</sup> B cells from AR nasal mucosa can induce skewed Th2 polarization. It may be a potential therapeutic target in the treatment of AR.

**Capsule summary** B cells plays an important role in the initiation of Th2 polarization

## Key Messages

- High frequency of B cells exists in nasal mucosa of allergic rhinitis
- These B cells express high levels of TIM4
- TIM4<sup>+</sup> B cells can initiate the skewed Th2 polarization

**Keywords** B lymphocyte · allergy · Th2 polarization · nasal mucosa · rhinitis

Shuqi Qiu and Yun Du equally contributed to this work and are the joined first authors

S. Qiu (✉) · X. Duan · X. Geng · H. Gao  
Shenzhen ENT Institute,  
Shenzhen, Guangzhou, China  
e-mail: qiuqi66858@163.com

S. Qiu · X. Duan · X. Geng · J. Xie · H. Gao  
Shenzhen Longgang Central Hospital,  
Shenzhen, Guangzhou, China

Y. Du  
Department of Pathology, Hebei Medical University,  
Shijiazhuang, China

P.-C. Yang  
Department of Pathology & Molecular Medicine,  
McMaster University,  
Hamilton, Canada

P.-C. Yang (✉)  
Room T3303, 50 Charlton Ave East,  
Hamilton, ON, Canada L8N 3Z5  
e-mail: yangp@mcmaster.ca

## Abbreviations

|          |                                       |
|----------|---------------------------------------|
| AR       | Allergic rhinitis                     |
| DC       | Dendritic cell                        |
| Th2 cell | T helper type 2 cell                  |
| CFSE     | Carboxyfluorescein succinimidyl ester |
| BSA      | Bovine serum albumin                  |

## Introduction

The role of B cells in the pathogenesis of allergic disorders has been noted recently [1, 2]. The underlying mechanism is not fully understood yet. B cells can capture antigens from the microenvironment, process the antigen and present to T cells to regulate the T cell's function [1]. Furthermore, B cells also express the costimulatory molecules, such as CD40 [3], CD80 and CD86 [4], that facilitate the antigen presentation by B cells. Yet, whether B cells contribute to the skewed T helper (Th)2 response are not fully understood.

The prevalence of allergic diseases is more than 20% of the population in the world. The mechanism of allergy is incompletely understood. It is clear that the major change in immune pathology of allergy is a skewed Th2 polarization [5, 6]. However, what causes such a skewed Th2 polarization is unknown. A number of previous studies indicate that Staphylococcal enterotoxin B (SEB) has a correlation with airway allergy [7–9]. Dendritic cells (DC) in the subepithelial region can recognize SEB via Toll like receptor (TLR) 2 [10, 11]. SEB-pulsed DCs produce T cell immunoglobulin mucin domain molecule (TIM)4 [12, 13]; the latter binds TIM1 on naïve CD4+ T cells to drive them to differentiate into Th2 cells [13, 14]. Since immature B cells have similar antigen presentation properties to DCs in the context of antigen presentation [15], we hypothesize that SEB may induce B cells to produce TIM4; the latter drives naïve T cells to become Th2 cells and to further induce the skewed Th2 polarization. In this study, we observed abundant CD19+ B cells and high levels of SEB in the nasal mucosa of patients with nasal allergy. Exposure to SEB induced naïve B cells to express TIM4 and costimulatory molecules. SEB-pulsed B cells could induce Th2 cell differentiation.

## Materials and Methods

### Patients

Patients with hypertrophic inferior turbinate together with or without allergic rhinitis (AR) were recruited into this study. AR was diagnosed following the criteria in our department that was also published elsewhere [16]. All the patients had the long term nasal obstruction; had been treated with multiple remedies without satisfactory results and asked for surgical treatment. The demographic data were presented in Table I. The skin allergen test results were presented in Table II. All the patients did not use any anti-allergy medicines at least 2 weeks before the surgery. Non-AR (nAR) patients included those with hypertrophic chronic rhinitis with or without sinusitis, but no AR (all control patients showed negative skin allergen test, undetectable serum antigen-specific IgE/IgG1, nasal secretion MBP and tryptase). All the patients were undergone partially inferior turbinectomy.

**Table I** Demographic data

|                  | AR               | nAR       |
|------------------|------------------|-----------|
| Male             | 10               | 12        |
| Female           | 14               | 10        |
| Age              | 31.6±8.3         | 29.5±11.2 |
| Disease history  | 5.2±2.8          | 6.6±3.5   |
| Surgical history | None             | None      |
| Sinusitis        |                  |           |
| Maxillary        | 21               | 19        |
| Ethmoid          | 11               | 12        |
| Frontal          | 6                | 5         |
| Sphenoid         | 1                | 1         |
| Specific IgE     | 35.6±11.5 ng/ml  | ND        |
| Specific IgG1    | 29.3±13.8 ng/ml  | ND        |
| Nasal MBP        | 58.6±22.4 pg/ml  | ND        |
| Nasal tryptase   | 128.6±44.8 pg/ml | ND        |

*Specific antigen* All AR patients were sensitized to one to several allergens, *Nasal MBP* The levels of major basic protein of extracted proteins from nasal mucosa (determined by ELISA), *Nasal tryptase* The levels of tryptase in extracted proteins from nasal mucosa (determined by ELISA). *ND* Non-detectable

The using human tissue in research was approved by the Human Research Ethic Committee at the Longgang Central Hospital. Informed consent was obtained from each patient.

### Reagents

Antibodies of SEB, TIM1 and TIM4 were purchased from Santa Cruz (Santa Cruz, CA). Fluorescence labeled antibodies of CD19, CD38, CD11c, CD4, CD80, CD86 and IL-4 were purchased from BD Biotect (Shanghai, China). RNA extraction kit, cDNA synthesis kit and Qiagen PCR kit were purchased from Qiagen (Shanghai, China). ELISA kits of IL-4 and SEB were purchased from R&D Systems (Shanghai, China). Immune cell isolating reagent kits were purchased from Miltenyi Biotec (Auburn, CA). Endotoxin levels in all reagents were detected using the Limulus assay (Limulus amoebocyte lysate QCL 1000, Bio Whittaker, Walkersville, MD, USA). The reagents used in this study contained <0.2U endotoxin/10 µg reagents.

