

# Specific immunotherapy suppresses Th2 responses via modulating TIM1/TIM4 interaction on dendritic cells

C.-Q. Zhao<sup>1\*</sup>, T.-L. Li<sup>2\*</sup>, S.-H. He<sup>3</sup>, X. Chen<sup>2</sup>, Y.-F. An<sup>1</sup>, W.-K. Wu<sup>1</sup>, X.-H. Zhou<sup>1</sup>, P. Li<sup>2</sup> & P.-C. Yang<sup>2</sup>

<sup>1</sup>Department of Otolaryngology, Head and Neck Surgery, the Second Hospital, Shanxi Medical University, Taiyuan, Shanxi, China; <sup>2</sup>McMaster Brain Body Institute, St. Joseph Healthcare and Department of Pathology & Molecular Medicine, McMaster University, Hamilton, ON, Canada; <sup>3</sup>The Clinical Experimental Center of the First Affiliated Hospital, Nanjing Medical University, Nanjing, China

**To cite this article:** Zhao C-Q, Li T-L, He S-H, Chen X, An Y-F, Wu W-K, Zhou X-H, Li P, Yang P-C. Specific immunotherapy suppresses Th2 responses via modulating TIM1/TIM4 interaction on dendritic cells. *Allergy* 2009; DOI: 10.1111/j.1398-9995.2009.02295.x.

## Keywords

allergy; dendritic cell; immune regulation; specific immunotherapy.

## Correspondence

Dr Chang-Qing Zhao, Department of Otolaryngology, the Second Hospital, Shanxi Medical University, Taiyuan 030001, China.  
Tel.: +86 351 3365786  
Fax: +86 351 3365725  
E-mail: fahyj@126.com

Dr Ping-Chang Yang, BBI-T3330, 50 Charlton Ave East, Hamilton, ON L8N 4A6, Canada.  
Tel: +905 522 1155 ext. 35828  
Fax: +905 540 6593  
E-mail: yangp@mcmaster.ca

\*Equally contributed to this paper.

Accepted for publication 11 November 2009

DOI:10.1111/j.1398-9995.2009.02295.x

Edited by: Hans-Uwe Simon

## Abstract

**Background:** Specific immunotherapy (SIT) is the only curable remedy for allergic disorders currently; however, the underlying mechanism is not fully understood yet. This study aimed to elucidate the mechanism of SIT on suppressing TIM4 (T cell immunoglobulin mucin domain molecule 4) expression in dendritic cells (DCs) and modulating the skewed T helper 2 (Th2) responses in patients with airway allergy.

**Methods:** Twenty patients with allergic rhinitis (AR) were treated with SIT for 3 months. Before and after SIT, the expression of TIM4 in peripheral DC and TIM1 in Th2 cells was examined. The role of Fc gamma receptor (FcγR) I and II in modulating the expression of TIM4 in DCs was investigated.

**Results:** The interaction of TIM1/TIM4 played a critical role in sustaining the polarization status of Th2 cells in AR patients. Cross-linking FcγRI by antigen/IgG complexes increased the production of TIM4 by dendritic cells via upregulating tumor necrosis factor-α in DCs. Exposure to microbial products promoted the expression of FcγRI in DCs that further increased the expression of TIM4. Exposure to specific antigens alone upregulated the expression of FcγRII in DCs, that suppressed the expression of TIM4.

**Conclusions:** We conclude that SIT suppresses the skewed Th2 responses via disrupting the interaction of TIM1/TIM4 in antigen-specific Th2 cells.

The prevalence of allergic diseases, such as asthma, allergic rhinitis (AR), and allergic dermatitis, keeps rising worldwide (1, 2). The main immune pathogenic features of allergic diseases include T helper 2 (Th2) polarization, elevated serum IgE levels, profound infiltration of eosinophils and mastocytosis in local tissue (1, 3, 4). In fact, allergic diseases have merged as a major health problem in the world. Although research on allergic diseases advanced rapidly in recent years, the etiology of allergic diseases remains unknown.

It is still an enigma how antigen-specific Th2 cells get skewed polarization and how they maintain a dominant status. The T-cell immunoglobulin and mucin domain molecule (TIM)1 is a newly described molecule that plays a critical role in Th2 cell development (5, 6). However, whether TIM1

expressed by Th2 cells has the capacity maintaining functional status by itself remains unclear. TIM4 is the natural ligand of TIM1 (6). It is expressed by dendritic cells (DCs) (6); the expression can be upregulated drastically upon activation (7–9). Dendritic cell-derived TIM4 can bind TIM1 on CD4<sup>+</sup> T cells and drive them to develop into Th2 cells. Still, whether the interaction of TIM1/TIM4 plays a role in the maintenance of Th2 polarization as shown in allergic disorders is to be further understood.

Dendritic cells express Fc gamma receptor (FcγR) including FcγRI, FcγRII, and FcγRIII (10, 11). FcγRs recognize antigen/IgG complexes on the surface of DCs (12). Ligating these receptors by their ligands, IgG/Ag complexes, induces intracellular signaling and various effector responses such as

antibody-dependent cellular cytotoxicity, mast cell degranulation, and phagocytosis (12–15). However, whether activation of FcγRs is involved in the expression of TIM4 in DCs is unknown.

It is proposed that production of antigen-specific IgG during specific immunotherapy (SIT) is the major effector molecule to ameliorate allergic symptoms (16, 17); however, how the IgG suppress polarized Th2 responses is less understood. Although we have known that IgG can bind FcγRs to initiate or inhibit cellular activities of FcγR-bearing cells (18), whether the interaction of IgG/FcγR is involved in therapeutic effect of SIT is not clear. As DCs are the master modulator of immune responses, DCs express FcγRI and FcγRII (10, 12), we hypothesize that SIT-induced antigen-specific IgG ligates FcγRs on DCs and modulates the skewed Th2 responses subsequently.

In this study, with isolated peripheral CD4<sup>+</sup> T cells and DCs from patients with AR as study platforms, we were able to examine the kinetics of TIM1 expression in Th2 cells. TIM1 expression had the feature of natural decay that could be prevented in the presence of TIM4. The SIT had the capacity to suppress both TIM1 and TIM4 expression. Exposure to microbial products was involved in the regulation of FcγR expression in DCs that further modulated the expression of TIM4.

## Materials and methods

### Diagnosis of AR and SIT

Patients with AR were recruited into this study at outpatient of the Department of Otolaryngology, Head and Neck Surgery of Shanxi Medical University. Informed consent was obtained from each patient. Using human material in this study was approved by the Human Study Ethic Committee at Shanxi Medical University. Diagnosis of AR specific to Der and treatment with Der (*Dermatophagoides pteronyssinus* 1, the major allergen of dust mite) SIT were based on our routine procedures (SIT was performed subcutaneously). Patients using anti-allergy drugs within the previous 4 weeks and those with documented allergy to non-Der allergens were excluded. The demographic data were presented in Table S1, in supporting information.

### Flow cytometry

Cells were stained with fluorescently labeled antibodies on ice for 30 min. The stained cells were analyzed by a FACSarray (BD Bioscience, San Jose, CA, USA). Data were analyzed by software FLOWJO (FlowJo flow cytometry analysis software; Tree Star, Inc., Ashland, OR, USA).

### Real-time quantitative reverse transcription polymerase chain reaction (qPCR)

Total RNA was extracted from the DCs using an RNeasy Mini kit (Qiagen, Mississauga, ON, Canada). cDNA was synthesized using iScriptTm cDNA Synthesis kit (Bio-Rad,

Mississauga, ON, Canada). The resulting cDNA was subjected to qPCR that was performed with a LightCycler using a SuperScript III Platinum SYBR Green Two-Step qPCR kit (Invitrogen, Burlington, ON, Canada). The amplified product was detected by the presence of an SYBR green fluorescent signal. The standard curve was designed with β-actin cDNA. The resulted amplicon was quantified with the standard curve. The primers are listed in supporting information.

### Co-immunoprecipitation and Western blotting

Total proteins were extracted from cultured DCs. The immune complexes were formed with antibodies against Der, IgG, FcγRI, or FcγRII and 100 μl extracts for 12 h at 4°C. The rest procedures were the same as we reported (12). The membranes were then blotted with antibodies against Der, IgG, FcγRI, or FcγRII respectively. The proteins were detected using horseradish peroxidase conjugated second antibodies. Horseradish peroxidase enzymatic reaction was detected with enhanced chemiluminescent reagents and recorded with X-ray films.

