



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

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Abstract

It has been suggested that T-cell immunoglobulin- and mucin-domain-containing molecule-1 (TIM-1) plays an important role in the development of allergic asthma, though its molecular mechanism remains unclear. Our aim was to examine the expression of TIM-1 and Th2-associated transcription factor GATA-3 in asthmatic mice, and to evaluate the correlation of TIM-1 and GATA-3 in the pathogenesis of allergic asthma. We examined TIM-1 expression in lung tissue and peripheral blood mononuclear cells (PBMCs) of asthmatic mice by real-time PCR and flow cytometry, respectively; we also investigated TIM-1 and GATA-3 expression in the spleen tissue of asthmatic mice by western blot. These results demonstrate that TIM-1 was significantly increased in pulmonary tissues and PBMCs in asthmatic mice after ovalbumin (OVA) challenge ($P < 0.05$), and that the production of TIM-1 as well as GATA-3 was upregulated in the spleen of asthmatic mice. The production of TIM-1 correlated significantly with the production of GATA-3 in the spleen of asthmatic mice ($r = 0.753$, $P < 0.05$). The results of this study provide the first evidence that increased expression of TIM-1 in asthmatic mice is associated with the Th2-associated transcription factor GATA-3. The findings suggest a possible mechanism for how HAV infection and TIM-1 upregulation influence the development of allergic asthma.

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Introduction

Asthma is an immunological disease caused by Th2-driven inflammation, which is characterized by increased mucus production in the lung, and by an inflammatory process involving eosinophils, basophils, mast cells, and Th2 lymphocytes, among others (Umetsu et al., 2002). The immunopathological hallmark of allergic asthma is the infiltration of eosinophils and Th2 cells in affected tissue. Immune deviation

in favor of Th2 is prominent in allergic asthma (Kay, 2001), but the immunologic mechanism remains unclear.

During the past few decades, the prevalence of allergic diseases has increased remarkably in Western countries indicating the importance of environmental influences (Holgate, 1999). It has been proposed by the hygiene hypothesis that the developing immune system is deprived of the microbial antigens that stimulate Th1 cells, because the environment is relatively clean and the use of antibiotics for minor illness in early life is widespread (Strachan, 1989). Correspondingly, epidemiologic data reveal that allergic diseases are less common among children who have had measles and hepatitis A, which suggests that repeated immune stimulation may protect against allergies (Shirakawa et al., 1997; Matricardi et al.,

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2002). For example, Matricardi and coworkers showed that exposure to hepatitis A is negatively associated with sensitization to any allergen except for cockroach, which is positively associated with the presence of antibodies to hepatitis A and represents one of the major etiological factors in asthma in US inner cities (McIntire et al., 2004). However, how hepatitis A virus (HAV) affects the development of asthma is not clear as yet.

Recently, a new gene encoding human hepatitis A virus cellular receptor (HAVCR), which was also identified as T-cell immunoglobulin- and mucin-domain-containing molecule-1 (TIM-1), has attracted much attention in the field of allergy and asthma (McIntire et al., 2004). TIM-1 is a type I transmembrane protein, with extracellular immunoglobulin and mucin domains and intracellular domains of various lengths. TIM-1 was first identified in the African green monkey, and later in human, as HAVCR, the receptor exploited by hepatitis A for viral entry. TIM-1 may be of relevance for asthma and allergic diseases for many reasons. For example, *Tapr*, a genetic locus regulating susceptibility to asthma or airway hyperactivity was recently mapped to the *Tim* locus (McIntire et al., 2001). It was reported that the frequency of the homozygous deletion variant (157delMTTTPV) in the fourth exon of the TIM-1 gene was higher among patients with asthma than in controls, suggesting that the genetic variants of TIM-1 contribute to asthma susceptibility in this African-American population (Gao et al., 2005) and in a Korean population (Chae et al., 2003a,b). Moreover, Chae and coworkers suggested a direct correlation between certain TIM-1 allelic variants in exon 4 of the gene and susceptibility to atopic diseases in a Korean population (Chae et al., 2003a,b), but Noguchi and colleagues have reported that insertion/deletion coding polymorphisms in hHAVcr-1 are not associated with atopic asthma in the Japanese population (Noguchi et al., 2003).