**Table II** Positive antigens from skin test

| Allergens    | Positive cases | %     |
|--------------|----------------|-------|
| Dust mite    | 24             | 100   |
| Cat debris   | 8              | 33.33 |
| Dog debris   | 6              | 25    |
| Grass pollen | 12             | 50    |
| Ragweed      | 9              | 37.5  |
| Cockroach    | 8              | 33.33 |

## Immune Cell Isolation from Surgically Removed Nasal Mucosa

Single cells were isolated from the surgically removed nasal mucosa of the inferior turbinate following published procedures [11]. Briefly, surgically removed nasal mucosa were cut into small pieces and treated with the predigestion solution ( $1 \times$  HBSS containing 5 mM EDTA and 1 mM DTT) at 37°C for 30 min and then incubated in the digestion solution (Dissolve 50  $\mu$ g collagenase D, 50  $\mu$ g DNase I and 0.3 g dispase II in 100 ml PBS) at 37°C for 60 min under slow rotation. Cells were filtered with a cell strainer. Mononuclear cells were further isolated from the single cells by the gradient density centrifugation.

### Peripheral Mononuclear Cell Preparation

About 30–40 ml elbow vein blood was collected from each healthy volunteer. Mononuclear cells were isolated by gradient density centrifugation. CD19<sup>+</sup> CD11c<sup>-</sup> B cells and CD4<sup>+</sup> CD25<sup>-</sup> T cells were isolated by magnetic cell sorting (MACS) respectively with commercial reagent kits following the manufacturer's instruction. The isolated cells were used for further experiments.

### Generation of the Expression of TIM4 by B Cells

Human B cells were cultured ( $10^4$  cells/ml) in completed RPMI1640 medium in the presence of SEB (0–100 ng/ml) at 37°C. The culture medium and reagents were changed every 3-day. Cells were collected on day 12 and used for further experiments.

### Generation of Th2 Cells

Naïve human CD4<sup>+</sup> CD25<sup>-</sup> T cells ( $10^4$  cells/ml) were cultured with generated TIM4<sup>+</sup> B cells ( $10^4$  cells/ml; pulsed by SEB) (or surgically removed nasal mucosa-isolated B cells) in RPMI1640 medium. Nine days later, the cells were collected to assess the frequency of Th2 cells (see below).

### Flow Cytometry

Cells were incubated with the primary antibodies (fluorescence-labeled; 1–6 antibodies could be stained concurrently; the concentrations of antibodies were 0.5 to 1  $\mu$ g/ml) on ice for 30 min (In the case of the intracellular staining, cells were fixed with 1% paraformaldehyde on ice for 30 min and incubated with permeabilization reagents for 30 min on ice). The stained cells were analyzed using a flow cytometer (Cando; BD Bioscience, San Jose, CA). The data were analyzed by software FlowJo. Positively stained cells were calculated by gating technique; one to six cell markers were

analyzed and recorded, and expressed as a percentage of the total cells.

### Protein Extraction

Surgically removed nasal mucosa was cut into small pieces and homogenated in lysis buffer at 4°C; the samples were kept at 4°C for 30 min. After centrifuged ( $17,600 \times g$ ) at 4°C for 20 min, the supernatant was collected. The protein contents were determined by Bradford assay.

### Western Blotting

Equal volume (40  $\mu$ g/well) of proteins was added to each well and separated using a precast NuPAGE gel system and blotted onto nitrocellulose membrane. The membrane was incubated with primary antibody (0.05–0.1  $\mu$ g/ml) in 5% nonfat milk in TBS at 4°C overnight. This was followed by incubation with HRP-conjugated second antibody for 1 h at room temperature with shaking. Reactions were developed using the Pierce ECL chemiluminescence substrate kit. Results were recorded with X ray film.

### Enzyme-linked Immunoassay (ELISA)

Levels of SEB, IL-4, IL-5 and IL-13 in the extracted protein of nasal mucosa or culture supernatants were determined by ELISA with commercial reagent kit following the manufacturer's instruction.

### Real-time Quantitative RT-PCR (qRT-PCR)

The expression of TIM4 in B cells was determined by qRT-PCR. 25 ng cDNA were amplified in 50  $\mu$ l  $1 \times$  SYBR-Green I PCR master mix, containing 200 nM primer for TIM4. Thermal cycling conditions were: 60°C for 2 min, 95°C for 10 min, 33 cycles of denaturation at 95°C for 15 s, and annealing/extension at 59.6°C for 1 min. All the experiments were performed in triplicate, using the qRT-PCR thermal cycler (Bio-Rad), and negative controls (master mix containing untranscribed total RNA or without any cDNA or RNA) were added to each experiment. The quantification was obtained using the relative standard curve method. The standard curve was created by amplification of 5  $\mu$ l control total RNA extracted from the cells at different dilutions (1.0–0.025 ng/ $\mu$ l by two-fold dilution). The relative amount of each unknown sample was calculated using the linear regression from the respective standard curve. The relative TIM4 gene expression value was normalized by dividing the target value by the housekeeping gene value. The primers of TIM4 using in this study were: Forward: gaaactgtctctcccagtg; reverse: ccaggctgaggagaagacac

(NCBI: NM\_138379; start code: 742; end code: 899; size: 158 bp).

### RNA Interference (RNAi) of TIM1

The mouse TIM1 shRNA carried by lentiviral vectors and control shRNA were purchased from Santa Cruz Biotech. A batch of naïve CD4<sup>+</sup> CD25<sup>-</sup> T cells were transduced with TIM1 shRNA or control shRNA following the manufacturer's instruction. The T cells were then activated in culture in anti-CD3/CD28 coated plates in order to assess the effect of RNAi of TIM1. As assessed by Western blotting, the TIM1 gene knockdown effect was reached to 90% at 48 h after the transduction. Transduction with control shRNA did not affect the expression of TIM4.

### Statistics

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

## Results

### CD19<sup>+</sup> B Cells are Increased in the Nasal Mucosa of AR Patients

Mononuclear cells were isolated from surgically removed nasal mucosa and analyzed by flow cytometry. Markedly more CD19<sup>+</sup> B cells were detected in the mononuclear cells isolated from AR nasal mucosa than that from the nAR nasal mucosa (Fig. 1a–d). The CD19<sup>+</sup> B cells were not plasma cells as they were CD38<sup>-</sup> (Fig. 1e–f). The results indicate that the frequency of B cell is increased in the AR nasal mucosa.