### Enzyme-linked immunosorbent assay (ELISA)

Cytokine and IgE levels were determined by ELISA from the instruction of reagent kits.

### T-cell proliferation assay

Isolated CD4<sup>+</sup> T cells were cultured in the presence or absence of specific antigen, Der with radiated DCs for 3 days. [<sup>3</sup>H] thymidine (1 μCi per well) was added for the last 16 h. The [<sup>3</sup>H] thymidine incorporation was detected by a scintillation counter.

### Statistic analysis

Data are presented as the means ± SD. Differences between two groups were evaluated with the Student's *t*-test; data among three or more groups were evaluated with ANOVA. Bonferroni adjustment was applied to *post hoc* group comparisons when required. A *P* < 0.05 was accepted as a significant criterion.

The procedures of RNA interference (RNAi), LightShift chemiluminescent electrophoretic mobility shift assay, chromatin immunoprecipitation assay, symptom scoring, serum antibody measurements, and details of reagents were presented in supporting information.

## Results

### Expression of TIM1 in polarized human Th2 cells

It is reported that Th2 cells express TIM1 that has the costimulatory activity to promote Th2 cell proliferation (5–7, 19). However, whether the expression of TIM1 on Th2 cells is stable or whether the expression requires support from other molecules is not clear. With isolated peripheral CD4<sup>+</sup>

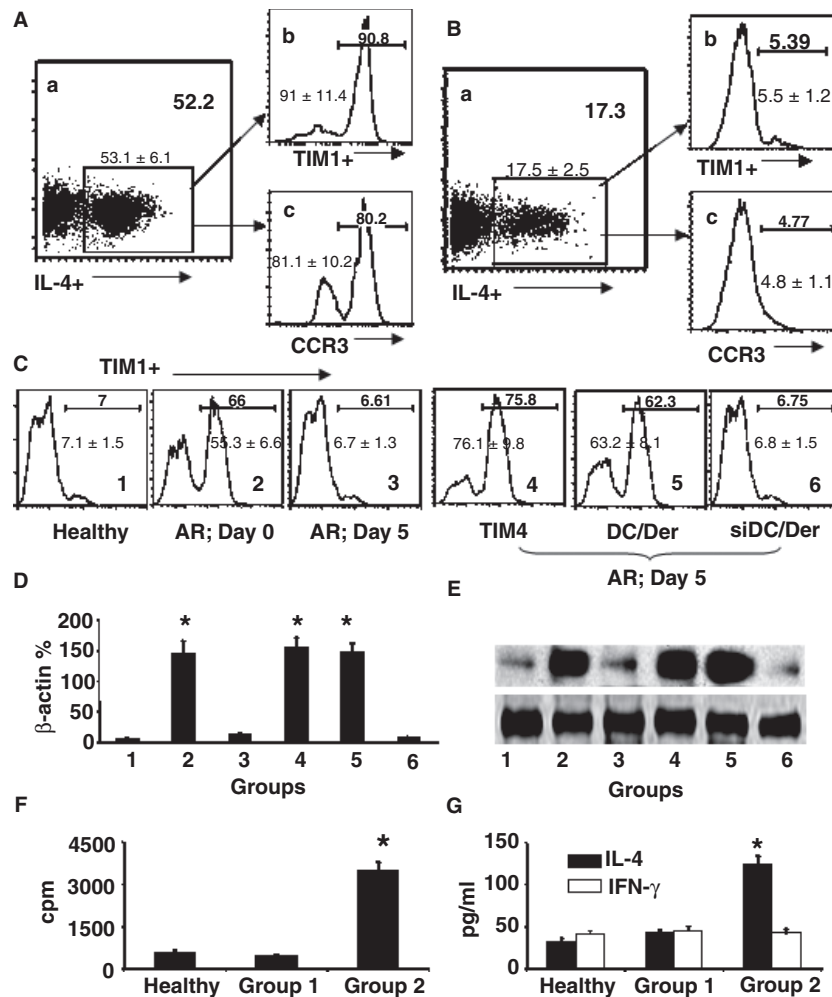


**Figure 1** T-cell immunoglobulin mucin domain molecule (TIM)1 is required in the maintenance of antigen-specific T helper 2 response. (A) and (B), peripheral CD4<sup>+</sup> CD25<sup>+</sup> T cells were isolated from 20 patients with allergic rhinitis (AR) (A) 20 healthy subjects (B) by magnetic cell sorting. The isolated cells were stained with antibodies against interleukin (IL)-4, TIM1, and CCR3, and analyzed by flow cytometry. Panels Aa and Ba show IL-4<sup>+</sup> T cells (the gated cells). Panels b and c in A and B show TIM1<sup>+</sup> cells (b) and CCR3<sup>+</sup> cells (c) in gated cells of A and B. (C) CD4<sup>+</sup> CD25<sup>+</sup> T cells from patients with AR were cultured for 0–5 days. Flow cytometry histograms show TIM1<sup>+</sup> cells (the right peaks). C1, cells were from healthy subjects. C2–C6, cells were from patients with AR. C2, day 0. C3–C6, cells were analyzed on day 5 (day 5). C4, addition of exogenous TIM4 to the culture. C5, added dendritic cells (DCs) and Der. C6, siDC: DCs were transfected with TIM4siRNA in the presence of Der. (D) Bars indicate the expression of TIM1 mRNA in cultured CD4<sup>+</sup> CD25<sup>+</sup> T

cells that was analyzed by quantitative real-time reverse transcription polymerase chain reaction. The legend numbers are the same as those in panel C. (E) Western blots show the TIM1 protein contents in cellular extracts from cultured CD4<sup>+</sup> CD25<sup>+</sup> T cells as described in panel C. The legend numbers are the same as those in panel C. (F) CD4<sup>+</sup> CD25<sup>+</sup> T cells were isolated from healthy subjects and patients with AR. Bars indicate the rate of T-cell proliferation representing by [<sup>3</sup>H] thymidine incorporation. (G) Bars indicate the levels of IL-4 and IFN-γ in culture media of CD4<sup>+</sup> CD25<sup>+</sup> T cells. Group 1: T cells were cultured for 5 days with media alone and then exposed to Der and DCs. Group 2: T cells were exposed to Der and DCs immediately after isolation. In E, F, and G, data are presented as the means ± SD from three separate experiments. Isotype IgG was employed as a control in each experiment; no positive staining was observed (data not shown). The control siRNA did not show any effect on target genes (data not shown).

CD25<sup>+</sup> T cells from 20 patients with AR, ratio of interleukin (IL)-4<sup>+</sup> T cells was analyzed by flow cytometry. The results showed that IL-4<sup>+</sup> T cells was markedly more in patients with AR (>50%) (Fig. 1Aa) than in healthy controls (20 subjects) (<20%) (Fig. 1Ba). Using flow cytometry gating technique, we further analyzed TIM1 expression in IL-4<sup>+</sup> T

cells. Significantly more TIM1<sup>+</sup> IL-4<sup>+</sup> T cells were detected in patients with AR (Fig. 1Ab) when compared with healthy subjects (Fig. 1Bb). CCR3 was proposed as a specific marker on Th2 cells of patients with allergic diseases (20). We thus analyzed the expression of CCR3 on IL-4<sup>+</sup> T cells. As shown by flow cytometry data, more than 80% IL-4<sup>+</sup> T cells was

