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ORIGINAL ARTICLE

Expression of the Th1-Specific Cell-Surface Protein Tim-3 Increases in a Murine Model of Atopic Asthma

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T-cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) is preferentially expressed on Th1-helper type T-cells and functions to repress the Th1-mediated immune response. However, the role of Tim-3 during the inflammatory pathogenesis of asthma remains unclear. This study determines the expression level of Tim-3 in CD4⁺ T-cells within the peripheral blood and bronchoalveolar lavage fluid (BALF) isolated from a murine model of atopic asthma and explores the potential role of Tim-3 during the inflammatory response. Mice were randomly divided into normal control, asthma day 1, and asthma day 7 groups, and peripheral blood T lymphocytes and BALF cells were collected. The ratio of Tim-3⁺/CD4⁺ cells among the total CD4⁺ cell populations from peripheral blood and BALF was determined by flow cytometry, and the expression of the Tim-3 mRNA was determined by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). In contrast with the normal control group, the ratio of Tim-3⁺/CD4⁺:CD4⁺ cells and the level of Tim-3 mRNA in both the peripheral blood T lymphocytes and BALF cells among the asthma day 1 and asthma day 7 groups were significantly increased ($p < 0.01$), and those in the asthma day 7 group were higher than the asthma day 1 group ($p < 0.05$). There was also a positive correlation between the ratio of Tim-3⁺/CD4⁺:CD4⁺ detected in BALF and that the ratio detected in peripheral blood T lymphocytes ($r = 0.84$, $p < 0.01$). Therefore, the expression of Tim-3 is increased in CD4⁺ T-cells following airway challenge and likely affects asthma-induced inflammation by repressing the Th1-mediated immune response.

Keywords asthma; inflammation; T lymphocyte; Th1/Th2 cells; Tim-3

INTRODUCTION

Naive helper T-cells are activated by signaling from antigen-presenting cells (i.e., dendritic cells), resulting in cell division and differentiation. Depending on the signals received, these cells can differentiate into a number of sub-lineages, including the CD4⁺ helper T-cells, Th1 and Th2, which function to orchestrate the host defence against invading pathogens (1–5). Th1 cells mediate the immune response against intracellular pathogens such as viruses and function to maximize the killing efficacy of macrophages and promote the proliferation of cytotoxic CD8⁺ T-cells (6). Th1 cells are able to affect these responses via the production of cytokines such as interferon- γ (IFN- γ), interleukin (IL)-2, lymphotoxin, and tumor necrosis factor- α (TNF- α) (7). In contrast, Th2 cells mediate the immune response to extracellular pathogens such as parasitic worms and do so by stimulating B-cell proliferation, increasing antibody production, and promoting eosinophilic inflammation. Th2 cells mediate these responses by producing the cytokines IL-4, IL-5, IL-6, IL-10, and IL-13 (7).

The cytokines produced by a given helper T-cell subset not only dictate the type of immune response, but they also function as effective negative regulators of the other subset by inhibiting their development (3, 7). Specifically, IFN- γ produced by Th1 cells can inhibit Th2 differentiation, and IL-4 produced by Th2 cells can inhibit Th1 differentiation (3). This type of cross-suppression allows for a significant amplification of a given T-cell response. However, preventing an inappropriately strong or prolonged Th-specific response requires regulatory pathways that mediate the re-establishment of the Th1/Th2 balance, as an uncontrolled response can have distinct and severe pathological effects. The pathological consequences of an inappropriate Th1 response include delayed-type hypersensitivity reactions and induction of organ-specific autoimmune diseases (8), whereas an inappropriate Th2 response can result in allergies and/or asthma (8–13).

Asthma is a chronic illness characterized by persistent bronchial hyper-reactivity in response to environmental allergens, resulting in obstructed breathing. In the airway, a balanced response between Th1 and Th2 cells prevents an inappropriate reaction against common environmental antigens. However, recent reports indicated that an abundance of Th2 specific cytokines, such as IL-4, IL-5, IL-9, and IL-13, is the initiator of the asthmatic response (9–11). Because this adverse reaction is due to an imbalance between the Th1 and Th2 responses in the airway, either enhancing the Th1

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response or inhibiting the Th2 response might be implemented as a novel treatment for asthma (14). Therefore, knowing how the Th1 and Th2 T-cell responses are regulated is vital for the understanding and treatment of this disorder.