These genetic studies in mice and humans, as well as epidemiological data that link infection with HAV and allergic diseases, indicate that TIM-1 has an important immunological function. In addition to the genetic linkage to asthma in mice, there are several lines of evidence suggesting that TIM-1 is involved in Th cell differentiation and immune regulation. First, TIM-1 mRNA is up-regulated by CD4⁺ T cells within 7 h of stimulation, a time when Th cells are undergoing differentiation to become effector cells. Also, retroviral transduction of the gene for Tim-1 in T cells, followed by T cell activation and differentiation, resulted in increased frequencies of cells producing IL-4, but not IFN- γ (de Souza et al., 2005). Second, it was reported that TIM-1 mRNA was expressed by Th2 cells but not Th1 cells in human and mouse. Furthermore, TIM-1 ligation enhanced proliferation of T cells and, in Th2 cells, production of IL-4. In vivo administration of anti-TIM-1 antibodies increased production of both IL-4 and IFN- γ in unpolarized cells (Umetsu et al., 2005). To investigate whether TIM-1 is associated with early expression of Th2 cytokines in the pathogenesis of asthma, we examined the expression of TIM-1 in a mouse model of asthma in this study; we also evaluated the association of transcription factor GATA-3 with TIM-1 in the development of allergic asthma.

Materials and methods

Sensitization and challenge of animal model

Female BALB/c mice (6–8 weeks of age, $n=14$) were provided by the Experimental Animal Center of Nanjing Medical University and housed in a specific-pathogen free (SPF) facility. All animal experiments were performed in compliance with a protocol approved by an institutional animal care and use committee.

Mice were divided into two groups ($n=7$ per group): control and asthma groups. The animal model of asthma was established according to previous reports (Kitada et al., 2001; Shen et al., 2006). Briefly, active sensitization was performed by injection of 100 μ g of ovalbumin (OVA) (Sigma, St Louis, MO, USA) in 2 mg of alum intraperitoneally on days 0 and 7. Mice in the control group were treated with saline only containing 2 mg of alum instead. One week after the second injection, mice were challenged with nebulized 1% OVA or saline (for control mice) for seven consecutive days.

Histological examination

Mouse pulmonary tissues were removed from mice 24 h after the final challenge, fixed with 10% formalin, and embedded in paraffin. Five-micrometer-thick sections were then made and stained with hematoxylin and eosin, rinsed, and examined by means of a light microscope with an eyepiece graticule at $\times 100$ or $\times 400$ magnification. To determine the number of stained cells in 1 mm² of tissue, ten randomly chosen reticules (10 $\times$ 0.1 mm²) of a single section were considered by two independent operators in a blinded fashion. A total of five sections from each sample were employed.

Enzyme-linked immunosorbent assay (ELISA)

Cytokine concentration was determined by ELISA. One hundred milligrams of fresh pulmonary tissue was weighed, chopped into 1 mm³ pieces, and homogenized in 1 ml 0.9% sodium chloride solution on ice. Suspension was centrifuged at 4 °C at 3000 rpm for 10 min, and supernatant was stored at –20 °C until used. IFN- γ and IL-4 cytokine concentrations were measured in duplicate by using a commercially available solid-phase sandwich ELISA kit (Invitrogen, Carlsbad, CA, USA) and set according to the manufacturer's instructions. Assays were performed in duplicate. Results are expressed in pg/mg tissue.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription was performed and the cDNA was synthesized from 2 μ g of total RNA by using an oligo (dT)18 primer and M-MLV reverse transcriptase (Takara, Syuzou, Shiga, Japan) for quantitative PCR.

Expression of mRNA was determined using an ABI PRISM 7300 Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix TaqTM (Takara, Syuzou, Shiga, Japan)

as described in our previous report (Xu et al., 2007). Briefly, the sequences of the primer were as follows: TIM-1 (GenBank accession No. NM134248) forward 5'-TTC TCC CAG GCA CTG TGG AT-3', reverse 5'-CAG GAATCT CCA CTC GAC AA-3'; β -actin (GenBank accession No. NM007393) forward 5'-CAT TGT GAT GGA CTC CGG AGA CGG-3', reverse 5'-CAT CTC CTG CTC GAA GTC TAG AGC-3'. PRISM samples contained 1 $\times$ SYBR Green Master Mix, 1.5 μ l of 5 μ M primers, and 25 ng synthesized cDNA in a 25- μ l volume. Reactions were heated to 95 $^{\circ}$ C for 10 min followed by 35 cycles of denaturation at 95 $^{\circ}$ C for 10 s, annealing at 56 $^{\circ}$ C for 5 s, and extension at 72 $^{\circ}$ C for 20 s. All PCR reactions were performed in duplicate. Melting curve analysis was used to control for amplification specificity.