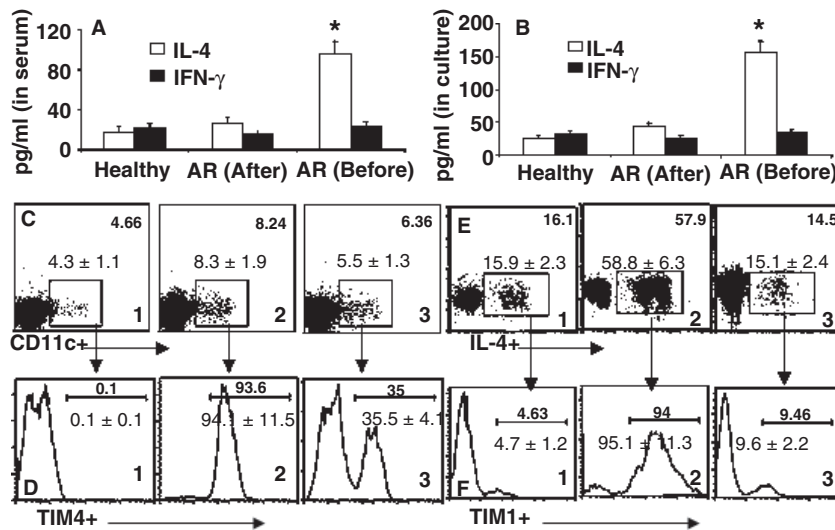


**Figure 2** Specific immunotherapy (SIT) suppresses T-cell immunoglobulin mucin domain molecule (TIM)1/TIM4 and modulates T helper 2 response. Twenty patients with allergic rhinitis (AR) were treated with SIT for 3 months. Serum levels of interleukin (IL)-4 and IFN- $\gamma$  were determined by enzyme-linked immunosorbent assay (ELISA) (A). Peripheral CD4<sup>+</sup> CD25<sup>+</sup> T cells were isolated and cultured with or without specific antigen Der for 3 days in the presence of dendritic cells (DCs) from the same patients. Levels of IL-4 and IFN- $\gamma$  in culture media were determined by ELISA (B). Data are presented as the means  $\pm$  SD. \* $P$  < 0.05, compared with healthy group. (C) and (D), peripheral mononuclear cells were isolated from the patients with AR and healthy subjects. Cells were stained with

anti-CD11c and anti-TIM4 and analyzed by flow cytometry. The flow cytometry dot plots show DCs (the gated cells). (D) The histograms show TIM4<sup>+</sup> DCs within the gated DCs (pointed by arrows). (E) and (F), peripheral CD4<sup>+</sup> CD25<sup>+</sup> T cells were isolated from the patients with AR and healthy subjects. Cells were stained with anti-IL-4 and anti-TIM1 and analyzed by flow cytometry. The flow cytometry dot plots show IL-4<sup>+</sup> T cells (the gated cells). (F) the histograms show TIM1<sup>+</sup> T cells within the gated DCs (pointed by arrows). AR: samples were taken from patients with AR. Before (or after): before (or after) SIT. (C–F), the digital numbers: 1, healthy controls; 2, patients with AR before SIT; 3, patients with AR after SIT. Mean  $\pm$  SD was presented in each flow cytometry graph.

CCR3<sup>+</sup> in patients with AR (Fig. 1Ac), whereas 5% IL-4<sup>+</sup> T cells was also CCR3<sup>+</sup> in healthy subjects (Fig. 1Bc).

To clarify whether the expression of TIM1 in polarized Th2 cells was stable, another batch of CD4<sup>+</sup> CD25<sup>+</sup> T cells from the same group of patients with AR was cultured for 1–5 days. As shown by flow cytometry data, the rate of TIM1<sup>+</sup> cells in total CD4<sup>+</sup> CD25<sup>+</sup> T cells decreased gradually that dropped down to control level on day 5 (Fig. 1C). The results were further confirmed by qPCR (Fig. 1D) and immunoblotting assay (Fig. 1E). The total cell number on day 0 and 5 was counted. The results showed that the total cell number increased  $1.56 \pm 0.66$  times on day 5. The fact indicates that the reduction of TIM1 in CD4<sup>+</sup> CD25<sup>+</sup> T cells was not caused by natural dying of TIM1<sup>+</sup> T cells, but caused by natural decay of TIM1 expression during the 5-day culture.

We next sought to determine whether the decay of TIM1 expression on Th2 cells was accompanied by suppression of Th2 response. CD4<sup>+</sup> CD25<sup>+</sup> T cells from the same group of patients with AR were divided to group 1 and group 2. Group 1 was cultured with media alone for 5 days and then exposed to specific mite antigen (Der) of these patients, and freshly isolated peripheral DCs (irradiated) for another 3 days. Group 2 was directly exposed to Der and DCs for 3 days. As shown by [<sup>3</sup>H] thymidine incorporation assay, T-cell proliferation was significantly higher in group 2 than

in group 1 (Fig. 1F). Meanwhile, we also examined Th1 and Th2 cytokines in culture media. As expected, the levels of IL-4, but not IFN- $\gamma$ , were markedly higher in group 2 when compared with group 1 (Fig. 1G).

#### DC-derived TIM4 contributes to the maintenance of skewed Th2 response

It is reported that TIM-1 is critical in Th2 response (21). The present data show a positive correlation between the expression of TIM1 and the maintenance of Th2 response (Fig. 1). Therefore, it is possible that interaction of TIM1/TIM4 is one of the approaches to sustain TIM1 activities in Th2 cells. Thus, we added exogenous TIM4 to the culture of another batch of CD4<sup>+</sup> CD25<sup>+</sup> T cells from patients with AR at a dose of 5  $\mu$ g/ml (the media and reagents were changed on day 3). The results showed that TIM1 expression in CD4<sup>+</sup> IL-4<sup>+</sup> T cells kept high levels throughout the 5-day culture (Fig. 1C–E, item 4). The fact indicates that TIM4 has the capacity to sustain Th2 activities in the presence of TIM1.

Dendritic cells express TIM4 in response to various stimuli (7–9) that can be the endogenous source of TIM4. We then examined the effect of DC-derived TIM4 in the maintenance of TIM1 expression in Th2 cells. CD4<sup>+</sup> CD25<sup>+</sup> T cells from patients with AR were cocultured with DCs from the same

patients in the presence of Der for 5 days. As expected, the expression of TIM1 in T cells as well as the Th2 responses was maintained at high levels (Fig. 1C–E, item 5). However, when the TIM4 gene was knocked down in DCs by RNAi, the expression of TIM1 could not be maintained in cocultured polarized Th2 cells (Fig. 1C–E, item 6).

### Specific immunotherapy suppresses TIM4 expression in DCs of patients with AR

Currently, SIT is the only specific therapy for the treatment of allergic diseases (1, 2). The underlying mechanisms of SIT are not fully understood yet. From the data we got from interaction of TIM1/TIM4 (Fig. 1), we reasoned that the interaction of TIM1/TIM4 might be one of the targets in SIT. To test the hypothesis, 20 patients with AR with Der sensitization were treated with the Der desensitization therapy for 3 months. The results of ELISA showed that levels of serum IL-4 (Fig. 2A) and IL-4-secretion by CD4<sup>+</sup> CD25<sup>+</sup> T cells in culture (Fig. 2B) were significantly inhibited by SIT. The expressions of TIM4 in peripheral DCs and TIM1 in peripheral CD4<sup>+</sup> CD25<sup>+</sup> T cells were examined by flow cytometry before and after SIT. TIM4 expression in gated CD11c<sup>+</sup> cells (Fig. 2C,D) and TIM1 expression in gated IL-4<sup>+</sup> T cells (Fig. 2E,F) were also significantly decreased after the 3-month therapy (Fig. 2). The downregulation of TIM1 and TIM4 had a correlation with decreased Th2

responses. Levels of IFN- $\gamma$  in culture media were not significantly changed.

In addition, we also examined the serum levels of Der-specific IgE and IgG4 before and after SIT. The AR clinical symptom scores were documented. The results indicated that SIT significantly suppressed the Der-specific IgE and increased Der-specific IgG4 levels in the sera (Fig. S1) as well as the clinical score (Fig. S2). Correlation assay was performed between the expression of TIM4 in peripheral DCs and serum Der-specific IgE (TIM4 and IgE), TIM4 and IgG4, TIM4 and symptom score respectively. The results showed significant correlation between TIM4 and IgE ( $r = 0.58$ ,  $P < 0.05$ ), TIM4 and IgG4 ( $r = -0.71$ ,  $P < 0.01$ , TIM4 and symptom score ( $r = 0.53$ ,  $P < 0.05$ ).

