

Dexamethasone Reduces IL-17 and Tim-3 Expression in BALF of Asthmatic Mice^{*}

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Summary: This study investigated the expression of interleukin-17 (IL-17) and T cell immunoglobulin mucin and domain-containing molecule-3 (Tim-3) in bronchoalveolar lavage fluid (BALF) of asthmatic mice and the effect of dexamethasone (DEX) on these factors. Thirty-six mice were randomly divided into three groups: normal group, asthmatic group and DEX group. The mouse model of asthma was established by sensitization with ovalbumin in both the asthmatic and DEX groups. The levels of IL-6, IL-10, IL-17 and TGF- β were measured in BALF by enzyme-linked immunosorbent assay (ELISA). The mRNA expression level of Tim-3 was detected by reverse transcription polymerase chain reaction (RT-PCR). The ratio of Tim-3+CD4⁺ cells to total CD4⁺ cells in BALF was determined by flow cytometry. Differential inflammatory cells in BALF were detected. The correlations among IL-17, IL-6, IL-10, Tim-3 and inflammatory cells were analyzed. The results showed that the levels of IL-17, IL-6 and Tim-3 were substantially increased and the IL-10 level decreased in BALF in the asthmatic mice, which was significantly reversed by DEX treatment. IL-17 expression was positively correlated with IL-6 and Tim-3 expression and the number of inflammatory cells but negatively with IL-10 expression. These results indicate that the increased expression of IL-17 and Tim-3 in BALF may be implicated in the occurrence and development of asthmatic inflammation; the mechanism by which DEX suppresses asthmatic airway inflammation involves down-regulation of IL-17 and Tim-3 levels.

Key words: asthma; Th17 cells; Tim-3; cytokines; corticosteroids

Asthma is a chronic inflammatory disease of the airways and many cell types are involved in this disease, in particular, T lymphocytes, mast cells and eosinophils. CD4⁺ T cells play critical roles in allergic responses and autoimmunity in asthma. During the activation of T cell receptors (TCRs) in a particular cytokine milieu, CD4⁺ T cells may differentiate into one of the several lineages of T helper type (Th) cells, including Th1, Th2, Th17 and iTreg^[1]. Th17 cell is a novel type of CD4⁺ T cell. These effector cells were named Th17, because they mainly produce cytokine interleukin-17A (IL-17A) (also called IL-17) and IL-17F^[2, 3]. Current studies indicate that Th17 cells play a central role in the development of various allergic and autoimmune diseases. IL-17 mRNA and/or protein increases have been reported in lung tissues, sputum, BALF and sera from asthmatics^[4-6], and the level of IL-17 expression is correlated with the degree of severity of airway hypersensitivity^[7]. In addition, in an asthmatic mouse model, both IL-17A and IL-17F were

found to induce neutrophilic airway inflammation^[8, 9]. IL-17R-deficient mice could exhibit a decrease in eosinophil recruitment, Th2 cytokine production, and IgE levels during induction of allergic airway inflammation^[10]. All these findings indicate that Th17 cells are critical in the induction of asthmatic airway inflammation.

T-cell immunoglobulin and mucin domain-containing molecule (Tim) is a new family, which plays emerging roles in allergy and autoimmune diseases^[11, 12]. It is a 281-amino-acid transmembrane protein preferentially expressed on activated Th1 and Th17 cells^[13]. As a ligand of Tim-3, Galectin-9 (Gal-9) is predominantly expressed on naive CD4⁺ T effector and CD4⁺CD25⁺Treg cells^[14, 15]. Several recent reports indicate that Tim-3 functions as a potential inhibitor of the Th17 immune response. Tim-3 was first detected on IL-17-expressing LN cells in the murine model of contact dermatitis^[16]. Thereafter, investigators found that the percentage of Tim-3+CD4⁺ cells in the spleen of Gal-9-deficient mice was significantly higher than that of wild-type mice in the CIA model, which confirmed Gal-9 controls the differentiation to Tim-3+Th17 cells *in vivo*^[17]. Moreover, Gal-9 treatment resulted in decreased number of Tim-3+CD4⁺ cells and down-regulation of IL-17^[17]. These findings imply that the interaction of Tim-3 with galectin-9 can decrease IL-17 secretion and suppress Th17 immunity, by inducing Th17 cell apoptosis and

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preventing the differentiation of CD4⁺ T cells to Th17 cells.

