Elevated Expression of Tim-3 and PD-1 Immune Checkpoint Receptors on T-CD4+ Lymphocytes of Patients with Asthma

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ABSTRACT

Asthma is a chronic disorder of the airways characterized by reversible airflow obstruction, inflammation and bronchial hyperresponsiveness. Different immune cells and molecules have been attributed to involve in pathogenesis of asthma. In the current case-control study, the expression of T cell Ig and mucin domain-containing molecule-3 (Tim-3) and programmed death-1 (PD-1) was studied on CD4+ T cells of patients with asthma and normal controls.

The frequency of Tim-3+/PD-1+/CD4+ T cells was determined by a three color flow cytometry method in 37 patients with asthma and 32 healthy controls. To evaluate the Th1/Th2 ratio, peripheral blood mononuclear cells were isolated from all samples and stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 18 h. IFN-γ and Interleukin-4 (IL-4) were measured in culture supernatants by ELISA. Serum total immunoglobulin E (IgE) was also measured in all samples.

Significant increase in percentage and absolute count of Tim-3+/PD-1+/CD4+, Tim-3+/CD4+ and PD-1+/CD4+ T cells was found in asthmatic patients compared to healthy controls (p=0.02, p<0.0001 and p=0.01, respectively). The IFN-γ/IL-4 ratio (Th1/Th2 ratio) was significantly higher in healthy controls than that of asthmatic patients (p=0.029).

Our data regarding the increased expression of PD-1 and Tim-3 on CD4+ T cells of patients with asthma suggest the potential roles of these immune checkpoint receptors in immune dys-regulation of asthma.

Keywords: Asthma; Interferon-γ; Interleukin-4; PD-1; Tim-3
INTRODUCTION

Asthma is a chronic disorder of the airways characterized by reversible airflow obstruction, inflammation and bronchial hyperresponsiveness. Different immune cells such as T cells, B cells, mast cells, eosinophils, macrophages and neutrophils have been attributed to involve in the pathogenesis mechanisms of asthma. While the Th2 paradigm and imbalance in Th1/Th2 profile have explained many features of asthma immunopathogenesis, this mechanism does not fully characterize the etiology of asthma and then possible roles of other CD4+ T cell subsets including Th1 cells, Th17 cells and regulatory T cells have been suggested in asthma pathogenesis.\(^1\)\(^,\)\(^3\) In this regard, several immunotherapeutic approaches such as blocking of Th2 cells specific transcription factor GATA binding protein 3 (GATA-3) or Th2 cytokines Interleukin-4 (IL-4) and Interleukin-13 (IL-13) were designed to regulate the balance between Th1 and Th2 cells and inhibition of Th2 cells differentiation in patients with asthma.\(^4\) Since these approaches were not effective in a major subset of patients, so other immune related mechanisms may involve in asthma pathogenesis.

In the last decades, a new set of immune regulatory molecules called immune checkpoint receptors have been introduced and widely studied in immune system disorders, mostly in the context of chronic inflammatory complications and cancers.\(^5\)\(^,\)\(^6\) Among different identified immune checkpoint molecules, programmed death-1 (PD-1) and T cell Ig and mucin domain-containing molecule-3 (Tim-3) are two important molecules which are expressed on a variety of immune cells and have attracted more attention in recent years. Galectin-9 was identified as the main ligand for Tim-3 and its interaction with Tim-3 was shown to induce cell death in Th1 cells.\(^7\) These results suggested Tim-3 as an inhibitory molecule which terminates Th1 immunity.\(^8\) Later on, the inhibitory function of Tim-3 was studied in several mouse models of autoimmune diseases and asthma. It was shown in an exacerbated experimental autoimmune encephalomyelitis (EAE) model that treatment of EAE mice with anti-Tim-3 antibody cause increasing in macrophage and aggravation of disease activity.\(^9\) Later on it was revealed that blocking Tim-3 signaling pathway resulted in enhanced Th1 cell proliferation and cytokines secretion.\(^10\) Other studies have also demonstrated that Tim-3 influences chronic autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).\(^11\)\(^,\)\(^12\) In this regard, association of Tim-3 polymorphisms with susceptibility to several autoimmune diseases has been identified.\(^13\)\(^,\)\(^14\) A study on allergic asthma patients has shown that up-regulation Tim-3 on T cells was accountable for Th1/Th2 imbalance in allergic asthma patients. Blocking of Tim-3 signaling increased Th1 cytokines response with suppression of Th2 cytokines responses.\(^15\) In a contradictory study on mouse model using Tim-1 and Tim-3 deficient mice, it was reported that Tim-1 and Tim-3 are not essential for the induction of the type-2 response in lung allergy.\(^16\) Similar to Tim-3, the current data supporting the role of PD-1 in Th2 polarized complications such as asthma is controversial. Following engagement with its ligands, PD-1 deliver inhibitory signals that regulate the balance between T- cell activation, tolerance and immunopathology.\(^17\) In a study on murine model of cockroach-induced allergic asthma, it has been demonstrated that blocking of PD-1 pathway resulted in substantially increasing of Th2 cytokines, together with increasing of anti-inflammatory cytokines and IL-10.\(^18\) In a mouse model of allergic inflammatory responses, it has been shown that PD-1-B7-H1-mediated inhibitory pathway may play a critical role in regulation of inflammatory responses and maintaining peripheral tolerance.\(^19\)