One of the best-characterized Th-cell regulatory proteins is the T-cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3). Tim-3 is a 281-amino-acid transmembrane protein predominantly expressed on the surface of terminally differentiated Th1 cells (15–17). Tim-3 was originally identified following extensive screening of antibodies to identify specific cell surface markers of Th1 or Th2 cells (18). Several recent reports indicate that Tim-3 functions as a potent inhibitor of the Th1 immune response. First of all, expression of the Tim-3 mRNA correlates with the activation of Th1 cells in the animal model for experimental autoimmune encephalomyelitis (15) and with the increased expression of the Th1-specific cytokines in patients with multiple sclerosis (19). Furthermore, treatment with a Tim-3-Ig fusion protein, which blocks Tim-3 function, results in increased proliferation of Th1 cells and production of Th1-specific cytokines (i.e., IFN- γ) (17). Also, anti-Tim-3 antibody treatment has been shown to enhance IFN- γ expression in control subjects (20). Similarly, Tim-3 knockout mice display hyperproliferation of Th1 cells and subsequent increased production of Th1-specific cytokines (16). Finally, treatment with galectin-9, the Tim-3 ligand, induced the cell death of Th1 cells in vitro, and administration of galectin-9 in vivo resulted in suppression of Th1 immunity (21). Together these results strongly indicate that the Tim-3/galectin-9 pathway serves to suppress Th1 immunity and does so by eliminating Th1 cells. Furthermore, this pathway is likely to play a role in attenuating or terminating an escalating Th1 response and may perform this function by inducing the death of Th1 cells.

In this study, we examined the role of Tim-3 in a murine model of atopic asthma. We demonstrated that eosinophils and leukocytes were increased in bronchoalveolar lavage fluid (BALF) following asthma challenge. We also determined that the ratio of Tim-3⁺ expressing cells among the CD4⁺ cell population in the peripheral blood and BALF was increased in our asthma model and that Tim-3 mRNA expression was similarly increased. Finally, we showed that increased expression of Tim-3 correlated with a heightened and systemic immune response. These observations are consistent with a strong and prolonged Th2-mediated immune response following the airway challenge, which was likely enhanced by a repressed Th1 response mediated by Tim-3. Together these results highlight the role of Tim-3 during the asthma immune response and suggest the potential of Tim-3 as a target for asthma therapeutic development.

MATERIALS AND METHODS

Animals

All animal experiments were conducted according to the Chinese Council on Animal Care guidelines, and the research protocol was reviewed and approved by the hospital institutional review board of the Tongji Hospital Committees on Animal Experimentation (Tongji Medical College, Huazhong University of Science and Technology, Wuhan, P. R. China). Twenty-four specific pathogen-free adult male Kunming mice (6 to 8 weeks of age and weighing 18 to 22 g)

were obtained from the animal center of Tongji Medical College (Wuhan, Hubei Province, P. R. China). All of the animals were housed at room temperature with a 12-hour light/dark cycle and access to standard chow and water ad libitum.

Induction of Airway Inflammation by Ovalbumin (OVA) Sensitization and Challenge

Airway inflammation was generated as previously described (22). Animals were randomly divided into three equal groups: normal control, asthma day 1, and asthma day 7 groups. Mice received an intraperitoneal injection of 0.1 mL 0.1% (wt/vol) OVA complexed with 0.4% (wt/vol) aluminium hydroxide on days 0 and 7. Two weeks after the first sensitization, the mice inhaled 1% (wt/vol) OVA for 30 minutes in an exposure chamber (50 cm $\times$ 30 cm $\times$ 25 cm) to challenge their airways. The mice in the asthma day 1 group were challenged once on day 14. The mice in the asthma day 7 group were challenged once per day between days 14 and 21. The mice in the normal control group received equal amounts of 0.9% NaCl, instead of OVA and aluminium hydroxide during the sensitization and challenge treatments. Positive responses include fidgeting, fast breathing, abdominal muscle twitching, and incontinence, indicating that the asthma model was successfully established.