In the present study, we examined the alteration of IL-17 and Tim-3 expression in an asthmatic murine model and the effect of dexamethasone (DEX) on these factors in an attempt to shed light on the future asthmatic therapy.

1 MATERIALS AND METHODS

1.1 Main Reagents

The main reagents included ovalbumin (OVA, Sigma, USA), fetal bovine serum (Gibco, USA), TRIzol[®] reagent (Promega, USA), MMLV reverse transcriptase (Promega, USA), phycoerythrin (PE)-conjugated anti-Tim-3 antibody, fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody (eBiosciences, USA), and ELISA kit (Dakewe Biotech Company Limited, China).

1.2 Animals

All animal experiments were conducted according to the guidelines of Chinese Council on Animal Care. Research protocols were reviewed and approved by the Institutional Review Board of Wuhan Women and Children Health Care Center Committees on Animal Experimentation. Thirty-six pathogen-free female mice (wild-type Balb/C, 6 to 8 weeks of age and weighing 18 to 22 g) were obtained from the Experimental Animal Center of Tongji Medical College (Wuhan, China). Mice were housed at room temperature under a 12-h light/dark cycle and allowed access to standard chow and water *ad libitum*.

1.3 Establishment of Murine Asthmatic Models and Grouping

Mice were exposed to OVA as described previously^[18]. Briefly, 36 mice were randomly divided into three groups: control group, asthmatic group (OVA), and dexamethasone group (DEX) ($n=12$ each). On day 0 and day 7, mice in the OVA group received an intraperitoneal injection of 0.1 mL 0.1% (wt/vol) OVA and 0.4 % (wt/vol) aluminium hydroxide. From day 14 to day 21, these mice were inhaled with 1% (wt/vol) OVA for 30 min in exposure chamber to challenge their airways once per day. Positive responses included fidgeting, fast breathing, abdominal muscle twitching and incontinence, indicating that the asthma model was successfully established. Mice in DEX group were intraperitoneally given 10 mg/kg DEX 0.5 h before each OVA challenge. Mice in control group were intraperitoneally administered with equal amount of saline, instead of OVA and aluminium hydroxide during sensitization and challenge phases.

1.4 BALF Preparation and Differential Cell Counting

Twenty-four h after the final OVA challenge, all mice were sacrificed to prepare BALF as described previously^[19]. Lungs were lavaged *in situ* with 1 mL of ice-cold phosphate buffer solution (PBS) via the tracheal cannula. After three times lavage, approximately 2 mL of BALF were recovered and centrifuged at 4°C (1500 r/min) for 10 min. The supernatants were rapidly collected and frozen for further cytokine measurement by ELISA. The pelleted cells were resuspended in 0.5 mL PBS. One hundred microliter of the solution was used to count total cell number, 200 μ L was stained with Wright-Giemsa and 200 cells counted for cell classification.

1.5 RNA Extraction and Reverse Transcription-polymerase Chain Reaction (RT-PCR)

Total RNA from mouse BALF cells was extracted by TRIzol[®] reagent according to the manufacturer's instructions. The first strand cDNA was generated from 1 mg of total RNA by using MMLV reverse transcriptase. RT-PCR primers were as follows: Tim-3 primers, 5'-GGTGACCCTCCATAATAAC-3' (sense) and 5'-TAATAAGGCTCAAACCTCG-3' (antisense), generating a 200 bp fragment; β -actin primers, 5'-TGCTGTCCCTGTATGCCTCT-3' (sense) and 5'-GGTCTTTACGGATGTCAACG-3' (antisense), generating a 460 bp fragment. PCR amplifications were performed under the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. RT-PCR products were analyzed by 2% agarose gel electrophoresis. The relative content of Tim-3 mRNA was expressed as the ratio of Tim-3/ β -actin.