### The CD19<sup>+</sup> B Cells Express TIM4 in the Nasal Mucosa of AR Patients

TIM4 is expressed by activated dendritic cells and plays a critical role in Th2 cell differentiation [13, 14]. Since B cells have some similar features of dendritic cells, such as antigen presentation and regulating T cell activities, we postulate that B cells may also express TIM4. Thus, with the same batch of isolated cells in Fig. 1, we assessed the expression of TIM4. As shown by flow cytometry, TIM4<sup>+</sup> CD19<sup>+</sup> B cells were detected in the cells isolated from the AR nasal mucosa that were significantly more than that from the nAR

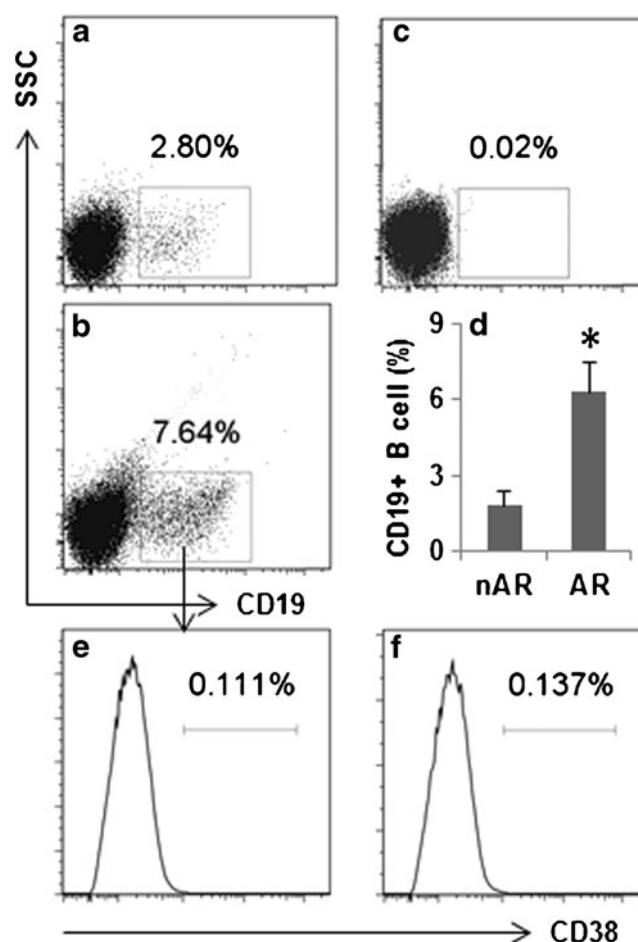


**Fig. 1** The number of B cells is increased in the AR nasal mucosa. Mononuclear cells were isolated from surgically removed nasal mucosa specimens, including 16 Non-AR patients (a; nAR) and 18 AR patients (b). The cells were stained with antibodies of CD19 and analyzed by flow cytometry. The representative dot plots show the frequency of CD19<sup>+</sup> B cells. C is an isotype control. The bars indicate the summarized data of A and B (mean  $\pm$  SD). \*,  $p < 0.01$ , compared with the nAR group. E, the histogram indicates the frequency of CD38<sup>+</sup> B cells in the gated CD19<sup>+</sup> B cells in panel C. F is an isotype control

nasal mucosa (Fig. 2a). Further analysis showed that the CD19<sup>+</sup> TIM4<sup>+</sup> B cells express high levels of CD80 (Fig. 2b) and CD86 (Fig. 2c). We then examined the frequency of CD19<sup>+</sup> TIM4<sup>+</sup> B cells in the peripheral mononuclear cells by flow cytometry. The results showed that the frequency of CD19<sup>+</sup> TIM4<sup>+</sup> B cells was below detectable levels in nAR subjects; 1.55 $\pm$ 0.68 CD19<sup>+</sup> B cells expressed TIM4 were detected in AR patients.

### The Frequency of TIM4<sup>+</sup> B Cells is Correlated with the Levels of SEB in the AR Nasal Mucosa

It is proposed that SEB plays a role in the pathogenesis of allergic disorders [17]. Whether the increase in TIM4<sup>+</sup> B cells has any association with SEB in the nasal mucosa is

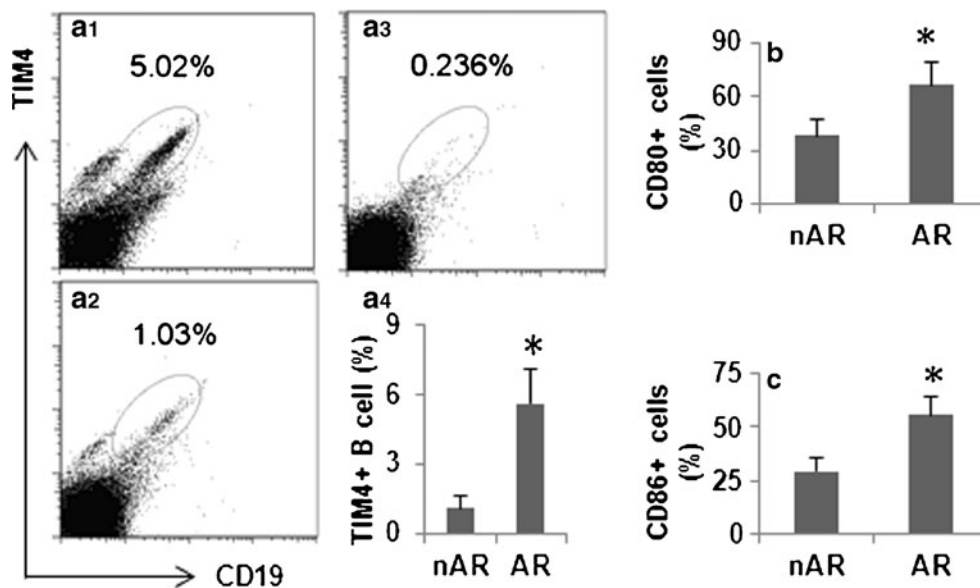


**Fig. 2** TIM4+ B cells are increased in the AR nasal mucosa. A portion of the isolated mononuclear cells in Fig. 1 was stained with antibodies of TIM4, CD19, CD80 and CD86, and analyzed by flow cytometry. A, the representative dot plots show the frequency of the TIM4+ CD19+ B cells (the gated cells) among the mononuclear cells of the specimens of AR (A1) and nAR (A2). A3 is an isotype control. A4, the bars

indicate the summarized data of A1 and A2. With gating technique, the TIM4+ CD19+ B cells in gated cells in panel A and B were further analyzed to show the frequencies of CD80+ cells (B) and CD86+ cells (C). The data in bar graphs are presented as mean  $\pm$  SD; \*,  $p < 0.01$ , compared with the nAR group

not clear. We thus measured the levels of SEB in the protein extracts from the surgically removed nasal mucosa of the same batch of specimens of Fig. 1. As shown by ELISA, SEB was detected in the AR nasal mucosa that was significantly more than nAR nasal mucosa (Fig. 3). There was also a positive correlation between SEB levels and the frequency of TIM4+ B cells ( $r = 0.596$ ,  $p < 0.05$ ). The results imply that SEB may play a role in the increase in TIM4+ B cells in the AR nasal mucosa.