### Interaction of antigen-specific IgG and Fc $\gamma$ RII downregulates TIM4 production by DCs

As shown by Figs 1 and 2, SIT reduced the expression of TIM4 on DCs and TIM1 on Th2 cells of patients with AR. We further explored the underlying mechanism. It is proposed that increase in antigen-specific IgG produced in SIT plays a major role in the amelioration of allergic symptoms (21, 22). However, the targets of antigen-specific IgG still remain obscure. As DCs express Fc $\gamma$ Rs that recognize IgG to form complexes on the surface (23), we hypothesized that SIT produced antigen-specific IgG bound Fc $\gamma$ Rs to

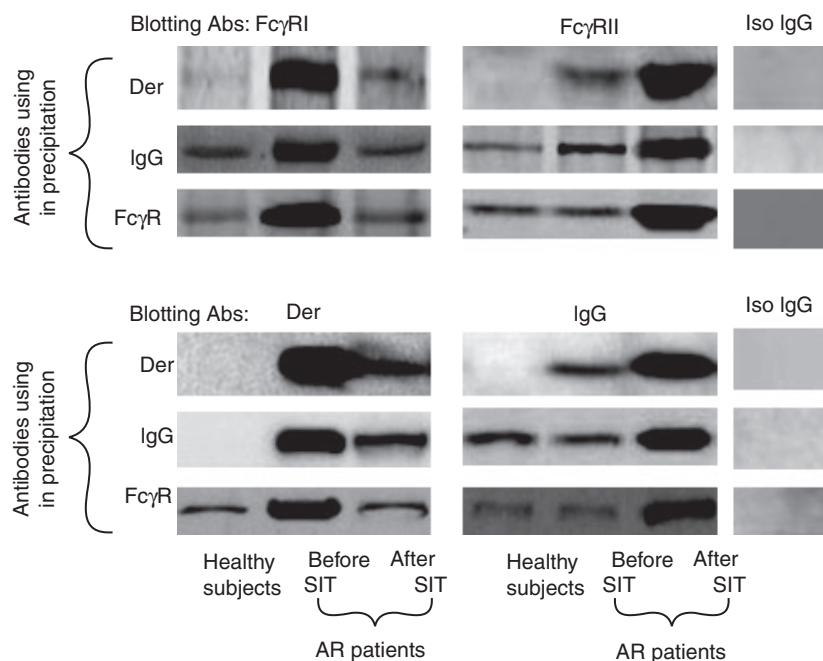


**Figure 3** Specific immunotherapy modulates expression of Fc gamma receptors (Fc $\gamma$ Rs) on dendritic cells (DCs). Isolated peripheral mononuclear cells were cultured in the presence of granulocyte colony-stimulating factor (20 ng/ml) for 3 days. DCs were isolated and cultured in the presence of Der (10  $\mu$ g/ml) for 6 h. The

cellular protein extracts of DCs were subjected to coimmunoprecipitation assay to identify the Der-specific IgG/Fc $\gamma$ R complex. Immune blots show Fc $\gamma$ RI or Fc $\gamma$ RII in DC cellular extracts precipitated by antibodies against IgG, Fc $\gamma$ RI, or Fc $\gamma$ RII.

prime DCs. Re-exposure to specific antigens, such as those using in SIT, activated DCs to suppress the production of TIM4 by DCs. To test the hypothesis, we prepared DCs from the peripheral mononuclear cells of patients with AR and healthy controls with our established protocols (the purity of DCs was over 96.5% as examined by flow cytometry). The first attempt was to identify the complexes of Der/IgG/Fc $\gamma$ R on DCs. DC cellular extracts were analyzed by coimmunoprecipitation assay with antibodies against Der, IgG, and Fc $\gamma$ Rs (including Fc $\gamma$ RI, Fc $\gamma$ RII and Fc $\gamma$ RIII) respectively. The separated proteins on membranes were blotted by antibodies against Der, IgG, Fc $\gamma$ RI, or Fc $\gamma$ RII. As shown in Fig. 3, before SIT, the abundance of Der/IgG/Fc $\gamma$ RI was significantly higher in AR group than healthy group; less Der/IgG/Fc $\gamma$ RII was found, whereas Fc $\gamma$ RIII was under detectable level (data not shown). The examination was carried out again after the 3-month SIT with DCs obtained from the same group of patients. The Der/IgG/Fc $\gamma$ RII complex was increased significantly, whereas the complex of Der/IgG/Fc $\gamma$ RI was decreased on DCs. Using as a comparison, another group of six patients with AR with Der sensitization were treated with Veramyst (a nasal steroid spray) alone for 3 months. Complexes of Der/IgG/Fc $\gamma$ RI and Der/IgG/Fc $\gamma$ RII were also detected in isolated peripheral DCs from these patients, which were similar to SIT group (before SIT treatment); but no detectable changes in the immune complexes were identified after treatment with Veramyst (data not shown).

To understand whether the complex of antigen/IgG/Fc $\gamma$ R plays any roles in TIM4 expression in DCs, TIM4 protein in isolated peripheral DCs were analyzed by Western blotting with the same set of antibodies in Fig. 3 and anti-TIM4. Indeed, we detected TIM4 in DC cellular extracts that was positively correlated with the amount of Fc $\gamma$ RI and negatively correlated with the amount of Fc $\gamma$ RII in samples before and after SIT (Fig. 4).

Collectively, the results indicate that antigen-specific IgG can bind both Fc $\gamma$ RI and Fc $\gamma$ RII to form complexes. IgG/Fc $\gamma$ RI complexes recognizing specific antigens increase the expression of TIM4, whereas IgG/Fc $\gamma$ RII complexes recognizing specific antigens decrease the expression of TIM4 in DCs.

#### Microbial products modulate Fc $\gamma$ Rs on DCs

Dendritic cells express both Fc $\gamma$ RI and Fc $\gamma$ RII. Antigen-specific IgG has equal opportunity to bind either Fc $\gamma$ RI or Fc $\gamma$ RII on DCs. The mechanism by which antigen-specific IgG choose to bind to Fc $\gamma$ RI or Fc $\gamma$ RII is unclear. Prompted by our previous studies that microbial products are involved in the initiation of Th2 responses (8, 19), we postulated that microbial products promotes Fc $\gamma$ RI activities in DCs that further promotes the expression of TIM4 in DCs. Thus, we next exposed naïve human DCs (generated from peripheral mononuclear cells) or exposed DCs from patients with AR to Der or microbial products [lipopolysaccharide (LPS), staphylococcal enterotoxin B, or cholera toxin] in culture for 48 h. As shown by qPCR and Western blotting, microbial products

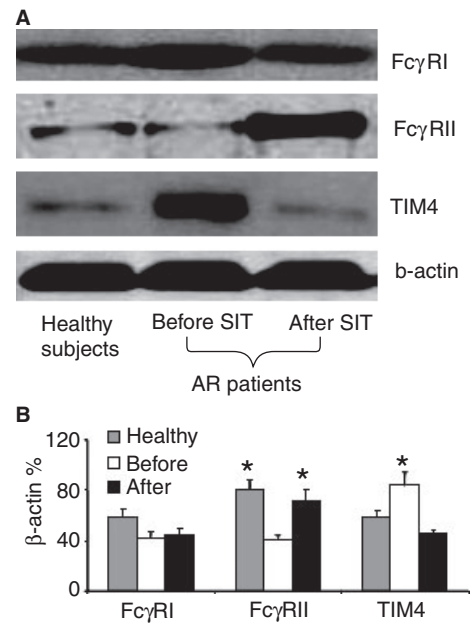


**Figure 4** Specific immunotherapy (SIT) suppresses T-cell immunoglobulin mucin domain molecule 4 (TIM4) in dendritic cells (DCs). Peripheral DCs were isolated from healthy subjects or patients with allergic rhinitis (AR) before and after SIT. Cellular protein extracts of DCs were analyzed by Western blotting to evaluate the expression of Fc gamma receptor (Fc $\gamma$ RI), Fc $\gamma$ RII, and TIM4. A. The immune blots show proteins of Fc $\gamma$ RI, Fc $\gamma$ RII, and TIM4 in DC cellular extracts. B. Bars indicate the data of densitometry of immune blots. Data were normalized to the percentage of  $\beta$ -actin blots (the internal control) and presented as mean  $\pm$  SD from 12 patients with AR and eight healthy controls. \* $P < 0.05$ , compared with healthy control.

markedly increased the expression of Fc $\gamma$ RI and TIM4 in DCs, while Der increased Fc $\gamma$ RII but not TIM4 in DCs (Fig. 5A,B). Because exposure to microbial products induces DCs to produce IL-12 and TNF- $\alpha$  that play important roles in immune inflammation (24), the autocrine IL-12 and TNF- $\alpha$  in DCs might be involved in the expression of Fc $\gamma$ Rs and TIM4. Thus, in further experiments, DCs were transfected with IL-12 small interference RNA (siRNA) or TNF- $\alpha$  siRNA prior to exposure to microbial products in separate experiments. The results showed that TNF- $\alpha$ -deficient DCs did not express TIM4 in response to stimulation of microbial products, whereas those IL-12-deficient DCs still had the capacity to produce TIM4 upon exposure to microbial products (Fig. 5C). To further confirm the role of TNF- $\alpha$  on TIM4 expression in DCs, a batch of DCs was exposed to TNF- $\alpha$  at graded doses for 48 h. The results showed that TIM4 was expressed by DCs in a TNF- $\alpha$  dose-dependent manner (Fig. 5D).