1.6 Flow Cytometry

Direct immunofluorescence was performed on BALF cells as described previously^[20]. Briefly, BALF cells were washed three times with ice cold PBS, and resuspended in FACS staining buffer (PBS+0.5% BSA) containing phycoerythrin (PE)-conjugated anti-Tim-3 antibody (eBiosciences, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody for 30 min at 4°C. All samples were analyzed for the level of cell surface marker Tim-3 and CD4 by flow cytometry. The relative expression level of Tim-3 protein was defined as the ratio of Tim-3+CD4⁺ double positive cells to total CD4⁺ cells (Tim-3+CD4⁺/CD4⁺). Flow cytometric data were collected in a logarithmic mode on light scatter-gated live cells using CellQuest software.

1.7 Cytokines Measurement by ELISA

Levels of IL-6, IL-10, IL-17 and TGF- β were quantified in BALF supernatants by ELISA according to the manufacturer's protocol.

1.8 Statistical Analysis

Statistical analysis was performed using SPSS 13.0 program (SPSS, USA). All data were expressed as $\bar{x} \pm s$. Significant differences between different groups were analyzed using one factor ANOVA. Pearson correlation was used for correlation analysis. A P value less than 0.05 was considered to be statistically significant.

2 RESULTS

2.1 Effect of DEX on Airway Inflammation

Infiltration by inflammatory cells is a characteristic of allergic airway inflammation. As expected, a significant increase in the recruitment of inflammatory cells to the airways was found in OVA-challenged mice compared with control mice ($P<0.05$). The increased inflammatory cells included eosinophils, lymphocytes, and neutrophils (fig. 1). Furthermore, it was noted that the total inflammatory cell number, and the number of eosinophils and lymphocytes were significantly reduced in DEX group compared with OVA group ($P<0.05$), whereas the number of neutrophils in the DEX group was not significantly different from that in the OVA group (fig. 1). These results suggested that DEX is able to decrease the number of inflammatory cells, especially that of eosinophils and lymphocytes in the BALF in the airway inflammation.

2.2 Effect of DEX on the Levels of IL-6, IL-17 and IL-10 in BALF

It has been shown that glucocorticoids can inhibit the production of IL-4, IL-5, and IL-13 from Th2 cells^[21]. To test the sensitivity of Th17 cells to glucocorticoids and the changes of related cytokines, we used ELISA to determine the effect of DEX on IL-6, IL-10, IL-17 and TGF- β levels in BALF supernatant. The TGF- β level in BALF underwent no significant change after OVA challenge (data not shown). However, the levels of IL-6 and IL-17 were 3.9-fold higher in the OVA group than in the control group (fig. 2), which were significantly attenuated in BALF supernatant by DEX treatment ($P<0.05$) (fig. 2A–2C). Contrast to IL-6 and IL-17, the level of IL-10 was reduced by OVA challenge, and this decrease could be partially reversed by DEX treatment ($P<0.05$). These results indicate that inflammatory cytokine responses are associated with OVA-challenged airway inflammation, and DEX can inhibit airway inflammation by regulating the expression of IL-6, IL-7 and IL-10.

2.3 Effect of DEX on Tim-3 Expression in BALF Cells

Tim-3 is specifically expressed in CD4⁺ Th1 and Th17 cells^[22–24]. To understand the role of Tim-3 in asthma, we examined the Tim-3 expression on the surface of cells in BALF in OVA-challenged mice. Flow cytometric analysis of BALF cells revealed that the pro-

portion of Tim-3⁺ expressing cells among the CD4⁺ T cell population was increased after OVA challenge. The Tim-3⁺CD4⁺/CD4⁺ ratio in BALF cells was significantly increased in OVA-challenged mice as compared with control mice ($P<0.05$) (fig. 3A, 3B), and this increase was suppressed by DEX treatment. Moreover, RT-PCR revealed that the mRNA expression level of Tim-3 was 6.4-fold higher in OVA group than in control group ($P<0.05$) (fig. 3C, 3D). DEX treatment could significantly decrease the OVA-induced Tim-3 mRNA expression ($P<0.05$).