Expression of TIM-1 was expressed as fold increase or decrease relative to the expression of β -actin. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (Ct). The amount of gene expression was then calculated as the difference (Δ Ct) between the Ct value of TIM-1 and the Ct value of β -actin. Fold changes in TIM-1 mRNA were determined as $2^{-\Delta\text{Ct}}$.

To confirm the specificity of the PCR reaction, PCR products were analyzed with 2% agarose gel electrophoresis and ethidium bromide staining, followed by visualization with an UV transilluminator.

Flow cytometric analysis

Peripheral blood was sampled in sodium citrate-containing cell preparation tubes and peripheral blood mononuclear cells (PBMCs) were isolated by lymphoprep density gradient separation. Flow cytometric analysis was performed according to manufacturer's instructions. Briefly, after washing with staining buffer, cell suspensions were labeled by biotin anti-mouse TIM-1 mAbs (eBioscience, San Diego, CA, USA). The samples were incubated in the dark at 4 $^{\circ}$ C for 30 min, washed twice with PBS, and labeled with streptavidin-phycoerythrin (PE)-conjugated secondary antibody (eBioscience). After the cells were washed and resuspended in 0.2 ml of PBS, cell fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA), and data were

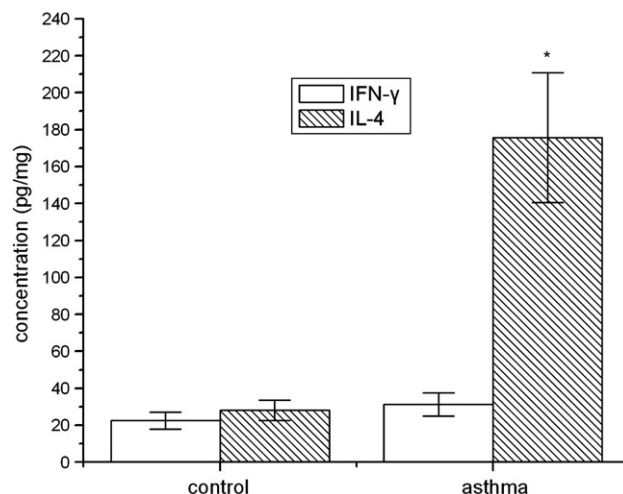


Fig. 2. Average concentration of IL-4 and IFN- γ measured by ELISA in allergic pulmonary specimens after OVA challenge.

analyzed using CellQuest software (BD Biosciences). Relative TIM-1 expression in the cells was quantified by determination of the normalized mean fluorescence intensity (MFI).

Western blot

One hundred milligrams of fresh spleen tissue was weighed, chopped into 1 mm³ pieces, and then lysed and stored at -80° C until needed. The protein concentration was determined by the Bradford method. Each sample contained 50 μ g of protein and was boiled and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% Tris-glycine gels; it was then transferred electrophoretically to polyvinylidene fluoride membrane, which was incubated with 5% fat-free skimmed milk in Tween buffered Solution (TBS) containing 0.05% Tween 20 (1 h, room temperature) and incubated (overnight, 4 $^{\circ}$ C) with rabbit-anti-mouse TIM-1 (Imgenex, San Diego, CA, USA), GATA-3 and β -actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at dilutions of 1:1000. The membrane was washed twice with TBS containing 0.05% Tween 20 and then incubated with horseradish peroxidase-

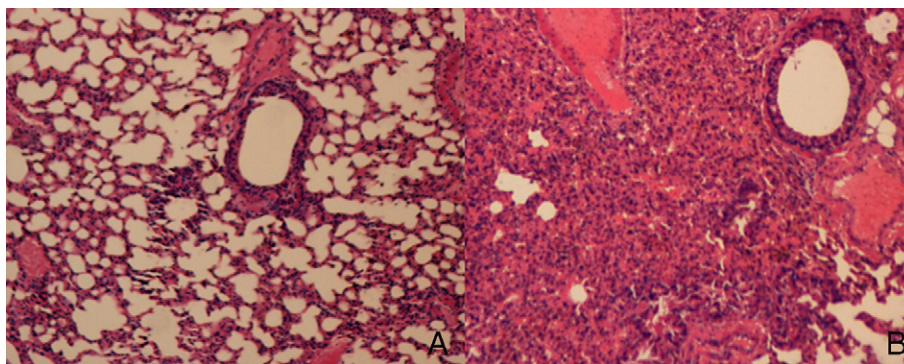


Fig. 1. Increased inflammatory cells were detected in allergic pulmonary specimens after OVA challenge (HE, original magnification 200 $\times$) (A control mice; B asthmatic mice).