Alternatively, a batch of peripheral DCs was cultured in IgG (100  $\mu$ g/ml)-coated plates for 48 h to cross-link the Fc $\gamma$ Rs on DCs. Unexpectedly, no increase in TIM4 was detected in these DCs. Considering both Fc $\gamma$ RI and Fc $\gamma$ RII might be

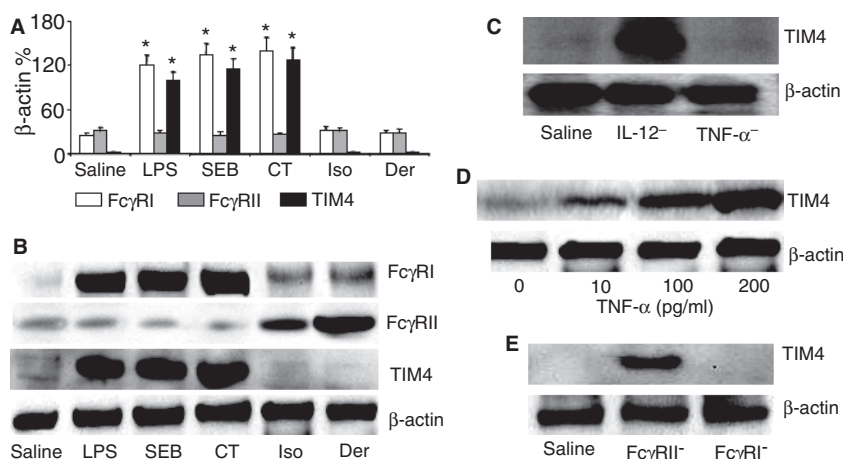


**Figure 5** Microbial products modulate T-cell immunoglobulin mucin domain molecule 4 (TIM4) expression in dendritic cells (DCs). Isolated DCs were exposed to lipopolysaccharide (LPS, 1 µg/ml), staphylococcal enterotoxin B (SEB, 5 µg/ml), or cholera toxin (CT, 5 µg/ml) for 24 h. (A) Bars indicate TIM4 mRNA level in DCs that was determined by quantitative real-time reverse transcription polymerase chain reaction. Data were presented as the percentage of β-actin (mean ± SD from three separate experiments). Iso: isotype IgG. (B) Immune blots show proteins of Fc gamma receptor (FcγRI), FcγRII, and TIM4 in isolated DCs after

exposure to microbial products for 24 h (the presented data were from CT-stimulated DCs; data from LPS- or SEB-stimulated DCs were not shown). (C) Immune blots show TIM4 protein in DCs after exposure to microbial products (CT). Interleukin (IL)-12<sup>-</sup> (or TNFα<sup>-</sup>): IL-12<sup>-</sup> (or TNFα<sup>-</sup>)deficient DCs [genes were knocked down by RNA interference (RNAi)]. (D) Immune blots show TIM4 protein in DCs after exposure to exogenous TNF-α. (E) Immune blots show TIM4 protein in DCs after cross-linking by IgG. FcγRII<sup>-</sup> (or FcγRI<sup>-</sup>): FcγRII- (or FcγRI-)deficient DCs (genes were knocked down by RNAi).

cross-linked simultaneously in response to the exposure to non-specific IgG, DCs with FcγRI- or FcγRII-knocked down were cultured in IgG (100 µg/ml)-coated plates for 48 h. The results showed that abundant TIM4-expression was detected in FcγRII-deficient DCs, but not in those FcγRI-deficient DCs (Fig. 5E). The fact further confirmed that the FcγRI activities were involved in TIM4 expression in DCs, whereas the FcγRII activities inhibit TIM4 expression in DCs.

## Discussion

The present study reports several novel findings from a study with human specimens in which we found that the expression of TIM1 in polarized Th2 cells from patients with AR decayed quickly when cultured *in vitro*. The decay of TIM1 expression in Th2 cells correlated with the decrease in Th2 responses; addition of TIM4 stabilized the TIM1 expression. Dendritic cell-derived TIM4 could be one of the endogenous sources of TIM4 in the body. We also found that SIT increased the activities of FcγRII, whereas microbial products increased FcγRI on DCs; interaction between specific antigen, specific IgG and FcγR modulated the expression of TIM4 in DCs.

Antigen-specific Th2 polarization plays a critical role in the clinical attacks and the recurrence of allergic disorders (25). One of the characteristics of antigen-specific Th2 cells is that once developed, this cell population sustains in the body. Currently, we do not have any recognized remedies to eliminate these cells (25). The discovery of TIM1 expression in Th2 cells shed a new light in the study of the

pathogenic mechanism of allergic diseases (5, 6). The present study reveals that TIM1 not only plays a critical role in the development of Th2 responses (5, 6) but also is important in the sustaining of antigen-specific Th2 responses as manifested that the natural decay of TIM1 expression correlates with the decreases in Th2 responses. Previous studies also showed that using blocking antibodies of TIM1 could suppress allergic responses in animal models (8, 19, 26, 27).

A correlation between the TIM1 expression in Th2 cells and allergic disorders was noted by several clinical studies (8, 28–34), so did the present study. The data show that over 90% CD4<sup>+</sup> IL-4<sup>+</sup> T cells in patients with AR express TIM1. TIM1 is also recognized in atopic dermatitis (28), AR (35), asthma (36), rheumatoid arthritis (37), and systemic lupus erythematosus (30). These findings indicate that TIM1 expression in Th2 cells of patients with immune diseases is relatively common; the fact implicates that there must have been one or more specific factors (34) in the body playing roles in the maintenance of TIM1 expression in Th2 cells under allergic environment. TIM4 is the natural ligand of TIM1 (6). It is possible that TIM4 is one of the factors to maintain TIM1's function. This notion is supported by our previous study (8) in which we recognized that TIM4 favors the expression of TIM1 in CD4<sup>+</sup> T cells. The present data further confirm that TIM4 is capable of maintaining the TIM1 expression in Th2 cells.

The endogenous source of TIM4 is gradually elucidated. Meyers et al. (6) found that DCs express TIM4. In response to microbial stimulation, the expression of TIM4 is highly

strengthened, as we reported recently (8). A recent study indicates that activated DCs show high expression of TIM4 in DCs (7). Although it is not exclusive, at least we have known that DCs have the capacity to produce TIM4. DCs have the tendency to migrate to draining lymph nodes where the DCs have opportunity to interact with T cells. Thus, it is feasible that DCs can transfer the TIM4 to T cells when necessary. In other words, polarized Th2 cells can get TIM4 from activated DCs to maintain the polarization status.