T-cell Isolation and Culture

All mice were killed 24 hours after the final challenge, and blood samples were collected from the heart under sterile conditions. Peripheral blood mononuclear cells were isolated from blood samples by Ficoll (Dakewei Biotech Co., China) density gradient centrifugation. Purification of the T lymphocytes from the peripheral blood mononuclear cells was performed as described previously (23). Trypan blue exclusion was used to count the viable cells, and the cell concentration was adjusted to 2×10^6 /mL with RPMI 1640 media containing 10% fetal calf serum.

BALF Cell Separation and Counting

BALF was obtained by the method described previously (24–26). Briefly, BALF was isolated by injection of 0.4 mL normal saline (37°C) through a tracheal cannula and then extracted into centrifuge tubes. This procedure was repeated 3 times. After centrifugation (800 g/min for 10 minutes), the pelleted cells were resuspended in 0.5 mL phosphate buffer solution (PBS), and 0.1 mL of the solution was used to determine the cell number, while 0.2 mL was stained with Wright-Giemsa and 200 cells were counted for cell classification.

RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from peripheral blood T-cells and BALF cells using Trizol reagent (Promega, USA) according to the manufacturer's instructions. cDNA was synthesized using MMLV reverse transcriptase (Promega, USA). The PCR reaction was performed in a 20- μ L volume containing 1 μ L of cDNA template, 0.5 μ L of each primer, and 10 μ L 2 $\times$ PCR mix. The primer sequences for Tim-3 were 5'-GGTGACCTCCATAATAAC-3' (sense) and 5'-TAATAAGGCTCAAACCTCG-3' (antisense), generating a

200 bp fragment. The control primer sequences for β -actin were 5'-TGCTGTCCCTGTATGCCTCT-3' (sense) and 5'-GGTCTTTACGGATGTCAACG-3' (antisense), generating a 460 bp fragment. Amplification was conducted in the linear range with the following cycling parameters: initial denaturation at 95°C (5 minutes); 35 cycles of denaturation at 94°C (45 seconds), annealing at 53°C (30 seconds), and extension at 72°C (1 minute); then a final extension at 72°C (10 minutes). RT-PCR products were analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet (UV) light.

Flow Cytometer Analysis

Peripheral blood T lymphocytes and BALF cells were stained and analyzed as previously described (27). The antibodies used were phycoerythrin (PE)-conjugated anti-Tim-3 mAb and fluorescein isothiocyanate (FITC)-conjugated anti-CD4 mAb (eBiosciences, USA). The relative expression level of the Tim-3 protein was calculated as the ratio of Tim-3⁺/CD4⁺ double positive cells to total CD4⁺ cells (Tim-3⁺/CD4⁺:CD4⁺).

Statistical Analysis

Data were presented as the median and interquartile range in box-plot format. Statistical comparisons were performed using Kruskal-Wallis test, followed by Wilcoxon rank sums test for two by two comparison adjusted with the Bonferroni method. Spearman's correlation was used to explore the correlation between variables. Data were analyzed using SAS 9.0 (SAS Institute Inc., Cary, NC, USA) and graphed with SPSS 15.0 statistics software (SPSS Inc, Chicago, IL); a p value of less than 0.05 was considered statistically significant. Unless otherwise noted, all experimental procedures were performed in triplicate.

RESULTS

Eosinophils and total leukocytes are increased in BALF after OVA challenge. To determine whether our OVA challenge protocol was properly inducing an immune response in our test mice, we measured the amount of eosinophils and total leukocytes present in the total BALF cell population isolated from both control and challenge groups. Both eosinophil and total leukocyte counts in the asthma day 1 group were significantly higher than the normal control group ($p < 0.05$), and those in the asthma day 7 group were also significantly higher than both the normal control group ($p < 0.05$) and the asthma day 1 group ($p < 0.05$) (see Figure 1). These data are consistent with a strong and prolonged Th2-mediated immune response after OVA challenge.