#### SEB Induces CD19+ CD38- B Cells to Express TIM4

The results in Fig. 3 imply that SEB may be the causative factor for the increase of the frequency of the TIM4+ B cells in the AR nasal mucosa. To test the hypothesis, we isolated CD19+ CD38- B cells from peripheral mononuclear cells and cultured in the presence of SEB for 7 days. As shown by flow cytometry, abundant TIM4+ cells were induced in the CD19+ CD38- B cells; the results were confirmed by the data from qRT-PCR and Western blotting (Fig. 4). The results indicate that SEB can induce the TIM4 expression in B cells in the nasal mucosa.

#### SEB-conditioned B Cells Induce Th2 Cell Differentiation

We and others have reported that TIM4 plays a role in the induction of skewed Th2 polarization [13, 14]; Th2 polarization plays an important role in the pathogenesis of allergic disorders. To investigate the effect of B cells in the induction

of Th2 cells, we isolated peripheral CD19+ CD38- B cells to be conditioned in the culture in the presence of SEB for 12 days. We then collected the B cells; a portion of the cells

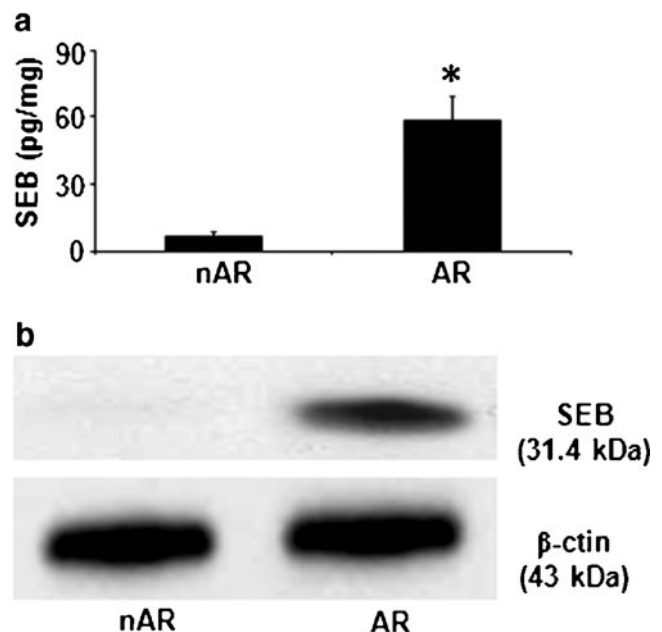


**Fig. 3** SEB levels in the nasal mucosa. Proteins were extracted from each surgically removed nasal mucosal specimen (20 AR, 20 nAR patients) and analyzed by ELISA and Western blotting. A, the bars indicate the levels of SEB in the nasal mucosa (The data were presented as mean  $\pm$  SD; \*,  $p < 0.01$ , compared with nAR group). B, the representative immunoblots indicate the levels of SEB in the protein extracts of nasal mucosa