Altogether, there are limited and controversial studies about the expression and role of Tim-3 and PD-1 in asthma. In addition, most of the previous studies have focused on mouse model of asthma and there are a few reports conducted on human samples. In the present study, for the first time co-expression of Tim-3 and PD-1 were evaluated on CD4+ T cells of patients with asthma.

MATERIALS AND METHODS

Patients and Controls

A total of 37 patients with asthma attending the Tooba Clinic affiliated to Mazandaran University of Medical Sciences and 32 healthy controls were enrolled in this study. The diagnostic criteria were based on the Global Initiative for Asthma (GINA) guidelines.\(^20\) Since, medications consumption by asthmatic patients may affect the frequency of lymphocytes and also expression of immune regulatory molecules, our
asthmatic patient received inhaled corticosteroids which have less systemic effects than oral corticosteroids. But, due to the ethical limitations, we were not allowed to stop taking medications before sampling. Lung function tests and routine lab tests were performed and all patients with asthma were selected based on their abnormal spirometry results (Table 1). The control group was individuals with normal pulmonary function and negative allergy tests. Written informed consent was obtained from all subjects and the study was approved by the Ethical Committee of Mazandaran University of Medical Sciences (N. 95.2633).

Peripheral Blood Mononuclear Cell Isolation
Heparinized venous blood was collected and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Ficoll-Histopaque (Biosera, Nuaille, France). Isolated PBMCs were washed twice with RPMI-1640 culture medium (Biosera, Nuaille, France) supplemented with penicillin (100 IU/mL) and streptomycin (100 μg/mL) (Biosera, Nuaille, France). The viability of isolated cells was >95% as determined by trypan blue staining.

Surface Staining and Flow Cytometry Analysis
For analysis of the expression level of Tim-3 and PD-1 on CD4+ T cells, PBMCs were incubated with fluorochrome-conjugated monoclonal antibodies: anti-CD4-FITC (eBioscience, San Diego, USA), anti-Tim-3-PE (eBioscience, San Diego, USA), anti-PD-1-PerCP/Cy5.5 (Biolegend San Diego, USA). Isotype-matched control antibodies were also applied for background subtraction. After washing PBMCs with washing buffer (PBS 0.15M pH: 7.4 with 0.5 % BSA), 1×10⁶ cells were resuspended in 100 μL of washing buffer and incubated with appropriate specific mAbs for 45 min in the dark at 4°C. Stained cells were then analyzed on a Partec PAS flow cytometer system (Partec GmBH, Munster, Germany) using the FlowMax software. For compensation analysis, the spillover amount of each fluorochrome into other channels was determined and subtracted.

Cell Culture and Stimulation
For analysis of the in vitro cytokines production, PBMCs were cultured in flat-bottomed 96-well microplates (2×10⁶/well) in 200 μL RPMI-1640 medium, supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL) and 10% heat inactivated fetal bovine serum (Biosera, Nuaille, France) and incubated in 37°C with 5% CO2. For measurement of cytokine production, PBMCs were stimulated with cell stimulation cocktail containing phorbol myristate acetate (PMA) and ionomycin (eBioscience; San Diego, USA) for 18 hours.

Enzyme Linked Immunosorbent Assays (ELISA)
Total serum Immunoglobulin E (IgE) levels were measured in all samples by ELISA-based IgE detection