The ratio of Tim-3⁺ expressing cells among the CD4⁺ cell population in the peripheral blood and BALF is increased following OVA challenge. To determine the relative number of Tim-3 expressing cells, we measured the ratio of Tim-3⁺/CD4⁺ cells among the total CD4⁺ cell population in peripheral blood T lymphocytes and BALF cells via flow cytometry. The Tim-3⁺/CD4⁺:CD4⁺ ratio in the peripheral blood T lymphocytes and BALF cells from the asthma day 1 group were both higher than the normal control group ($p < 0.05$), and those in asthma day 7 group were also higher than both the normal control group ($p < 0.05$) and the asthma day 1 group ($p < 0.05$) (see Figure 2). These data are consistent

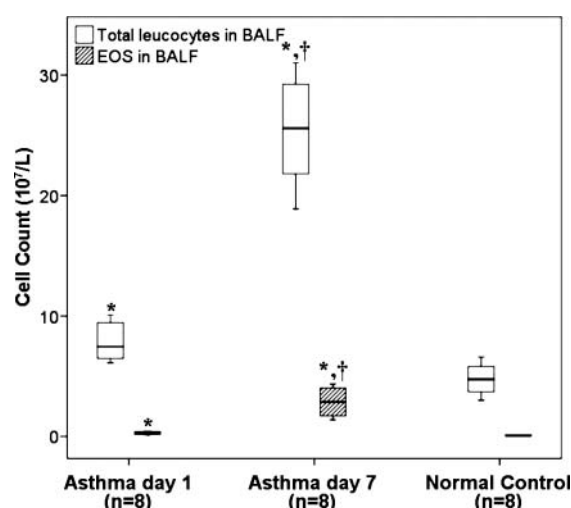


FIGURE 1.—The amount of eosinophils (EOS) and total leukocytes in the BALF cell population in asthma day 1 group, asthma day 7 group, and normal control group. * $p < 0.05$ compared to the normal control group, † $p < 0.05$ compared to the asthma day 1 group.

with an increased number of CD4⁺ cells that express Tim-3 following OVA challenge.

Tim-3 mRNA expression is increased in peripheral blood and BALF after OVA challenge. To determine whether the increased expression of Tim-3 observed in the previous experiment was a result of increased transcription of the Tim-3 gene or another mechanism (e.g., increased proliferation of Tim-3 expressing cells), we measured Tim-3 mRNA expression in peripheral blood T lymphocytes and BALF cells via RT-PCR. All results were normalized relative to a β -actin mRNA control. The Tim-3 mRNA levels in peripheral blood T lymphocytes and BALF cells from the asthma day 1 group were higher than the normal control group ($p < 0.05$), and those in the asthma day 7 group were also higher than both the normal control group ($p < 0.05$) and the asthma day 1 group ($p < 0.05$) (see Figure 3). These data are consistent with an increase in Tim-3 mRNA transcription following OVA challenge.

Increased expression of Tim-3 correlated with a heightened and systemic immune response following OVA challenge. To determine if the increased expression of Tim-3 observed in the above experiments was related to the immune response elicited following OVA challenge, we performed univariate analysis on the data presented above. Our analysis indicated a positive correlation between the Tim-3⁺/CD4⁺:CD4⁺ ratio and eosinophile number in the BALF ($r = 0.9991$, $p < 0.0001$), as well as a positive correlation between Tim-3 mRNA expression and eosinophile number in the BALF ($r = 0.9985$, $p < 0.0001$). These observations suggested that the increased expression of Tim-3 had a direct relationship with the immune response induced by OVA challenge.