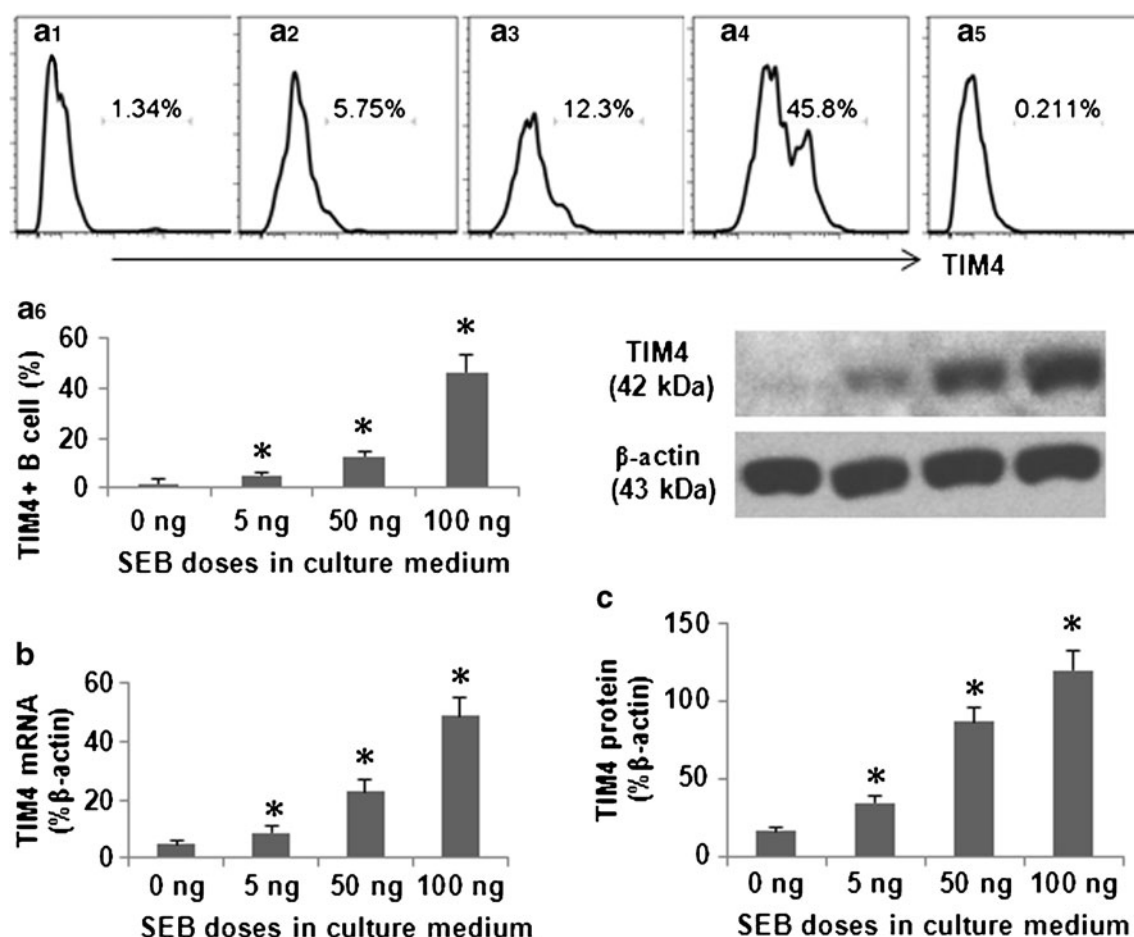


**Fig. 4** SEB induces TIM4 expression in B cells. Peripheral CD19+ CD20+ CD38- B cells were isolated from six healthy subjects by MACS. The B cells were cultured in the presence of SEB at a dose of 0–100 ng/ml for 12 days. Cells were collected to analyze the expression of TIM4 by flow cytometry. A, the representative histograms show the frequency of TIM4+ B cells at SEB doses of 0 ng/ml (A1), 5 ng/ml (A2), 50 ng/ml (A3) and 100 ng/ml (A4) respectively. A5 is an isotype control. A6, the bars indicate the summarized data

from 3 experiments of A1–A4. A portion of B cells was analyzed by qRT-PCR (B) and Western blotting (C). B, the bars indicate the levels of TIM4 mRNA in B cells. C, the immune blots show the TIM4 protein levels in B cells. The bars below the gel graph show the summarized data of the integrated density of the immune blots from 3 experiments. The data in bar graphs were presented as mean  $\pm$  SD; \*,  $p < 0.01$ , compared with the “0 ng” group

was checked by flow cytometry to ensure the expression of TIM4; high levels of TIM4 ( $86.3\% \pm 6.8\%$ ) were detected in the B cells. The conditioned B cells were also expressed high levels of CD80 ( $88.4\% \pm 5.5\%$ ) and CD86 ( $71.9\% \pm 9.2\%$ ). The TIM4+ B cells were cultured with naïve peripheral CD4+ CD25- T cells (isolated by MACS) for 9 days. As analyzed by flow cytometry, significant increase in IL-4+ cells was detected in CD4+ T cells; the cytokines of IL-4, IL-5 and IL-13 were also markedly increased in the supernatants of the T cells cultured with conditioned B cells. To confirm the results, we knocked down the gene of TIM1 (the receptor of TIM4 [14]) in a batch of naïve T cells with shRNA of TIM1. The TIM1-deficient CD4+ T cells did not respond to the stimulation of TIM4+ B cells (Fig. 5). The results indicate that the conditioned B cells can convert naïve CD4+ T cells to Th2 cells. In addition, we also observed several other cytokines in the culture media.

Although these cytokines play important role in other immune reactions, the results showed no significant difference between AR group and nAR group (Table III). Using as negative controls, some B cells were stimulated by bovine serum albumin. The results showed no detectable response in the B cells was induced (data not shown).

#### B Cells from the Allergic Nasal Mucosa Induce Th2 Cell Differentiation

To confirm the results of Fig. 5, we isolated B cells from the surgically removed nasal mucosa and cocultured the B cells with naïve CD4+ CD25- T cells for 9 days. As shown by flow cytometry, abundant IL-4+ CD4+ T cells were induced in those cocultured with the CD19+ B cells from the AR mucosa, which was much more than those cocultured with the CD19+ B cells from the nAR mucosa (Fig. 6).