| Table 1. General clinical and paraclinical characteristics of patients with asthma and healthy controls |
|---------------------------------|-----------------|----------------|
| Asthma group (n=37) | Control group (n=32) | p value |
| Gender (m/f) | 17/20 | 13/19 | >0.05 |
| Age (yrs) * | 44.30±1.637 | 40.69±2.15 | >0.05 |
| FEV1% * | 59.89±2.6 | 95.69±1.25 | <0.0001 |
| FVC% * | 75.57±2.7 | 89.63±0.87 | <0.0001 |
| FEV1 / FVC * | 63.5±6.9 | 77.5±4.5 | <0.0001 |
| Total serum IgE (IU/mL) ** | 190.9 (45.06-373.8) | 53.23 (19.5-278.3) | 0.03 |
| Eosinophil absolute count (μL) ** | 142 (82.5-474.5) | 64.5(44.38-138.5) | 0.0002 |
| Neutrophil absolute count (μL) * | 4508±1473 | 3457±923.8 | 0.0008 |
| Asthma duration (yrs) * | 20.67±13.04 | - | - |
| Age onset (yrs) * | 23.43±11.97 | - | - |
| Mild | 10 | - | - |
| Moderate | 20 | - | - |
| Severe | 7 | - | - |

Data are represented as mean±SEM (*) or median+interquartile ranges (**). The level of total IgE was determined by ELISA. Neutrophil and eosinophil absolute counts were calculated based on CBC-Diff results obtained from cell counter system. FEV1: forced expiratory volume in one second. FVC: forced vital capacity.
kit according to the manufacturer’s instruction (Euroimmun, Luebeck, Germany). The concentrations of IFN-γ and IL-4 were measured in the culture supernatants by ELISA according to the manufacturer’s protocol (eBioscience, San Diego, USA). All samples were measured in duplicate. The sensitivity of IFN-γ and IL-4 ELISA kits was 4 and 2 pg/mL, respectively.

Statistical Analysis
Statistical analyses were performed with GraphPad Prism 6 and SPSS version 20 softwares. Data are expressed as the Mean±SEM or Median±interquartile ranges. T-test and non-parametric Mann-Whitney U test were used to calculate the mean difference between the groups. Pearson and spearman’s rank correlation tests were used to calculate the correlation coefficients. P-values of less than 0.05 were considered significant.

RESULTS
General Characteristics of the Asthmatic Patients and Controls
The characteristics of the studied groups are summarized in Table 1. There was no significant difference in age and gender between asthmatic patients and healthy controls. The concentration of serum total IgE and the number of eosinophils and neutrophils (Table 1) were significantly higher in asthmatic patients (p=0.03, p=0.0002 and p=0.0008, respectively).

Elevated Expression of Tim-3 and PD-1 on CD4+ T Cells of Patients with Asthma
Surface expression of Tim-3 and PD-1 on CD4+ T cells was investigated by a three-color flow cytometry method. Representative flow cytometry dot plots for a patient with asthma and a normal control are illustrated in Figure 1a. Immunophenotyping results showed significant increasing in percentage and absolute count of Tim-3 and PD-1 co-expressing CD4+ T cells in asthmatic patients compared to healthy controls (Figure 1b). When we looked at single expression of either Tim-3 or PD-1 on CD4+ T cells, we found the significant higher percentage and absolute count of Tim-3+ CD4+ T cells and PD-1+ CD4+ T cells in asthmatic patients compared to healthy controls (Figures 1c and 1d).

Evaluation of IFN-γ and IL-4 Production by PBMCs of Asthmatic Patients and Healthy Controls
PBMCs from all samples were cultured and stimulated with PMA/ionomycin. In the asthmatic group, the supernatant level of IL-4 (79 pg/mL) was significantly higher than that of the healthy control group (48 pg/mL) (Figure 2a). But the level of IFN-γ was relatively similar in patients and controls (Figure 2b). As expected, the ratio of IFN-γ/IL-4 (Th1/Th2 ratio) was significantly higher in healthy controls in comparison to asthmatic patients (Fig. 2c) which shows the polarization of the Th2 cells in asthma. A significant positive correlation was found between the IL-4 concentration and the levels of total IgE (r=0.45, p=0.0048; Figure 3a). A significant negative correlation was also found between IFN-γ/IL-4 ratio and neutrophil absolute count in the asthma patients (r=-0.43, p=0.006; Figure 3b). Correlation analysis between the frequency of PD-1 or Tim-3 expressing cells and concentrations of IFN-γ, IL-4 and IgE did not show any significant associations.

Frequency of Tim-3+/PD-1+/CD4+ T Cells and Ratio of IFNγ/IL-4 among the Clinical Subgroups of Asthmatic Patients
Following our previous observations that Tim-3 and PD-1 are upregulated on CD4+ T cells of asthmatic patients and might influence the disease progression, the frequency and cytokine production of CD4+ T cells were studied in subgroups of asthmatic patients with different clinical presentation. Patients were categorized based on the GINA guidelines; mild, moderate and severe. The concentration of total IgE and eosinophil absolute count were higher in patients with severe asthma than those of moderate and mild asthma, but the difference was not significant maybe due to the low sample size in each group (Figures 4a and 4b).