Similarly, we determined that there was a positive correlation between the Tim-3⁺/CD4⁺:CD4⁺ ratio in BALF cells and that in peripheral blood T lymphocytes ($r = 0.9991$, $p < 0.0001$), as well as a positive correlation between Tim-3 mRNA expression in BALF cells and that in peripheral blood T lymphocytes ($r = 0.9950$, $p < 0.0001$). These

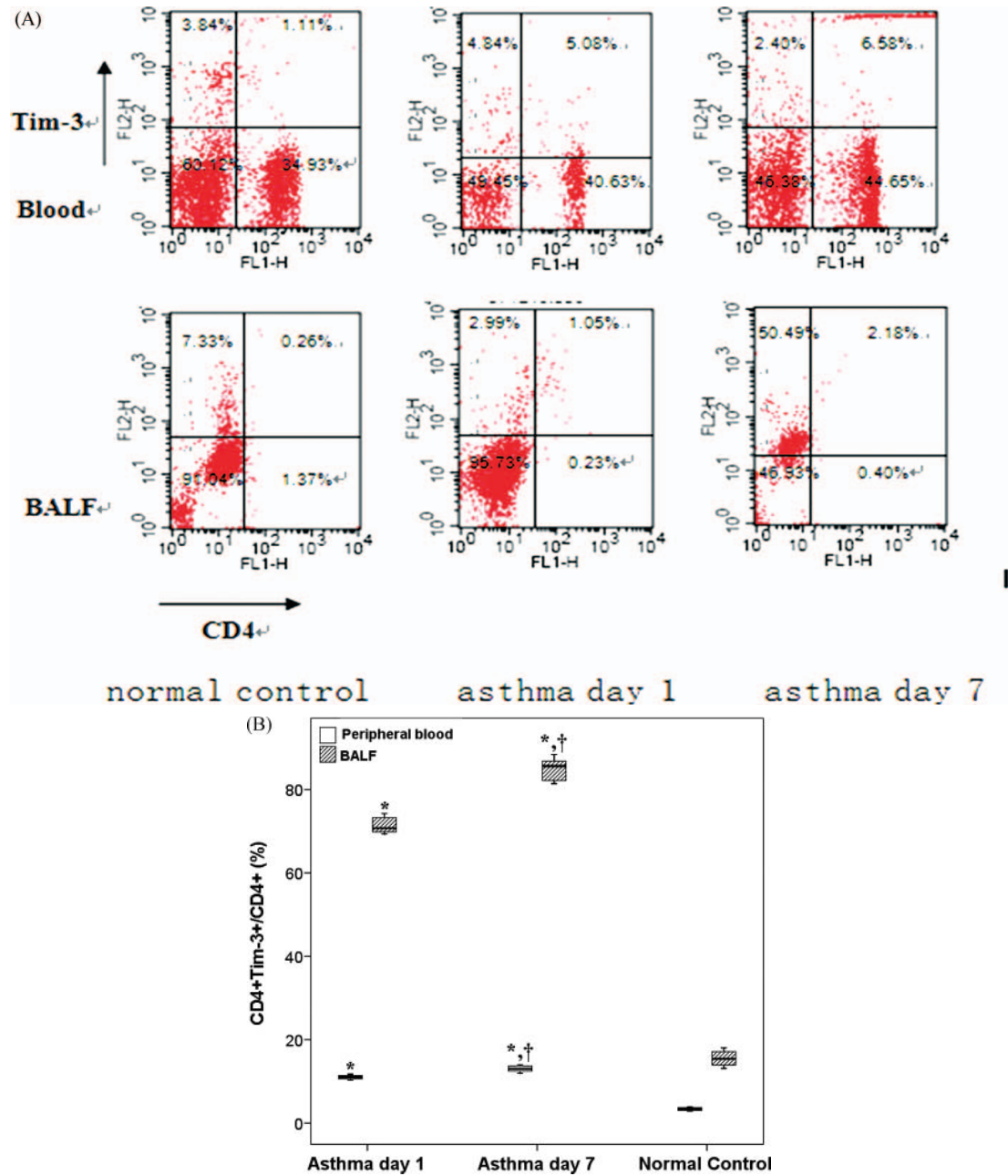


FIGURE 2.—(A) Flow cytometer analysis using PE-conjugated anti-Tim-3 mAb and FITC-conjugated anti-CD4 mAb. Quadrants were determined based on the relative expression levels of Tim-3 and CD4 in relation to background fluorescence of unstained negative controls. (B) Quantitative analysis of the ratios shown in A as detected by RT-PCR and gel electrophoresis imaging (densitometric analysis). The ratio of Tim-3⁺/CD4⁺:CD4⁺ in peripheral blood T lymphocytes and BALF cells in asthma day 1 group, asthma day 7 group, and normal control group. **p* < 0.05 compared to the normal control group, †*p* < 0.05 compared to the asthma day 1 group. PE-Phycoerythrin.

observations suggested that the increased expression of Tim-3 in the BALF cells and the peripheral blood T lymphocytes were both related to the OVA challenge, suggesting the possibility that OVA challenge to the airway of mice may have induced a systemic immune response.

DISCUSSION

Our data indicate that the OVA challenge protocol in our atopic-asthma mouse model was eliciting a Th2 cell-specific

immune response. Our initial observation, after a BALF differential cell count, indicated that both eosinophils and total leukocytes were dramatically increased in BALF after OVA challenge (see Figure 1). This not only indicated a strong inflammatory response in the airway of challenged mice, but because Th2 cells can promote eosinophilic inflammation, it also suggests that this response might be Th2 mediated. These data suggest that Th2 cells were most likely mediating the immune response in our asthma model.