The clinical effect of SIT on amelioration of allergic diseases has been recognized via several administration routes including subcutaneous injection, epicutaneous route, inhalation and sublingual route (16, 17); but the mechanism is not completely understood yet. The present data add novel information to the mechanism of SIT. The fact that after 3-month SIT, levels of TIM1 in Th2 cells and TIM4 in DCs of patients with AR markedly reduced indicates that SIT modulates the allergic status, at least partially, via suppressing the expression of TIM1 and TIM4. The fact of the maintenance of TIM1 on Th2 cells relies on the activities of TIM4 implicates that the downregulation of TIM1 by SIT is a consequence of TIM4 suppression. The data provide supportive evidence to this event. As shown by present study, as well as reported by others (10, 11), human DCs express both Fc $\gamma$ RI and Fc $\gamma$ RII. Fc $\gamma$ Rs have the capacity to bind IgG or complexes of antigen/IgG. Specific immunotherapy produces antigen-specific IgG in the body to bind to Fc $\gamma$ RI or Fc $\gamma$ RII to form complexes on DCs to make the DCs primed as shown by the present data. Re-exposure to specific antigen forms triple complexes, the antigen/IgG/Fc $\gamma$ RI or antigen/IgG/Fc $\gamma$ RII. The present data and our recent publication (12) also show these complexes on DCs. It is suggested that activation of Fc $\gamma$ RII generates negative signal, and activation of Fc $\gamma$ RI conducts positive signal (11). The present data are in agreement with these studies by showing that more Der/IgG/Fc $\gamma$ RI was detected in DCs from patients with AR before SIT, while high amount of Der/IgG/Fc $\gamma$ RII was detected in DCs after SIT.

This study collected a group of patients with AR sensitized to mite antigen, Der. We also treated these patients with Der during SIT. In fact, naturally exposed Der and therapeutic Der that we used in SIT have the same properties. Then, what makes the consequences different that the natural exposure to Der evokes attacks of AR, while the injection with Der in SIT inhibits AR attacks remains to be elucidated. As an extension of our recent studies (8, 19) that microbial products promote the expression of TIM4 in DCs, the present study further revealed that exposure to microbial products favors the expression of Fc $\gamma$ RI in DCs, which in turn promotes the expression of TNF- $\alpha$ , the latter is involved in the expression of TIM4 as shown by the present study. Meanwhile, we also found that exposure to antigen (such as Der) alone promote the expression of Fc $\gamma$ RII in DCs. Thus, it is conceivable that natural antigens (such as Der) are inevitable to be contaminated by microbial products such as LPS that distributes in natural environment ubiquitously. The present study suggests that such contaminated antigens have the capacity to activate DCs to express Fc $\gamma$ RI, to produce TIM4 and to process anti-

gens that favor the antigen-specific Th2 polarization. During SIT, however, injection with pure antigen induces Fc $\gamma$ RII expression in DCs that inhibits TIM4 expression resulting in the TIM1 decaying subsequently in antigen-specific Th2 cells and stops AR attacks eventually.

Our study also revealed the direct mechanism by which cross-linking Fc $\gamma$ Rs modulates the expression of TIM4 in DCs. TNF- $\alpha$  is the critical molecule that mediates the expression of TIM4 in DCs induced by Fc $\gamma$ Rs; its production by DCs can be upregulated by cross-linking Fc $\gamma$ RI and inhibited by cross-linking Fc $\gamma$ RII. Our finding is in line with previous reports (38) that TNF- $\alpha$  is a critical molecule in the development of optimal antigen-specific immune responses (39). The results implicate that modulating TNF- $\alpha$  expression in immune cells has potential to develop a novel approach in the therapeutics of allergic diseases.

In summary, the present study reports that TIM1 is required in the maintenance of antigen-specific Th2 responses. DC-derived TIM4 maintains TIM1 in Th2 cells in a stable status that plays a critical role in sustaining the Th2 polarization. Fc $\gamma$ RI recognizes antigen/IgG complexes to increase, while Fc $\gamma$ RII recognizes antigen/IgG complexes to decrease the expression of TIM4 in DCs that was confirmed in a group of patients with AR after treatment with SIT. Finally, we found that exposure to microbial products increases the expression of Fc $\gamma$ RI, while exposure to pure antigens increases the expression of Fc $\gamma$ RII in DCs. Activation of Fc $\gamma$ RI favors the expression of TIM4 in DCs.

### Acknowledgments

This study was supported by grants from the Natural Science Foundation of China, the Canadian Institute of Health Research (CIHR), and the Natural Science Engineering Research Council of Canada. Dr P. Yang holds a New Investigator Award of CIHR.

### Conflict of interest

The authors do not have any conflict of interest on this paper.

### Supporting information

Additional Supporting Information may be found in the online version of this article:

#### Methods.

**Figure S1.** Specific immunotherapy modulates the serum levels of IgE and IgG4 in allergic rhinitis (AR) patients.

**Figure S2.** Specific immunotherapy (SIT) reduces the allergic rhinitis (AR) clinical symptom score.

**Table S1.** Demographic data.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

## References

- Kay AB. Allergy and allergic diseases. First of two parts. *N Engl J Med* 2001;**344**:30–37.
- Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature* 2008;**454**:445–454.
- Munitz A, Seidu L, Cole ET, Ahrens R, Hogan SP, Rothenberg ME. Resistin-like molecule alpha decreases glucose tolerance during intestinal inflammation. *J Immunol* 2009;**182**:2357–2363.
- Burke SM, Issekutz TB, Mohan K, Lee PW, Shmulevitz M, Marshall JS. Human mast cell activation with virus-associated stimuli leads to the selective chemotaxis of natural killer cells by a CXCL8-dependent mechanism. *Blood* 2008;**111**:5467–5476.
- Umetsu SE, Lee WL, McIntire JJ, Downey L, Sanjanwala B, Akbari O et al. TIM-1 induces T cell activation and inhibits the development of peripheral tolerance. *Nat Immunol* 2005;**6**:447–454.
- 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**:455–464.
- Rodríguez-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**:4706–4713.
- 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**:1522–1533.
- Liu T, He SH, Zheng PY, Zhang TY, Wang BQ, Yang PC. Staphylococcal enterotoxin B increases TIM4 expression in human dendritic cells that drives naïve CD4 T cells to differentiate into Th2 cells. *Mol Immunol* 2007;**44**:3580–3587.
- Hulse KE, Reefer AJ, Engelhard VH, Sattinover SM, Patrie JT, Chapman MD et al. Targeting Fel d 1 to FcγRI induces a novel variation of the T(H)2 response in subjects with cat allergy. *J Allergy Clin Immunol* 2008;**121**:756–762.
- Laborde EA, Vanzulli S, Beigier-Bompadre M, Isturiz MA, Ruggiero RA, Fourcade MG et al. Immune complexes inhibit differentiation, maturation, and function of human monocyte-derived dendritic cells. *J Immunol* 2007;**179**:673–681.
- Chen X, Feng BS, Zheng PY, Liao XQ, Chong J, Tang SG et al. Fc gamma receptor signaling in mast cells links microbial stimulation to mucosal immune inflammation in the intestine. *Am J Pathol* 2008;**173**:1647–1656.
- Desai DD, Harbers SO, Flores M, Colonna L, Downie MP, Bergtold A et al. Fc gamma receptor IIB on dendritic cells enforces peripheral tolerance by inhibiting effector T cell responses. *J Immunol* 2007;**178**:6217–6226.
- Ravetch JV, Clynes RA. Divergent roles for Fc receptors and complement in vivo. *Annu Rev Immunol* 1998;**16**:421–432.
- Gessner JE, Heiken H, Tamm A, Schmidt RE. The IgG Fc receptor family. *Ann Hematol* 1998;**76**:231–248.
- Rodríguez-Pérez N, Penagos M, Portnoy JM. New types of immunotherapy in children. *Curr Allergy Asthma Rep* 2008;**8**:484–492.
- Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy. *J Allergy Clin Immunol* 2007;**119**:780–791.
- Heyman B. Regulation of antibody responses via antibodies, complement, and Fc receptors. *Annu Rev Immunol* 2000;**18**:709–737.
- 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**:55–61.
- Plewako H, Holmberg K, Oancea I, Gotlib T, Samoliński B, Rak S. A follow-up study of immunotherapy-treated birch-allergic patients: effect on the expression of chemokines in the nasal mucosa. *Clin Exp Allergy* 2008;**38**:1124–1131.
- Nathan RA. Management of patients with allergic rhinitis and asthma: literature review. *South Med J* 2009;**102**:935–941.
- Wachholz PA, Durham SR. Mechanisms of immunotherapy: IgG revisited. *Curr Opin Allergy Clin Immunol* 2004;**4**:313–318.
- Benitez-Ribas D, Tacke P, Punt CJ, de Vries IJ, Figs CG. Activation of human plasmacytoid dendritic cells by TLR9 impairs Fc gammaRII-mediated uptake of immune complexes and presentation by MHC class II. *J Immunol* 2008;**181**:5219–5224.
- Hirata N, Yanagawa Y, Ebihara T, Seya T, Uematsu S, Akira S et al. Selective synergy in anti-inflammatory cytokine production upon cooperated signaling via TLR4 and TLR2 in murine conventional dendritic cells. *Mol Immunol* 2008;**45**:2734–2742.
- Neurath MF, Finotto S, Glimcher LH. The role of Th1/Th2 polarization in mucosal immunity. *Nat Med* 2002;**8**:567–573.
- Encinas JA, Janssen EM, Weiner DB, Calarota SA, Nieto D, Moll T et al. Anti-T-cell Ig and mucin domain-containing protein 1 antibody decreases TH2 airway inflammation in a mouse model of asthma. *J Allergy Clin Immunol* 2005;**116**:1343–1349.
- Sizing ID, Bailly V, McCoon P, Chang W, Rao S, Pablo L et al. Epitope-dependent effect of anti-murine TIM-1 monoclonal antibodies on T cell activity and lung immune responses. *J Immunol* 2007;**178**:2249–2261.
- Graves PE, Siroux V, Guerra S, Klimecki WT, Martinez FD. Association of atopy and eczema with polymorphisms in T-cell immunoglobulin domain and mucin domain-IL-2-inducible T-cell kinase gene cluster in chromosome 5 q 33. *J Allergy Clin Immunol* 2005;**116**:650–656.
- Wu WK, An YF, Zhao CQ. Immunoglobulin domain and mucin domain-1 in helper T lymphocytes in allergic rhinitis. *Zhonghua Yi Xue Za Zhi* 2008;**88**:3392–3396.
- Wang Y, Meng J, Wang X, Liu S, Shu Q, Gao L et al. Expression of human TIM-1 and TIM-3 on lymphocytes from systemic lupus erythematosus patients. *Scand J Immunol* 2008;**67**:63–70.
- Liu Q, Shang L, Li J, Wang P, Li H, Wei C et al. A functional polymorphism in the TIM-1 gene is associated with asthma in a Chinese Han population. *Int Arch Allergy Immunol* 2007;**144**:197–202.
- McIntire JJ, Umetsu SE, Macaubas C, Hovte EG, Cinniglu C, Cavalli-Sforza LL et al. Immunology: hepatitis A virus link to atopic disease. *Nature* 2003;**425**:576.
- Marsh DG, Neely JD, Breazeale DR, Ghosh B, Freidhoff LR, Ehrlich-Kautzky E et al. Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. *Science* 1994;**264**:1152–1156.
- McIntire JJ, Umetsu SE, Akbari O, Potter M, Kuchroo VK, Barsh GS et al. Identification of Tapr (an airway hyperreactivity regulatory locus) and the linked Tim gene family. *Nat Immunol* 2001;**2**:1109–1116.
- Xu G, Cheng L, Lu L, Zhu Y, Xu R, Yao X et al. Expression of T-cell immunoglobulin- and mucin-domain-containing molecule-1 (TIM-1) is increased in a mouse model of asthma and relationship to GATA-3. *Life Sci* 2008;**82**:663–669.
- Chae SC, Song JH, Heo JC, Lee YC, Kim JW, Chung HT. Molecular variations in the promoter and coding regions of human Tim-1 gene and their association in Koreans with asthma. *Hum Immunol* 2003;**64**:1177–1182.
- Chae SC, Park YR, Song JH, Shim SC, Yoon KS, Chung HT. The polymorphisms of Tim-1 promoter region are associated with rheumatoid arthritis in a Korean population. *Immunogenetics* 2005;**56**:696–701.

38. Skarica M, Wang T, McCadden E, Kardian D, Calabresi PA, Small D et al. Signal transduction inhibition of APCs diminishes th17 and Th1 responses in experimental autoimmune encephalomyelitis. *J Immunol* 2009;**182**:4192–4199.
39. McLachlan JB, Shelburne CP, Hart JP, Pizzo SV, Goyal R, Brooking-Dixon R et al. Mast cell activators: a new class of highly effective vaccine adjuvants. *Nat Med* 2008;**14**:536–541.

Supplementary materials

**Specific immunotherapy suppresses Th2 responses via modulating TIM1/TIM4 interaction on dendritic cells**

Chang-Qing Zhao<sup>\*†</sup>, Tong-Li Li<sup>¶†</sup>, Shao-Heng He<sup>ξ</sup>, Xiao Chen<sup>¶</sup>, Yun-Fang An<sup>\*</sup>, Wen-Kui Wu<sup>\*</sup>, Xiao-Hui Zhou<sup>\*</sup>, Ping Li<sup>¶</sup>, Ping-Chang Yang<sup>¶</sup>.

<sup>\*</sup>Department of Otolaryngology, Head and Neck Surgery, the Second Hospital, Shanxi Medical University, Taiyuan, Shanxi, China. <sup>ξ</sup>The Clinical Experimental Center of the First Affiliated Hospital, Nanjing Medical University, Nanjing, China. <sup>¶</sup>McMaster Brain Body Institute, St. Joseph Health Care and Department of Pathology & Molecular Medicine, McMaster University, Hamilton, ON, Canada

Running title: Suppression TIM4 inhibits Th2 responses

<sup>†</sup>Equally contributed to this paper

Correspondence to: Dr. Chang-Qing Zhao or Dr. Ping-Chang Yang  
Dr. Chang-Qing Zhao, Department of Otolaryntology, the Second Hospital, Shanxi Medical University, Taiyuan, 030001, China. Tel: 351-307-4231; Email: [fahyj@126.com](mailto:fahyj@126.com)

Dr. Ping-Chang Yang, BBI-T3330, 50 Charlton Ave East, Hamilton, ON, Canada L8N 4A6. Tel: (905) 522-1155 ext. 35828. Fax: (905) 540-6593. Email: [yangp@mcmaster.ca](mailto:yangp@mcmaster.ca)

## Methods

**Reagents:** Anti-TIM1 (AF2929), anti-TIM4 (MAB1750), ELISA kit for IL-4 and IFN- $\gamma$  (R&D Systems, Burlington, ON, Canada). Anti-Fc $\gamma$ RI (HI16a) (Abbiotec, San Diego, CA). Anti-Fc $\gamma$ RII (AT10); Anti-Fc $\gamma$ RI (10.1) (Abcam; Cambridge, MA). Magnetic cell sorting reagents (Miltenyi Biotec, Auburn, CA). Der p 1 (Union Hospital Immunolab, Beijing, China). Enhanced chemiluminescent reagents (GE Health care, Baie d'Urfe, QC, Canada). Antibodies against IL-4 (3010.211), CD11c (B-ly6), CCR3 (5E-8), permeable reagent kit (BD Bioscience, Mississauga, ON Canada). Cell culture reagents, precast NuPAGE gel system (Invitrogen, Mississauga, ON, Canada). Nitrocellulose membrane (Bio-Rad, Mississauga, ON, Canada). [ $^3$ H] thymidine (GE Healthcare, Baie d'Urfe, QC, Canada). Endotoxin levels in all reagents were detected using the limulus assay (Limulus amebocyte lysate QCL 1000, Bio Whittaker, Walkersville, MD, USA). The reagents used in this study contained <0.2U endotoxin/10  $\mu$ g reagents. IgE kit (R&D System China, Shanghai, China).