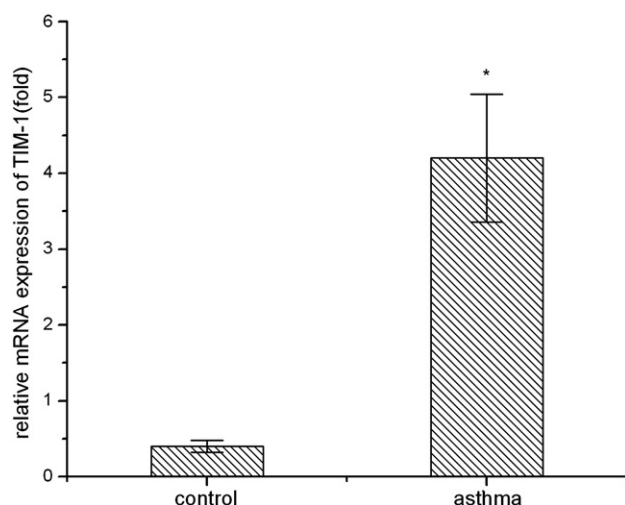


Fig. 3. Expression of TIM-1 mRNA was measured by real-time quantitative RT-PCR in the pulmonary tissues of asthmatic mice.

linked secondary antibody (goat anti-rabbit IgG, 1:1000). The membrane was washed twice with TBS. Immunoreactivity was determined using the ECL chemiluminescence reaction kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions and exposed on medical film. The band density was quantified with Bio-Rad Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data are expressed as means ± SEM. The statistical significance of the differences was assessed with the Student *t* test. Correlation coefficients were determined by using the Spearman rank correlation test. Differences with a *P* value of less than 0.05 were considered significant.

Results

Histological examination of pulmonary inflammation

Pulmonary tissues were obtained from 7 mice sensitized and challenged with OVA and 7 normal controls. Increased numbers

of inflammatory cells were detected in allergic pulmonary specimens compared with controls (Fig. 1). Inflammatory cells were significantly higher in asthmatic mice ($37.1 \pm 8.2/\text{mm}^2$) compared with normal control subjects ($4.9 \pm 0.3/\text{mm}^2$), with significant difference ($P < 0.05$).

ELISA

The average concentration of IFN- γ was 31.3 ± 9.9 pg/mg in the asthmatic group and 22.5 ± 10.9 pg/mg in the control. The average concentration of IL-4 was 175.6 ± 12.7 pg/mg in the asthmatic group and 28.1 ± 5.5 pg/mg in the control. The asthmatic mice showed markedly increased production of IL-4 but not IFN- γ after ovalbumin challenge (Fig. 2). There was a significant difference in the production of IL-4 in asthmatic mice compared with normal control subjects ($P < 0.05$).

Real-time RT-PCR

Expression of TIM-1 mRNA was measured by real-time quantitative RT-PCR in the pulmonary tissues of mice. RT-PCR analysis of total RNA extracted from samples demonstrated the presence of TIM-1 and β -actin in all subjects. After normalization of the data according to the expression of β -actin mRNA, relative expression levels of TIM-1 were presented as fold numbers. There was markedly increased production of TIM-1 in asthmatic mice compared with normal control subjects (4.2 ± 0.8 vs 0.4 ± 0.1), with significant difference ($P < 0.05$) (Fig. 3).

Flow cytometric analysis

A representative result of flow cytometric analysis of TIM-1 expression is shown (Fig. 4). Background fluorescence was determined by incubation of the cells with PE-conjugated secondary antibody alone (Fig. 4 A). Surface expression of TIM-1 in the control group (Fig. 4 B) or asthmatic group (Fig. 4 C) was determined by subtracting the MFI of the background. The relative TIM-1 expression (MFI) was 25.7 ± 9.8 in the asthmatic group, as compared to 6.4 ± 3.2 in the control group (Fig. 5), with significant difference ($P < 0.05$).