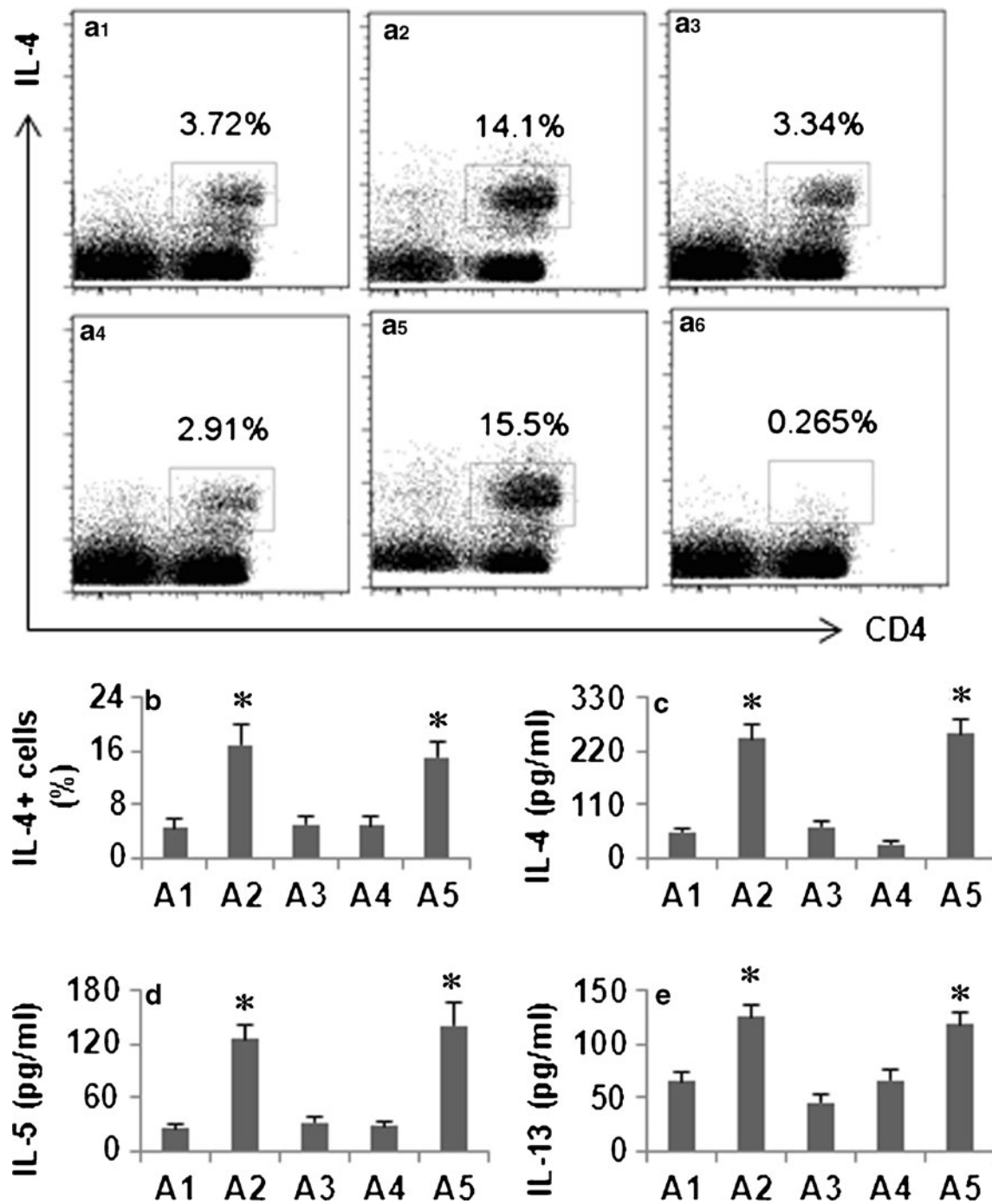


**Fig. 5** SEB-pulsed B cells induce Th2 cell development. Peripheral B cells were purified from isolated mononuclear cells by MACS. The B cells were conditioned by SEB (100 ng/ml) in culture for 12 days or by medium alone. The conditioned B cells were cultured with naïve CD4<sup>+</sup> CD25<sup>-</sup> T cells (B cell:T cell=1:1) for 3 rounds (3 days/round). Cells were collected and stained with antibodies of IL-4 and CD4, and analyzed by flow cytometry. A, the representative dot plots indicate the frequency of CD4<sup>+</sup> IL-4<sup>+</sup> T cells among: A1, T cells were cultured alone; A2, T cells were cultured with SEB-conditioned B cells; A3, T

cells were cultured with medium-conditioned B cells; A4, TIM1-deficient T cells were cultured with SEB-conditioned B cells and A5, control shRNA-treated T cells were cultured with SEB-conditioned B cells. A6 is an isotype control. B, the bars indicate the summarized data of panel A. C-E, the bars indicate the levels of IL-4 (C), IL-5 (D) and IL-13 (E) in supernatants that were determined by ELISA. The data in bar graphs were presented as mean  $\pm$  SD from 3 experiments. \*,  $p < 0.01$ , compared with group A1

**Table III** Levels of cytokine in culture

| Cytokine | AR             | nAR            |
|----------|----------------|----------------|
| IL-10    | 35.6±5.3       | 38.5±4.9       |
| IFN-g    | 24.3 ±         | 23.3±4.2       |
| TNF-a    | 19.5±3.2       | 23.2±5.4       |
| TGF-b    | Not detectable | Not detectable |

## Discussion

In this study, abundant non-plasma B cells were detected in the nasal mucosa of patients with AR. The B cells expressed high levels of TIM4 and costimulatory molecules, CD80

and CD86. The frequency of TIM4<sup>+</sup> B cells was correlated with the levels of SEB in the nasal mucosa. SEB-pulsed B cells could induce Th2 cell differentiation.

The role of B cells in the pathogenesis of allergic diseases has been recognized [1]. Previous reports suggest that the B cells contribute to allergic diseases mainly via producing IgE [18] since the major inflammatory cells at sites are mast cells, which bear the high affinity receptor of IgE [19]. Recent studies have unraveled that besides producing IgE, B cells were also involved in the immune regulatory responses during allergic reactions in animal studies [1]. The present study has provided human data that B cells accumulated on the sites of allergic reactions. Moreover, these B cells express CD19, but not CD38, which indicates that these B cells are not plasma