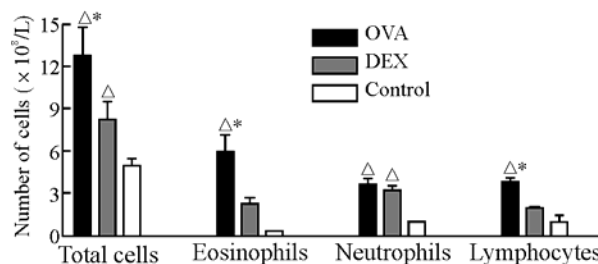


Fig.1 Effect of DEX treatment on the number of inflammatory cells in BALF

$\Delta P<0.05$ vs. control group; * $P<0.05$ vs. DEX group

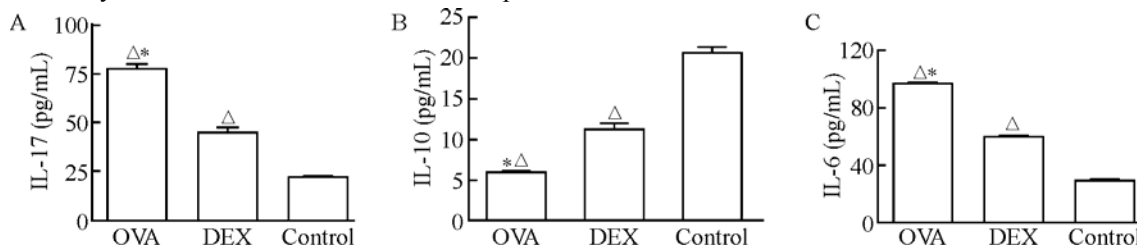


Fig. 2 Effect of DEX on the levels of IL-17 (A), IL-10 (B) and IL-6 (C) in BALF supernatant