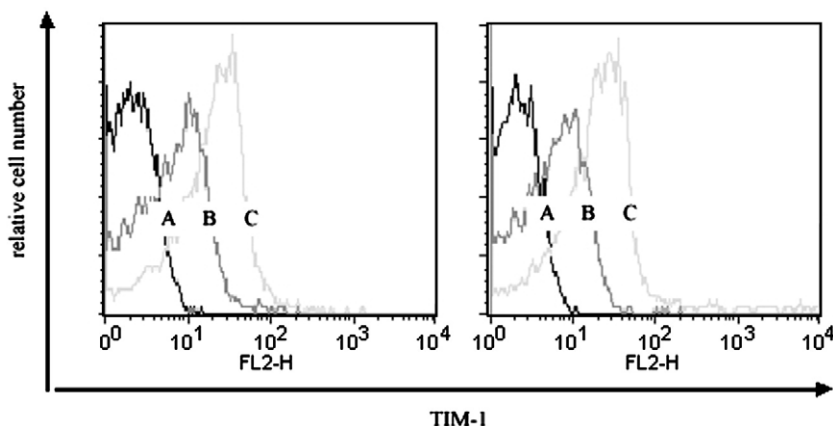


Fig. 4. Representative example of TIM-1 in PBMCs of asthmatic mice detected by flow cytometry. (A background; B control mice; C asthmatic mice).

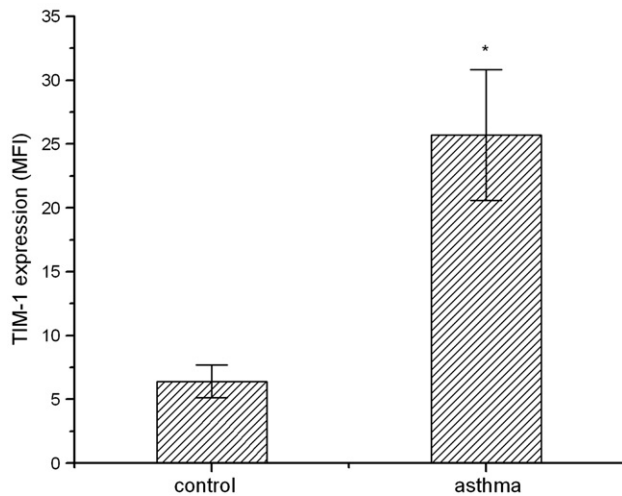


Fig. 5. The relative TIM-1 expression in PBMCs of asthmatic mice detected by flow cytometry.

Western blot

The relative amount of target protein was quantified as the density of the target protein vs β -actin. After normalization of the data according to the expression of β -actin protein, the relative density of TIM-1 is presented as fold numbers (Fig. 6). The mean values of normalized TIM-1 and GATA-3 were 0.31 ± 0.11 and 0.14 ± 0.03 in asthmatic subjects, while in the control subjects the values were 0.1 ± 0.03 and 0.06 ± 0.01 , respectively. There was a significant difference in the production of TIM-1 as well as GATA-3 in asthmatic mice compared with normal control subjects ($P < 0.05$) (Fig. 7).

Correlation of TIM-1 and GATA-3 expression in asthmatic mice

The production of TIM-1 as well as GATA-3 was up-regulated in asthmatic mice. The production of TIM-1 correlated significantly with the production of GATA-3 in the spleen tissue of asthmatic mice ($r = 0.753$, $P < 0.05$) (Fig. 8).

Discussion

In the past few decades, the prevalence of asthma and all atopic diseases has essentially doubled in industrialized countries, while the incidence of a number of infectious diseases has decreased dramatically. This inverse relationship between

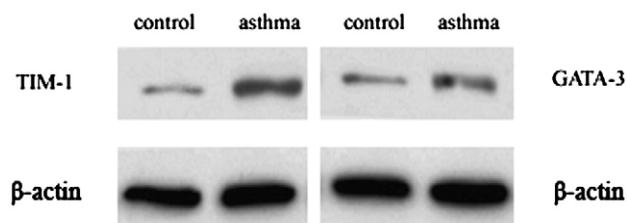


Fig. 6. Representative results of TIM-1 and GATA-3 in the spleen tissues of asthmatic mice detected by western blot.