The percentage and absolute count of Tim-3+/PD-1+/CD4+ T cells were higher in patients with mild asthma than those of moderate and severe asthma which was not statistically significant (Figure 4c). Interestingly, the IFNγ/IL-4 ratio was higher in patients with mild asthma compared to moderate asthma and then was increased in patients with severe asthma (Figure 4d), but the difference was not significant.
Figure 1. Frequency and absolute count of T-cell Ig and mucin domain – containing molecule – 3 (Tim-3+)/ programed death – 1 (PD-1+)/CD4⁺ T cells in peripheral blood of patients with asthma and healthy controls. Peripheral blood mononuclear cells (PBMCs) were obtained from all samples and stained for CD4, Tim-3, and PD-1. Representative flow cytometric dot plots obtained from an asthmatic patient and a normal control are shown (a). To analyze the graphs, CD4⁺ cells were initially gated from lymphocyte population and then the Tim-3⁺/PD-1⁺ cells were determined. The percentage and absolute count of Tim-3⁺/PD-1⁺/CD4⁺ T cells (b), Tim-3⁺/CD4⁺ T cells (c) and PD-1⁺/CD4⁺ T cells (d) from all asthmatic patients and healthy controls are represented. Data are represented as Mean±SEM for PD-1⁺/CD4⁺ T cells and Median±interquartile range for other populations. p values < 0.05 were considered significant.
Figure 2. Levels of IL-4 and IFN-γ in supernatants obtained from stimulated PBMCs of patients with asthma and healthy controls. PBMCs were obtained from all participants and stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 18 hours. Culture supernatants were harvested and the levels of IL-4 (a) and IFN-γ (b) were measured by ELISA. The IFN-γ/IL-4 ratio was also calculated for all samples by dividing the IFN-γ to IL-4 concentration which represents the Th1/Th2 ratio (c). Data are represented as Mean±SEM for IFN-γ and Median±interquartile range for IL-4 and IFN-γ/IL-4 ratio. p values < 0.05 were considered significant.

Figure 3. Correlation of IL-4 concentration with serum total IgE level (a) and IFN-γ/IL-4 ratio with neutrophil absolute count (b) in patients with asthma.

Figure 4. Serum total IgE level (a), eosinophil absolute count (b), Tim-3+/PD1+/CD4+ T cells absolute count (c) and IFN-γ/IL-4 ratio (d) in different clinical stages of patients with asthma. Asthmatic patients were clinically categorized into mild, moderate and severe based on the Global Initiative for Asthma (GINA) guidelines.
DISCUSSION

Multiple mechanisms are involved in the regulation of immune responses and deregulation of these mechanisms may lead to the development of hypersensitivity disorders including allergies and asthma. One of the mechanisms responsible for the regulation of immune response and tolerance is signaling via immune checkpoint or inhibitory receptors. Tim-3 and PD-1 are two important immune checkpoint receptors having critical roles in the maintenance of immune homeostasis which their functions in regulation of pro-inflammatory T cell response have been confirmed. Nevertheless, there are controversial findings regarding the role of Tim-3 and PD-1 in asthma.\textsuperscript{15,16,21,22} In the current study, for the first time, the co-expression of Tim-3 and PD-1 on CD4\(^+\) T cells has been examined in asthmatic patients and healthy controls. We demonstrated that the frequency of Tim-3\(^+/\)PD1\(^+/\)CD4\(^+\) T cells in patients with asthma were significantly higher than that of healthy controls. Besides the higher percentage of Tim-3\(^+/\) PD-1\(^+/\)CD4\(^+\) cells in asthma patients, significant increasing in the frequency of either Tim-3\(^+/\) CD4\(^+\) or PD-1\(^+/\)CD4\(^+\) cells was also observed in asthma patients. Similarly, Tang et al. have reported a significant elevated expression of Tim-3 in asthma patients compared to healthy controls.\textsuperscript{15} Administration of anti-Tim-3 antibody during pulmonary inflammation was previously examined in a mouse model of allergic asthma. It has been shown that blocking of Tim-3 signaling has a beneficial effect during pulmonary inflammation through polarization of the Th2 response toward the Th1 response. In addition, a suppression in IL-5 expression and increasing in IFN-\(\gamma\) levels have been also demonstrated suggesting a potential role for Tim-3 in the regulation of allergic asthma.\textsuperscript{23} Controversially, in a study on mouse model of allergic asthma, Hiraishi et al. demonstrated that Tim-3 mRNA is constitutively expressed in the lungs of wild-type mice and its level did not change in acute airway inflammation. In addition, the progression course of acute airway inflammation was similar in Tim-3\(^+/\) mice and wild-type mice, indicating that Tim-3 is not essential for acute airway inflammation.\textsuperscript{21} In another study using Tim1\(^+/\) and Tim3\(^+/\) mice, Barlow et al. have investigated the role of Tim-1 and Tim-3 in the regulation of type-2 responses during lung allergy. They found no important role for Tim-3 in this model of allergic asthma.\textsuperscript{16}