$\Delta P<0.05$ compared to control group; * $P<0.05$ compared to DEX group

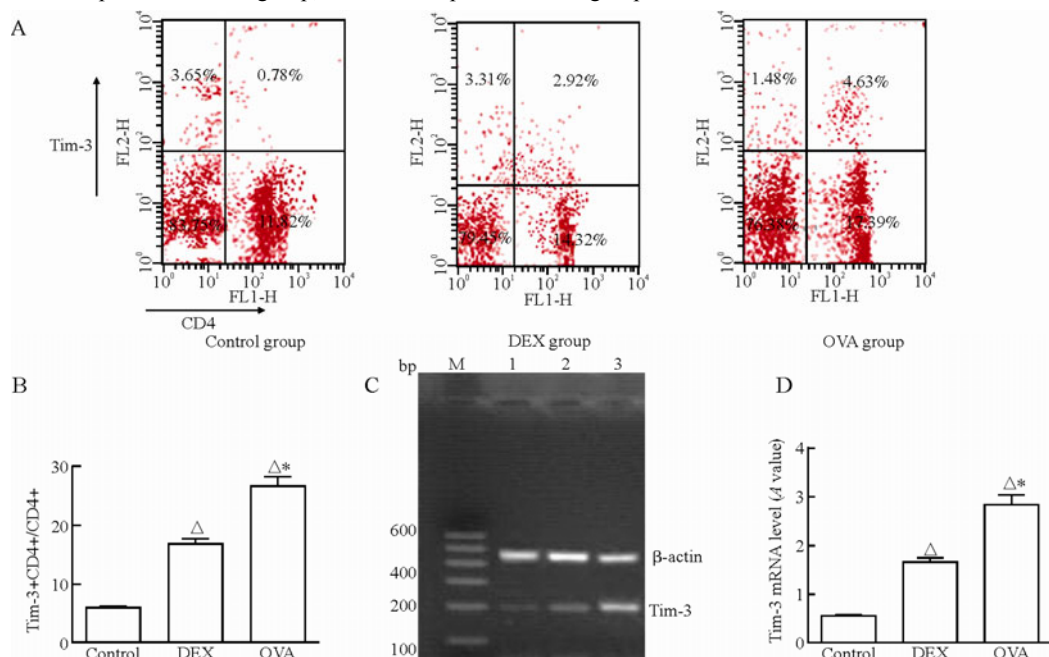


Fig. 3 Effect of DEX on Tim-3 expression in BALF cells

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: The ratio of Tim-3⁺CD4⁺/CD4⁺ in BALF cells in different groups; C: RT-PCR analysis of Tim-3 mRNA in BALF cells. Lane 1: Control group; Lane 2: DEX group; Lane 3: OVA group; D: Quantitative analysis (Δ values) of the mRNA expression level of Tim-3. $\Delta P<0.05$ compared with control group; * $P<0.05$ compared with DEX group

2.4 Relationship between IL-17 Level and Tim-3 Expression in BALF Cells

The IL-17 level was positively correlated with Tim-3 expression at both mRNA and protein levels ($r=0.87$ and $r=0.79$, respectively, $P<0.05$ for both). Furthermore, there was a positive correlation between IL-17 and IL-6 ($r=0.85$, $P<0.05$), and a negative correlation between IL-17 and IL-10 ($r=-0.91$, $P<0.05$). The IL-17 level was also found to positively correlate with the number of eosinophils, lymphocytes, and neutrophils ($r=0.79$, $r=0.81$ and $r=0.89$, respectively, $P<0.05$ for all).

3 DISCUSSION

The present study demonstrated the up-regulation of the cytokines IL-6 and IL-17 in BALF supernatant and the increased Tim-3 mRNA and protein expression in BALF cells in asthmatic mice. Moreover, IL-17 was found to positively correlate with the expression of Tim-3 and IL-6, and the number of eosinophils, lymphocytes, and neutrophils. Additionally, we found that DEX treatment significantly reduced the expression of IL-6, IL-17 and Tim-3 in the BALF of asthmatic mice. TGF- β expression, however, remained unchanged after OVA challenge.

Asthma is a chronic airway inflammation disease characterized by the reversible airflow obstruction, airway hyper-responsiveness (AHR) to a wide variety of specific or non-specific stimuli, and airway remodeling^[25]. CD4⁺ T cells play a crucial role in controlling airway inflammation in asthma. They are the predominant lymphocyte population that infiltrates the airways and are activated in these sites in asthmatics^[26]. During the past several decades, studies indicated that the pathogenesis of asthma was closely associated with a disturbed balance between Th1 and Th2 mediated immune responses. Th2 type cells were activated and produced a different panel of cytokines including IL-4 and IL-13, to promote the development of airway inflammation, whereas the function of Th1-type cells was inhibited^[27]. Investigators focused on how to restore the balance and induce a shift in cytokine responses from a Th2 to a Th1 dominant pattern to inhibit Th2-mediated immune responses^[28]. However, more and more studies have shown that many phenomena can not be explained by an imbalance of Th1/Th2 paradigm. For example, blockade on Th2 cell and cytokines production in asthma can not ameliorate the disease^[29]. Recently, a novel member of the T helper set, IL-17-producing CD4⁺ T cell (now called Th17 cells) is described as a distinct lineage and involved in the development of autoimmune and allergic diseases in both humans and mice. Pappu *et al*^[30] elucidated that IL-17 acts as a potent pro-inflammatory cytokine and is responsible for the release of several chemokines and cytokines, which synergize with other pro-inflammatory cytokines in enhancing the inflammatory responses. IL-17 can orchestrate local inflammation by inducing the release of TNF- α , IL-1b, and IL-6 by human bronchial fibroblasts, epithelial, and airway smooth muscle cells. In addition to stimulating airway structural cells, IL-17 can also enhance Th2-cell-mediated eosinophilic airway inflammation^[6, 31, 32]. In our study, we observed increased

IL-17 expression in asthmatic mice, which was consistent with previous investigations^[4-6]. Furthermore, DEX treatment attenuated OVA-induced IL-17 expression significantly. These findings indicate an important role of IL-17 in the pathogenesis of asthma.