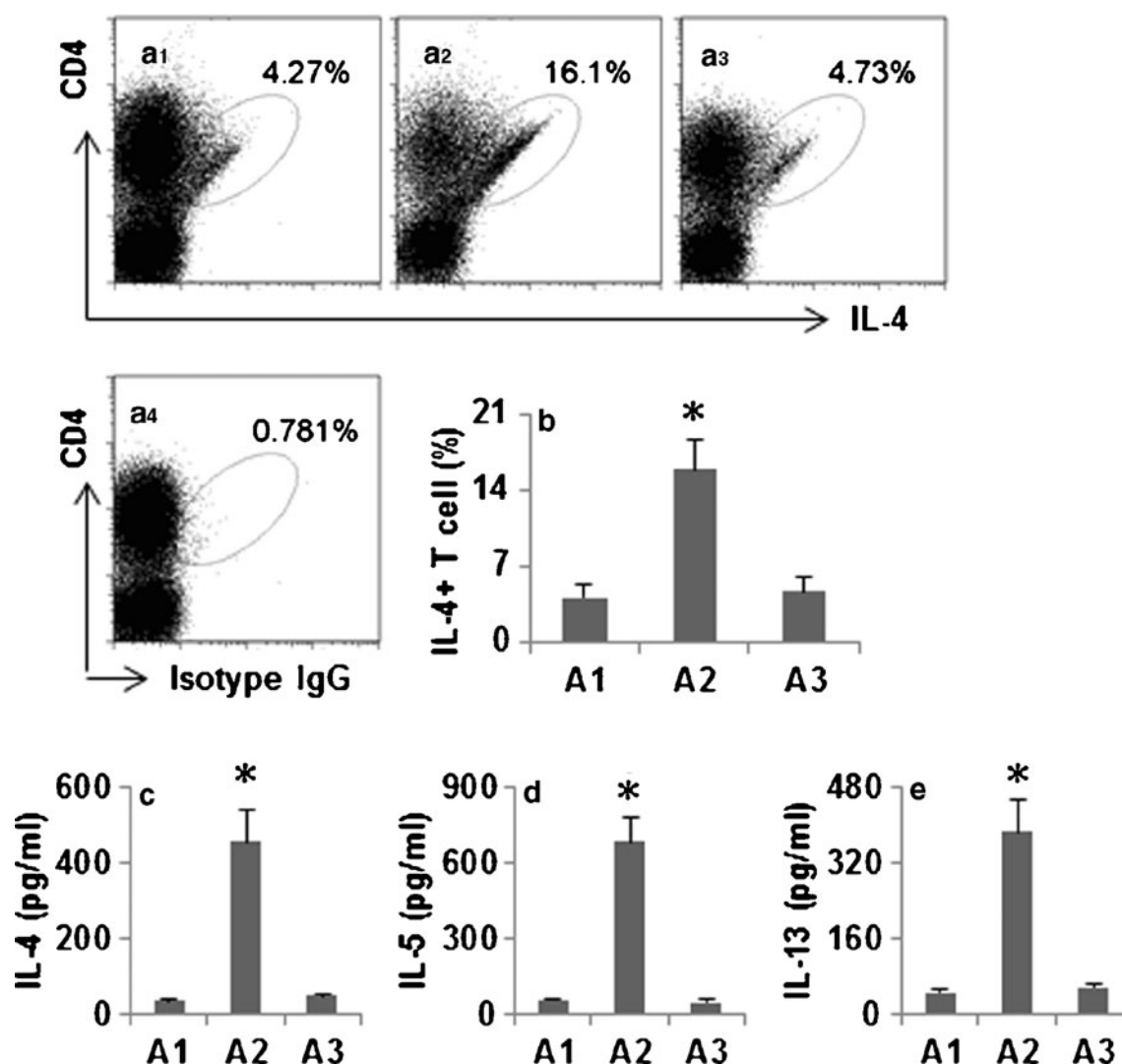


**Fig. 6** AR nasal mucosa-derived B cells induce Th2 cell development. CD19<sup>+</sup> CD38<sup>-</sup> B cells were isolated from the surgically removed nasal mucosa from 6 AR patients and 6 nAR patients respectively. The B cells were cocultured with naïve CD4<sup>+</sup> CD25<sup>-</sup> T cells (isolated from the peripheral blood) at a ratio of 1:1 for 9 days. The cells were analyzed by flow cytometry. A, the representative dot plots show the

frequency of IL-4<sup>+</sup> CD4<sup>+</sup> T cells. A1, T cells were cultured alone. A2, AR-mucosa-derived B cells were cocultured with naïve T cells. A3, nAR-mucosa-derived B cells were cocultured with naïve T cells. A4 is an isotype control. B, the bars show the summarized data of panel A. C-E, the bars indicate the levels of IL-4 (C), IL-5 (D) and IL-13 (E) in the culture supernatant that were determined by ELISA

cells. Our data imply that these B cells accumulating on allergic reaction sites of the nasal mucosa may be involved in immune regulation; the inference is in line with previous reports, in which the authors indicated that B cells could promote Th2 responses [20]. The increases in non-plasma B cells may be referred by clinical immunologists in the diagnosis of upper airway allergic disorders.

TIM4 is a newly described molecule [14] that is expressed by activated DCs [13, 21]. One of the functions of TIM4 is to initiate the Th2 cell differentiation [12, 14] to contribute to the initiation of allergic diseases via interacting with TIM1 on naïve T cells [13, 14]. Apart from DC, here we have newly found that CD19+ B cells also produce TIM4 in the AR nasal mucosa. The data indicate that the B cells we observed in the AR nasal mucosa have the potential to induce skewed Th2 response. Since the CD19+ B cells were mainly recognized in the patients with AR, but not in nAR, the phenomenon may be a pathological sign in AR.

Under physiological conditions, the invaded antigens can be captured by either DCs or B cells or macrophages. After processing, the cells present antigen information together with costimulatory molecules to T cells, either induce naïve T cells to differentiate into Th1 or Th2 cells or activate antigen specific T cells. However, it is still an enigma how the skewed Th2 polarization initiates and how allergic disorders develop eventually. The present study has revealed a previously undescribed phenomenon that a relatively large number of TIM4+ B cells in the nasal mucosa of AR patients. Since TIM4 plays a role in the initiation of Th2 cell differentiation as we and others reported [13, 14], the TIM4+ B cells may be involved in the initiation of skewed Th2 polarization in AR. To prove this inference, further clinical observation is needed, such as observe the frequency of TIM+ B cells in the nasal mucosa before and after specific immunotherapy.

In the present study, the TIM4+ B cells also expressed high levels of CD80 and CD86, which indicate that those B cells have the potential to activate naïve CD4+ T cells to differentiate into either Th1 or Th2 cells. The subsequent results addressed the postulation by showing that in co-culturing with TIM4+ B cells induced abundant IL-4+ expression in TIM1-sufficient CD4+ T cells, but not in TIM1-deficient CD4+ T cells. The results are in line with previous studies [13, 14] in which the interaction of TIM1 and TIM4 plays a critical role in the development of Th2 polarization in animal model studies. Whether this phenomenon also occurs in human AR needs additional clinical studies in AR patients.

In the TIM1-driven Th2 cell differentiation, whether is the TIM4-bearing cell physically contacting the naïve CD4+ T cell necessary? We observed this point in separate experiments with transwell system. The results showed there was no difference between the culture systems with or without transwells (data not shown). Also, the TIM4 could be detected in culture media [13].

In the present study, we observed that SEB could modify the properties of B cells. Since SEB is a superantigen; it has many more bioactivities in the regulation of immune responses; such as directly bind to the T cell receptors to activate T cells. Our previous study also showed that SEB could induce cytotoxic T cell differentiation [22]; suppressed the expression of integrin  $\alpha\text{v}\beta 6$  in the nasal mucosa and resulted in breaching the immune tolerance in the nasal mucosa [23]. These data demonstrate that SEB has a critical role in the compromising the immune homeostasis of the nasal mucosa.

In summary, the present study reports a subpopulation of B cells in the AR nasal mucosa that express TIM4 and costimulatory molecules, can present antigen to naïve CD4+ T cells to induce Th2 cell differentiation. We conclude that TIM4+ CD19+ B cells are a specific cell population that plays an important role in the pathogenesis of AR. The TIM4+ CD19+ B cells may be a potential target in the treatment of AR.

**Acknowledgement** This study was supported by grants from Shenzhen Scientific Foundation and the Canadian Institutes of Health Research (#191063, #220058, #177843), Natural Sciences and Engineering Research Council of Canada (#371268).

This study was supported by grants from the Shenzhen-Hong Kong Innovation circle of Shenzhen Technology Program (SG200810220171A) and the Canadian Institutes of Health Research (CIHR #191063, #220058, #177843), Natural Science & Engineering Research Council of Canada (#371268).