There are limited studies conducting on the role of PD-1 in immune regulation of allergic asthma. A reverse association between the frequency of PD-1\(^+/\)CD4\(^+\) T cells and IgE serum concentration of patients with allergic asthma was reported by Bratke et al.\textsuperscript{24} In contrast to above mentioned study, we did not observe a significant correlation between PD-1\(^+/\)CD4\(^+\) T cells frequency and total serum IgE concentration in our asthmatic patients. Depping et al. have examined the allergic response of PD-1\(^+/\) mice and showed an increasing in inflammatory cells recruitment in the bronchoalveolar lavage of the PD-1\(^+/\) mice compared to wild-type. They found that PD-1 decline the duration of allergic airway inflammation by inhibiting of Th2 cells.\textsuperscript{25} Similar to our study, Wang et al. have investigated co-expression of PD-1 and Tim-3 on CD4\(^+\) T cells of peripheral blood and decidual samples of individuals with normal pregnancy, as a Th2-biased phenomenon, and miscarriage. They demonstrated that decidual CD4\(^+\) T cells from women with normal pregnancy show higher expression of PD-1 and Tim-3 in comparison with miscarriage cases. While the production of Th1 cytokines was similar in PD-1\(^+/\)Tim-3\(^+/\) and PD-1\(^+/\)Tim-3\(^+/\)CD4\(^+\) T cells, the production of Th2 cytokines by PD-1\(^+/\)Tim-3\(^+/\)CD4\(^+\) T cells were much higher than that of PD-1\(^+/\)Tim-3/CD4\(^+\) T cells at the maternal–fetal interface.\textsuperscript{26} Our results regarding increasing in Tim-3\(^+/\)PD1\(^+/\)CD4\(^+\) T cells and IL-4 levels in asthmatic patients together with similar production of IFN-\(\gamma\) by asthmatic patients and healthy controls are in concordance with the mentioned study conducting on women with normal pregnancy and abortion.\textsuperscript{26} These observations suggest that PD-1 and Tim-3 may have no effects on the production of the IFN-\(\gamma\) and induce the production of IL-4. Dys-regulation of Tim-3 and PD-1 expression has been reported in various autoimmune disorders like multiple sclerosis,\textsuperscript{27} systemic lupus erythematosus\textsuperscript{28} and rheumatoid arthritis\textsuperscript{29} and it was shown that Tim-3 and PD-1 expression are inversely correlated with disease progression confirming their protective roles. Similarly, it seems that the higher expression of Tim-3 and PD-1 in asthmatic patients may be due to the feedback mechanisms and autoregulation of the immune responses to control the immunopathological events in this immune related disorder. Further studies are
required to confirm this hypothesis and more explore the role of these immune checkpoint pathways in asthma.

Significant negative correlation between IFN-\(\gamma\)/IL-4 ratio and neutrophil absolute count in our asthmatic patients is consistent with the results of Nandi et al. who studied the regulatory effect of IFN-\(\gamma\) on neutrophils during tuberculosis infection and have shown that IFN-\(\gamma\) suppresses neutrophil accumulation and recruitment by inhibiting IL-17 production.\(^{30}\)

Therefore, our results suggest that the imbalance of IFN-\(\gamma\)/IL-4 ratio may be accompanied with increasing in eosinophils and neutrophils in asthmatic patients. In this study, asthmatic patients were clinically categorized into mild, moderate and severe stages based on the GINA guidelines.\(^{20}\)

The absolute count of Tim-3+/PD1+/CD4+ T cells show a decreasing trend in mild, moderate and severe stages of asthma which suggest that Tim-3 and PD-1 may play potential roles in regulating the severity of asthma. Future studies are required to more confirm and extend this finding.

In summary, our data regarding the increased expression of PD-1 and Tim-3 on CD4+ T cells of patients with asthma suggest the potential roles of these immune checkpoint receptors on the pathogenesis of asthma and promotion of Th2-mediated immune responses in this disease.

ACKNOWLEDGEMENTS

This study was financially supported by Mazandaran University of Medical Sciences; grant number MCBRC-MAZUMS- 2633.

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Tim-3 and PD-1 Expression in Asthma