The Tim gene family plays a central role in regulating immune responses, including autoimmunity, asthma, allergy, and transplant tolerance. Tim-3, as one of the critical members of this family, was originally identified as a surface molecule expressed on Th1-polarized cells^[22]. More recently, investigators found that Tim-3 is expressed not only on *in vitro* Th1-polarized cells, but also on Th-17 cells, and act as a negative regulator of T cells and regulates Th-1 and Th-17 cytokine secretion^[13]. Using a murine asthma model, we examined the role of Tim-3 in asthmatic airway inflammation. Our investigation indicated that Tim-3 is an important molecule participating in up-modulating asthmatic inflammation, which involves Th17 cell-mediated immune response. We observed that the expression of Tim-3 mRNA and protein was significantly up-regulated in asthmatic mice compared with controls, which was consistent with previous work^[33]. Our previous study exhibited the relationship between Tim-3 and the imbalance of Th1/Th2^[33]. As a supplement to the previous study, our current data showed a positive correlation between IL-17 and Tim-3 expression. Furthermore, the response of Tim-3 to corticosteroids looked similar to that of IL-17, and their expression levels were significantly decreased by DEX. Moreover, we found that the level of IL-10 was decreased in asthmatic mice and this down-regulation was restored by DEX treatment. IL-10 is a key cytokine secreted by regulatory T (Treg) cells. Given these findings, we speculate that when mice are challenged by OVA, CD4⁺ T cells are activated and differentiated into Th17 cells, and Tim-3 expression is increased. Simultaneously, the production of Treg cells is suppressed and the level of IL-10 is decreased. These changes will promote the development of airway inflammation and exacerbate asthma. DEX can induce Th17 cells to undergo apoptosis and inhibit the airway inflammation by down-regulating Tim-3 expression.

IL-6 is a product of an ongoing innate immune response and can be produced by hematopoietic cells, epithelial cells, endothelial cells and fibroblasts after exposure to specific stimuli^[34]. However, recent studies suggest that IL-6 plays an important role in determining the type of adaptive immune response. Specifically, recent reports suggest that IL-6, together with TGF- β , induces CD4⁺ T cells to differentiate into Th17^[35, 36]. In this study, we detected an IL-6 increase in asthmatic mice compared with controls, and found that the IL-6 increase was significantly suppressed by DEX treatment. Further study showed that IL-17 was positively correlated with IL-6. Therefore, IL-6 may be a critical regulator and it determines the balance of CD4⁺ T cells differentiating to Treg/Th17 cells.

TGF- β is an important mediator involved in the process of airway inflammation and remodeling. However, TGF- β levels in asthma vary among different studies, and whether its expression correlates with disease severity is controversial^[37, 38]. In this study, there was no significant change in TGF- β expression between asth-

matic mice and controls, which was consistent with a previous study by Minshall^[39]. Thus, we speculate that TGF- β is produced mainly by activated eosinophils and epithelial cells in the airway, and is more closely related to airway remodeling which requires protracted exposure to allergen. Therefore, no significant change of TGF- β would be observed in BALF.

In summary, mechanisms of the immune function disorders in asthma are complicated. The role and the exact mechanism of Tim-3 involved in asthmatic airway inflammation still remain unclear. Our study demonstrated that corticosteroids could inhibit airway inflammation by suppressing IL-17 and Tim-3 expression, suggesting that Tim-3 may act as a promising target for immuno-regulation therapy.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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