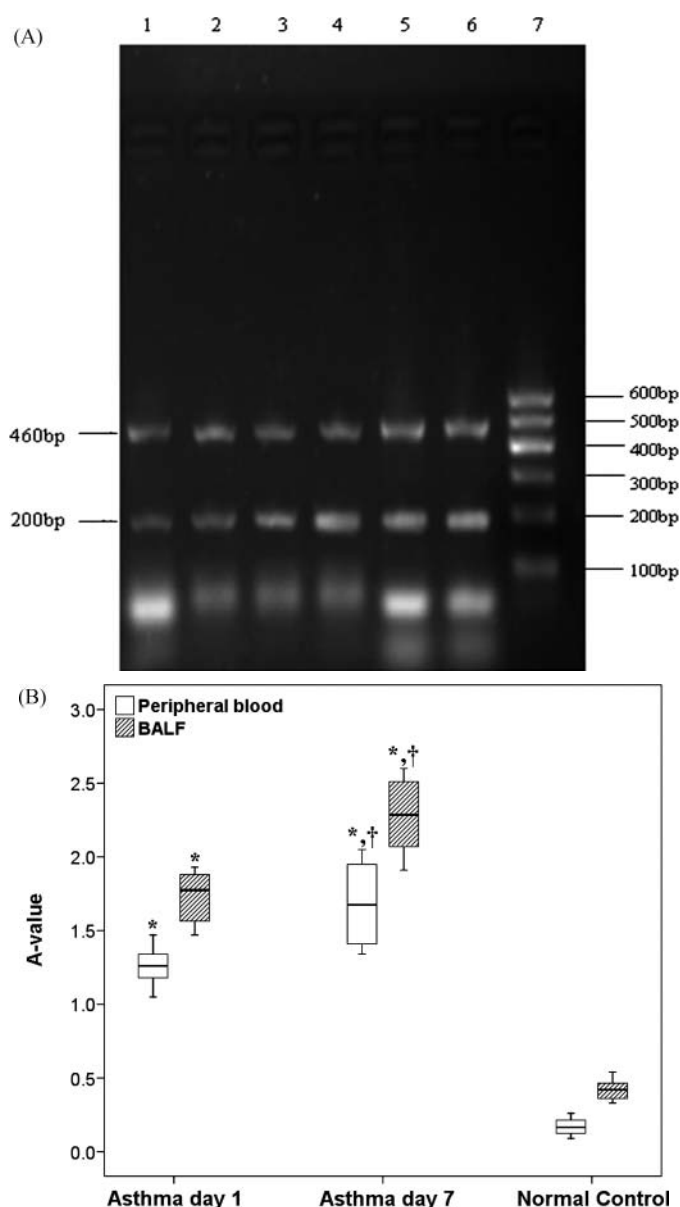


FIGURE 3.—(A) RT-PCR analysis of Tim-3 mRNA in peripheral blood T lymphocytes and BALF cells. Lanes 1: normal control group peripheral blood, 2: normal control group BALF, 3: asthma 1 day group peripheral blood, 4: asthma 1 day BALF, 5: asthma 7 day group peripheral blood, 6: asthma 1 day BALF, 7: size markers. (B) Quantitative analysis (A-values) of the mRNA bands shown in A as detected by gel electrophoresis imaging (densitometric analysis). * $p < 0.05$ compared to the normal control group, † $p < 0.05$ compared to the asthma day 1 group.

Three lines of evidence indicate that Tim-3 expression was increased in our asthma model. First, the ratio of Tim-3⁺ expressing cells among the CD4⁺ cell population in both the peripheral blood and BALF samples was shown to be increased after OVA challenge, and that this increase was heightened with prolonged challenge as evidenced in the asthma day 7 groups (see Figure 2). Second, Tim-3 mRNA expression was increased in both the peripheral blood and BALF samples in the asthma day 1 groups, and again this increase was heightened with prolonged challenge as

evidenced in the asthma day 7 groups (see Figure 3). Finally, both the increased ratio of Tim-3⁺ expressing cells among the CD4⁺ cell population and the increase in Tim-3 mRNA expression correlated with both eosinophil and total leukocyte counts in BALF from challenged mice ($p < 0.0001$ in both cases). Together these observations indicate that expression of Tim-3 is dramatically increased in response to our airway-challenge protocol and that this increase is likely due to changes at the level of transcriptional regulation or mRNA stability.

Previous reports indicate that Tim-3 is preferentially expressed in Th1 cells (15) and functions as a potent inhibitor of the Th1-mediated immune response. Furthermore, IFN- γ produced by Th1 cells can inhibit Th2 differentiation (3), and suppression of this mechanism would promote a significant amplification of the Th2 response. Together with our results, this suggests a model in which upregulation