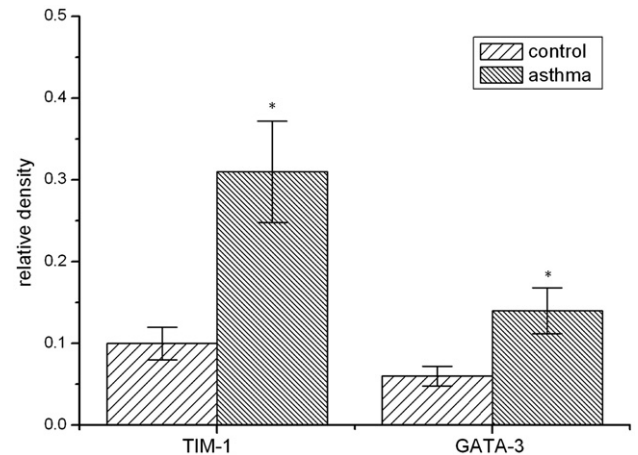


Fig. 7. The relative TIM-1 and GATA-3 expression in the spleen tissues of asthmatic mice detected by western blot.

infection and atopy is the basis for the “hygiene hypothesis,” but the specific infectious agents responsible for this relationship are not fully known (Ramsey and Celedon, 2005).

The hygiene hypothesis has given an immunological framework, where there is a pivotal balance between Th1 (associated with bacterial and virus infections and autoimmune diseases) and Th2 (associated with helminth infections and allergic diseases) immune responses (Romagnani, 2004). This hypothesis has now gained wide acceptance as the most plausible explanation for the increase in the prevalence of allergic diseases. On the other hand, although epidemiological data suggest that exposure to HAV reduces the risk of asthma, it is still difficult to interpret how HAV affects the balance between Th1 and Th2 subpopulations. This study shows that gene expression of TIM-1 is significantly increased in the airway of an asthmatic mouse model compared with normal control subjects, in addition to GATA-3, which is demonstrated in other reports (Christodouloupoulos et al., 2001; Erpenbeck et al., 2006). Our results confirm the role of TIM-1 in the pathogenesis of allergic asthma. To our knowledge, this is the first report about the association of TIM-1 and the Th2-associated transcription

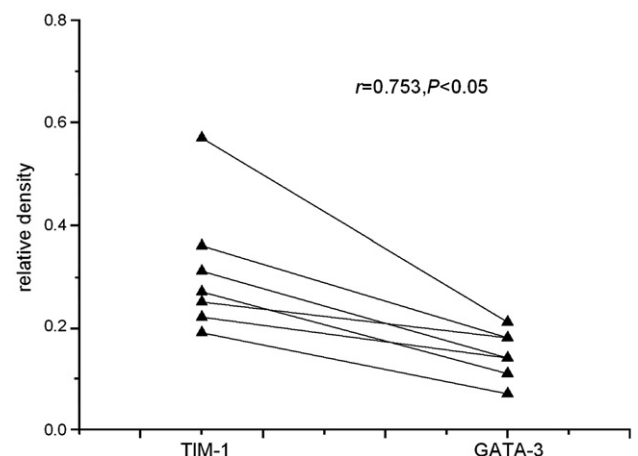


Fig. 8. Correlation of TIM-1 and GATA-3 expression in the spleen tissue of asthmatic mice.

factor GATA-3 in asthma, which implies that TIM-1 may be involved in the differentiation of Th cells.

Asthma is a chronic disease associated with reversible airway obstruction, bronchial hyperreactivity and airway eosinophilia, and Th2 cells play a critical role in eosinophilia and pulmonary dysfunction. According to the classification by Mosmann and coworkers, Th1 cells produce IL-2 and promote cellular immune responses, whereas Th2 cells produce IL-4, IL-5 and IL-13, which are associated with allergic diseases. IL-4 drives naïve Th cells toward the Th2 phenotype and induces B cells to switch isotype to IgE. IL-5 is essential for eosinophil differentiation, accumulation at the sites of inflammation and survival (Mosmann et al., 1986). It is well accepted that production of IL-4 and IL-5 is mediated by the transcription factor GATA-3. GATA-3 appears particularly important for development of the Th2 subpopulation (Ray and Cohn, 1999). Nakamura and colleagues reported a significant increase in GATA-3 expression in asthmatic airways compared with that in control subjects (Nakamura et al., 1999). In keeping with this, Finnoto and coworkers demonstrated that blockage of GATA-3 suppressed the production of several Th2 cytokines and mucus production, and prevented airway inflammation in a murine model of asthma in vivo (Finnoto et al., 2001).