**Declare of conflict of interests** None.

## References

1. Lindell DM, Berlin AA, Schaller MA, Lukacs NW. B Cell antigen presentation promotes Th2 responses and immunopathology during chronic allergic lung disease. *PLoS One*. 2008;3(9):e3129.
2. Arnaboldi PM, Behr MJ, Metzger DW. Mucosal B cell deficiency in IgA-/- mice abrogates the development of allergic lung inflammation. *J Immunol*. 2005;175(2):1276–85.
3. Jain S, Chodiseti SB, Agrewala JN. CD40 signaling synergizes with TLR-2 in the BCR independent activation of resting B cells. *PLoS One*. 2011;6(6):e20651.
4. Rau FC, Dieter J, Luo Z, Priest SO, Baumgarth N. B7-1/2 (CD80/CD86) direct signaling to B cells enhances IgG secretion. *J Immunol*. 2009;183(12):7661–71.
5. Koyasu S, Moro K. Type 2 innate immune responses and the natural helper cell. *Immunology*. 2011;132(4):475–81.
6. Nakanishi K. Basophils as APC in Th2 response in allergic inflammation and parasite infection. *Curr Opin Immunol*. 2010;22(6):814–20.
7. Pérez Novo CA, Jedrzejczak-Czechowicz M, Lewandowska-Polak A, Claeys C, Holtappels G, Van Cauwenberge P, et al. T cell inflammatory response, Foxp3 and TNFRS18-L regulation of peripheral blood mononuclear cells from patients with nasal polyposis after staphylococcal superantigen stimulation. *Clin Exp Allergy*. 2010;40(9):1323–32.
8. Zhang N, Holtappels G, Gevaert P, Patou J, Dhaliwal B, Gould H, et al. Mucosal tissue polyclonal IgE is functional in response to allergen and SEB. *Allergy*. 2011;66(1):141–8.

9. Hellings PW, Hens G, Meyts I, Bullens D, Vanoirbeek J, Gevaert P, et al. Aggravation of bronchial eosinophilia in mice by nasal and bronchial exposure to *Staphylococcus aureus* enterotoxin B. *Clin Exp Allergy*. 2006;36(8):1063–71.
10. Huvenne W, Callebaut I, Plantinga M, Vanoirbeek JAJ, Krysko O, Bullens DMA, et al. *Staphylococcus aureus* enterotoxin B facilitates allergic sensitization in experimental asthma. *Clin Exp Allergy*. 2010;40(7):1079–90.
11. Liu T, Xie C, Chen X, Zhao F, Liu AM, Cho DB, et al. Role of muscarinic receptor activation in regulating immune cell activity in nasal mucosa. *Allergy*. 2010;65(8):969–77.
12. Feng BS, Chen X, He SH, Zheng PY, Foster J, Xing Z, et al. Disruption of T-cell immunoglobulin and mucin domain molecule (TIM)-1/TIM4 interaction as a therapeutic strategy in a dendritic cell-induced peanut allergy model. *J Allergy Clin Immunol*. 2008;122(1):55–61.
13. Yang PC, Xing Z, Berin CM, Soderholm JD, Feng BS, Wu L, et al. TIM-4 expressed by mucosal dendritic cells plays a critical role in food antigen-specific Th2 differentiation and intestinal allergy. *Gastroenterology*. 2007;133(5):1522–33.
14. Meyers JH, Chakravarti S, Schlesinger D, Illes Z, Waldner H, Umetsu SE, et al. TIM-4 is the ligand for TIM-1, and the TIM-1-TIM-4 interaction regulates T cell proliferation. *Nat Immunol*. 2005;6(5):455–64.
15. Gonzalez SF, Kuligowski MP, Pitcher LA, Roozendaal R, Carroll MC. The role of innate immunity in B cell acquisition of antigen within LNs. In: Frederick WA, editor. *Advances in Immunology*. Volume 106 ed. Academic Press; 2010. p. 1–19.
16. Bousquet J, Reid J, Van Weel C, Baena Cagnani C, Canonica GW, Demoly P, et al. Allergic rhinitis management pocket reference 2008\*. *Allergy*. 2008;63(8):990–6.
17. Shiomori T, Yoshida Si, Miyamoto H, Makishima K. Relationship of nasal carriage of *Staphylococcus aureus* to pathogenesis of perennial allergic rhinitis. *J Allergy Clin Immunol*. 2000;105(3):449–54.
18. Hamelmann E, Tateda K, Oshiba A, Gelfand EW. Role of IgE in the development of allergic airway inflammation and airway hyperresponsiveness  $\Gamma\hat{\text{C}}\hat{\text{o}}$  a murine model. *Allergy*. 1999;54(4):297–305.
19. Korsgren M, Erjefält JS, Korsgren O, Sundler F, Persson CGA. Allergic eosinophil-rich inflammation develops in lungs and airways of B cell  $\Gamma\hat{\text{C}}\hat{\text{o}}$ deficient mice. *J Exp Med*. 1997;185(5):885–92.
20. Admyre C, Bohle B, Johansson SM, Focke-Tejkl M, Valenta R, Scheynius A, et al. B cell-derived exosomes can present allergen peptides and activate allergen-specific T cells to proliferate and produce TH2-like cytokines. *J Allergy Clin Immunol*. 2007;120(6):1418–24.
21. Rodriguez-Manzanet R, Meyers JH, Balasubramanian S, Slavik J, Kassam N, Dardalhon V, et al. TIM-4 expressed on APCs induces T cell expansion and survival. *J Immunol*. 2008;180(7):4706–13.
22. Qiu S, Du Y, Duan X, Geng X, Xie J, Gao H, et al. Cytotoxic T lymphocytes mediate chronic inflammation of the nasal mucosa of patients with atypical allergic rhinitis. *N Am J Med Sci*. 2011;3(8):378–83.
23. Liu T, Liang X, Li TL, Ma J, Yang JF, Yang PC. *Staphylococcal enterotoxin B* compromises the immune tolerant status in the airway mucosa. *Clin Exp Allergy*. 2011. doi:[10.1111/j.1365-2222.2011.03869.x](https://doi.org/10.1111/j.1365-2222.2011.03869.x). [Epub ahead of print] PubMed PMID: 22093045.