**RNAi:** Human siRNA of TIM4, TNF- $\alpha$ , IL-12, Fc $\gamma$ RI, Fc $\gamma$ RII was designed and synthesized by Invitrogen. The sequences of siRNA are available upon request. Transfection was performed following our established procedures (19). Briefly, siRNA was added to culture of DCs ( $10^6$  cells/ml) at a concentration of 60 pMol. The efficiency of siRNA transfection was determined by Western blotting. The peak inhibitory effect was reached 48 h post-transfection that sustained for another 48 h and declined thereafter;

the expression of TIM4 and TNF- $\alpha$  in these DCs returned to normal levels 4 days after transfection. The control siRNA did not affect TIM4 and TNF- $\alpha$  expression in DCs. The transcription efficiency was over 90% and the inhibition was also over 90% and reproducible in all experiments. The sequences of siRNA were listed below:

**siRNA sequences**

| Molecules       | siRNA sequences         | NCBI number |
|-----------------|-------------------------|-------------|
| TIM4            | acactccagtgtcaaaata     | NM_138379   |
| TNF-alpha       | aatggcgtggagctgagagataa | NM_000594   |
| IL-12           | tgaccctgcagctctacaa     | U03187      |
| Fc $\gamma$ RI  | gaaatgtcaggaacaaaaa     | NM_000566   |
| Fc $\gamma$ RII | acagcaggattgttaaaaa     | NG_012066   |
| Scramble siRNA  | ggacatatgggtagggatcaaga |             |

**TIM4 mRNA sequence:**

Forward: gaaactgtcctccccagtga.  
Reverse: ccaggctgaggagaagacac.

**LightShift chemiluminescent EMSA.** The reagent kit of lightShift chemiluminescent EMSA was purchased from Thermo Scientific (Chicago, USA). TIM4 promoter sequence probe was synthesized by the Research Center at Shanxi Medical University and labeled with biotin. Biotin end-labeled duplex DNA is incubated with a protein extract and electrophoresed on a native gel. The DNA is then transferred to a positive nylon membrane, UV-crosslinked, probed with streptavidin-HRP conjugate. HRP enzymatic

reaction was detected with enhanced chemiluminescent reagents and recorded with X-ray films.

### ChIP assay

Isolated peripheral DCs were fixed by 1% formaldehyde at room temperature for 10 min. After washing, cells were incubated with the lysis buffer for 20 min at 4 °C. The chromatin was sheared by sonication (Branson 250) at a power setting of 2 and 30% duty cycle. Input samples were stored at -20°C until use. Other samples were precipitated by anti-acetyl histone H4 antibody (Upstate Biotechnology, Lake Placid, NY). A part of the samples was used for the detection of acetyl histone H4 and activated AP-1 by immune blotting. DNA was isolated by phenol/chloroform extraction. 1 µl of immunoprecipitated DNA was used for each real time PCR. The following primers specific for the TIM4 promoter -1458- to -1347-bp (122 bp) region were used: forward primer (5'-aggctgcttagccacatg-3') and reverse primer (5'-gtgggaggagggggca-3').

### Symptom Scoring

Subjects recorded symptom scores daily during the study period at baseline and while on SIT treatment. Subjects rated symptoms of runny nose, stuffy nose, itchy nose, sneezing, on a scale of 0 to 4. A score of 0 represented no symptoms, 1 was mild symptoms, 2 was moderate symptoms, 3 was between 2 and 4, and 4 was severe symptoms. In each evening, subjects were instructed to record their symptoms reflecting the worst part of their day. Daily symptom scores were averaged each week. To calculate average

1  
2  
3 symptom and medication scores for each season, the total symptom scores for the study  
4  
5 period were calculated, added together, and divided by number of days.  
6  
7  
8  
9

10 **Serum antibody measurements**

11  
12 Serum levels of specific IgE and IgG4 were determined by ELISA. Plates were coated  
13  
14 with Der antigen (5 µg/ml) overnight at 4°C and then incubated with patient sera (1:10  
15  
16 dilution) at room temperature for 1 h. After washing, biotinylated anti-human IgG4 or  
17  
18 IgE mAb was added to the plates and incubated for another 1 h at room temperature. The  
19  
20 immune products were developed by horseradish peroxidase-labeled streptavidin  
21  
22 (1:1000) and recorded by a microplate reader (Bio-Tek ELx800™, London, UK) at  
23  
24 wavelength 450 nm. The standards for IgG4 and IgE ELISAs consisted of a pool of equal  
25  
26 volumes of sera from 10 AR patients before SIT. The highest standard serum was given  
27  
28 an arbitrary value of 100 U and the corresponding units for the test serum are read from  
29  
30 the standard curve as arbitrary units per ml (AU/mL).  
31  
32  
33  
34  
35  
36  
37  
38

39 **DC and CD4 T cell preparation.** Peripheral mononuclear cells (PBMC) were isolated  
40  
41 by density gradient centrifugation. For DC separation, PBMC were incubated with  
42  
43 magnetic beads coated anti-CD11c/MHC II antibodies. For separation of CD4<sup>+</sup>CD25<sup>+</sup>  
44  
45 and CD4<sup>+</sup>CD25<sup>-</sup> T cells, negative selection were performed by incubating the positively  
46  
47 selected CD4<sup>+</sup> T cells with anti-CD8, anti-B220, anti-MHC class II antibodies, followed  
48  
49 by depletion with magnetic beads coated second antibodies. Cells were then incubated  
50  
51 with a biotinylated anti-CD25 mAb, and positively selected using streptavidin-conjugated  
52  
53 microbeads; the remainder were CD4<sup>+</sup>CD25<sup>-</sup> T cells. Cell separation was performed on  
54  
55  
56  
57  
58  
59  
60

MS columns. Cell purity was checked with FACS. The purity of isolated cells was over 95%.

For Peer Review

Results

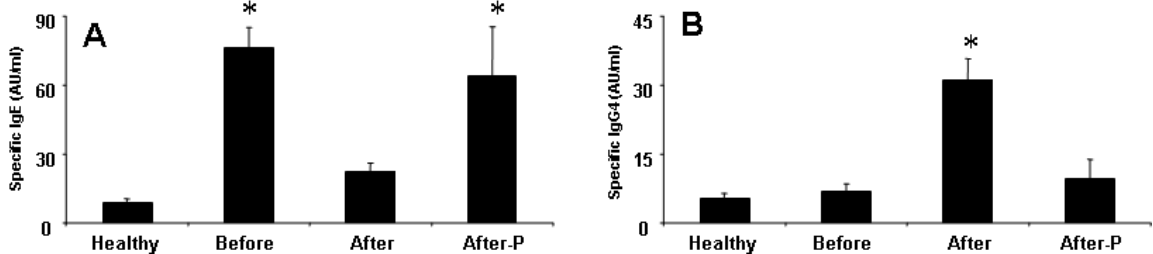


**Fig.S1.SIT modulates the serum levels of IgE and IgG4 in AR patients.** Sera were collected from 20 AR patients an 10 healthy controls and analyzed by ELISA.. Bars indicate the levels of Der-specific IgE (A) and Der-specific IgG4 (B). \*, p<0.05, compared with healthy controls. After-P, 5 AR patients were treated with placebo instead of specific antigen Der.

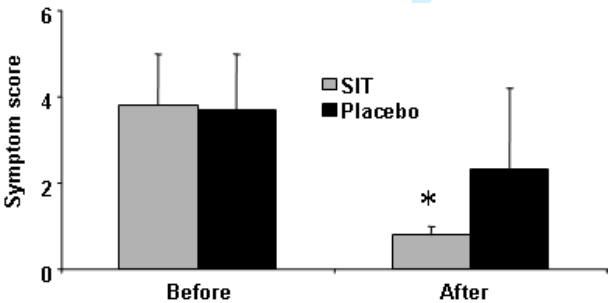


**Fig.S2. SIT reduces the AR clinical symptom score.** AR clinical symptom score was recorded before and after SIT with specific antigen Der or placebo. Bars indicate the difference of clinical score after the treatment. \*, p<0.05, compared with SIT group.

**Table 1. Demographic data**

|                  |        | Allergic Rhinitis  | Healthy Control         |
|------------------|--------|--------------------|-------------------------|
| Gender           | Male   | 10                 | 10                      |
|                  | Female | 10                 | 10                      |
| Age              |        | 18~42 (mean=28.6)  | 20~40 (mean=24.5)       |
| Total serum IgE  |        | 560 ± 160 U/ml     | 105 ± 86 U/ml           |
| Der specific IgE |        | 334.8 ± 112.4 U/ml | Under detectable levels |
| Duration         |        | 5.3~15.7 years     | -                       |
| Nasal polyposis  |        | No                 | No                      |
| Nasal surgery    |        | No                 | No                      |