In the present study, we have examined the expression of TIM-1 in OVA-sensitized mice challenged intranasally with OVA. We have also examined the pulmonary tissues and evaluated pulmonary inflammation by histological examination. In this study, increased inflammatory cells (mainly lymphocytes and eosinophils) have been detected in allergic pulmonary specimens compared with controls, which confirmed the allergic response in asthmatic mice. Based on these findings, we have examined IFN- γ and IL-4 production in the pulmonary tissue by ELISA, the expression of TIM-1 in pulmonary tissues by RT-PCR, and the surface expression of TIM-1 in PBMCs by means of flow cytometry. In addition, we have examined the expression of TIM-1 as well as GATA-3 in the spleen tissue by western blot.

Our results have clearly indicated that accumulation of eosinophils and lymphocytes occurred in the pulmonary tissues of asthmatic mice, and that IL-4 was upregulated significantly in asthmatic mice after OVA challenge. Our results have also demonstrated that TIM-1 was upregulated significantly in asthmatic mice after OVA challenge, which was detected by real-time RT-PCR and flow cytometry both in pulmonary tissues and PBMCs.

As a type I membrane protein, TIM-1 has a transmembrane domain, an intracellular domain, and a tyrosine phosphorylation motif, suggesting that it is involved in transmembrane signaling. In mice, TIM-1 encodes a 305-amino acid protein, which may regulate the development of asthma and allergies by preventing respiratory tolerance. Considering the genetic studies in mice and humans, as well as the epidemiological data that link infection with HAV and allergic diseases, it would be of interest to know how TIM-1 affects the development of allergic inflammation.

Recent studies have examined the expression and function of murine TIM-1 in some detail by using an anti-TIM-1 antibody. Encinas et al. (2005) found that administration of an anti-TIM-1

antibody to mice during OVA sensitization (Day 11, 13 and 15) results in a significant decrease in inflammatory cells in bronchoalveolar lavage fluid and a lower production of Th2 cytokines. These results indicate that anti-TIM-1 might offer a novel approach to treating asthma (Encinas et al., 2005). However, the study by Umetsu et al. (2005) reports that activated CD4⁺ T cells had a high expression of TIM-1. By using a monoclonal antibody specific for mouse TIM-1 before OVA sensitization, they showed that crosslinking of TIM-1 on CD4⁺ T cells provided a stimulatory signal that greatly enhanced T cell proliferation as well as the production of IL-4 and IFN- γ . They believed that TIM-1 alone provides a strong costimulatory signal for T cell activation that augments the TCR and CD28 signal (Umetsu et al., 2005).

These contradictory results may primarily be due to differences in study design (in vitro or in vivo trial, antibody administrated at different time points) rather than differences in the function of the antibodies used. Thus the effects of anti-TIM-1 on Th2 immune response may be related to the developmental state of the antigen responding cells on exposure to the antibody or related to the state of the antigen and antigen present cells during the treatment.

Our results in this study provide the first evidence of increased expression of TIM-1 in asthmatic mice in association with the Th2-associated transcription factor GATA-3. The findings of elevated TIM-1 levels in asthmatic mice and a correlation between TIM-1 and GATA-3 expression support the notion that TIM-1 may be associated with the differentiation of Th2 cells and the development of allergic asthma, suggesting the new approach that TIM-1 influences the inflammatory cascades of allergic asthma. However, it is not clear how TIM-1 is associated with GATA-3 transcription and Th2 cell differentiation. We believe that further study is still needed to clarify this.

Conclusion

This study shows that both TIM-1 and GATA-3 were increased in experimental asthma. It also provides a correlation between increased TIM-1 and GATA-3 in the allergic asthma model, which may partially explain the inverse linkage between infection of HAV and risk of asthma. To confirm the role of TIM-1 in the pathogenesis of asthma, further studies to investigate the signaling pathway of TIM-1 regulation are still necessary.

Acknowledgement

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