of Tim-3 expression following airway challenge contributes to a depression in Th1-cell function. Subsequently, this alleviates the Th1 suppressive effect on Th2-cell proliferation and allows the formation and maintenance of Th2-dominant immune response, resulting in chronic airway inflammation. Consistent with this idea, a previous investigation that used a functional block of Tim-3 (i.e., anti-Tim-3 antibody) administered during pulmonary inflammation showed that blocking Tim-3 function reduced airway hyper-reactivity, decreased eosinophile numbers and Th2 cells in the lungs, and increased IFN- γ levels (28). Thus, blocking Tim-3 function can result in a restoration of the Th1/Th2 balance in the airway and is consistent with our model.

Our data also indicate that a systemic immune response was elicited following airway challenge. This is indicated by the increased expression of Tim-3 (i.e., increased Tim-3⁺/CD4⁺:CD4⁺ ratio and Tim-3 mRNA levels) in the peripheral blood T lymphocytes following OVA challenge (see Figures 2 and 3). This supports the validity of indirect evaluation of Tim-3 expression after airway challenge via the measurement of Tim-3 expression levels in peripheral blood samples. This approach may help streamline future investigations into the role of Tim-3 during the asthma immune response.

The mechanisms affecting the immune function disorders in asthma are complex. However, it is known that an imbalance between the Th1- and Th2-mediated immune response that favors Th2-specific cytokines is closely related to the pathogenesis of asthma (9–11). The role of Tim-3 and how it might affect this imbalance during an asthma response is only now being investigated, but our investigations suggest that Tim-3 may be the key to restoring this balance. Further study will be necessary to fully understand the role of Tim-3 in the asthmatic response; however, these results further support the notion that Tim-3 is a promising target for immuno-regulation therapeutics.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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