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Anti-TIM1 suppresses airway inflammation and hyperresponsiveness via the STAT6 /STAT1 pathways in mice with allergic asthma

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T cell immunoglobulin and mucin domain-1 (TIM-1) is a transmembrane glycoprotein and has been reported as an molecular mechanism of allergic diseases. This study aimed to explore the effects of anti-TIM-1 monoclonal antibodies (anti-TIM1) on the development of allergic asthma. Female C57BL/6 mice were induced and challenged with ovalbumin (OVA) and received subsequent intranasal administration of anti-TIM1. The airway resistance of all mice was evaluated using a Buxco PFT system. Flow cytometry was used to detect the expression of TIM-1 in peripheral blood mononuclear cells. The level of cytokine production in the bronchial alveolar lavage fluid and serum was determined using ELISA. Mucous cells were observed using Alcian blue and periodic acid-Schiff staining. In addition, B-cell lymphoma gene 2 (BCL2), T-box transcription factor (T-bet), GATA binding protein-3 (GATA3), signal transducer and activator of transcription (STAT) 1, STAT6 were analyzed by western blot analysis. Their corresponding mRNA expression levels were determined by quantitative PCR. The mRNA expression level of Mucin 5AC in the lung tissues was also detected using quantitative RT-PCR. The results showed that the intranasal administration of anti-TIM1 ameliorated airway inflammation and hyperresponsiveness in an acute model of asthma. Following administration of anti-TIM1, both the mRNA and protein levels of T-bet were upregulated, while those of BCL2 and GATA3 were downregulated. Moreover, the phosphorylation levels of STAT1 and STAT6 were increased. Taken together, these findings demonstrated that intranasal administration of anti-TIM1 ameliorated allergic lung inflammation and remodeling in mouse models of asthma by repairing both the STAT1 and STAT6 pathways.

1. Introduction

Asthma is a chronic airway inflammatory condition with multi-genetic heritability that is influenced by environmental factors and characterized by reversible airway obstruction, mucous metaplasia, and hyper-responsiveness (AHR) (Broide 2001; Barnes 2017; Xu et al. 2020). Although some medicines such as corticosteroids and bronchodilators were used to relieve asthma symptoms, asthma is not readily preventable or curable (Jones et al. 2018; Wu et al. 2019). Therefore, there is a great need for new prevention and intervention therapies for asthma.

T cell immunoglobulin and mucin domain-1 (TIM-1) is a transmembrane glycoprotein identified to be a member of the Tim family of genes, which are related to the regulation of various immune responses (Kuchroo et al. 2008). In the immune system, TIM-1 was first identified to be expressed on T cells and DCs, where it plays an important role in regulating important cellular functions (Curtiss and Colgan 2007; Kuchroo et al. 2008; Rennett 2011; Xiao et al. 2011). TIM-1 is also an important susceptibility gene for both asthma and allergy (Brooks and Bonventre 2015; Du et al. 2016; Aravena et al. 2017). In addition, while TIM-1 is expressed on activated CD4⁺ T cells, it is preferentially expressed on Th2 cells, and regulates T cell activation as a potent costimulatory molecule (Rodríguez-Manzanet et al. 2009). The expression of TIM-1 results in a significant increase in the number of

IL-4-producing cells (Nakae et al. 2007). Moreover, TIM-1 can be activated by the exposure of phosphatidylserine (PtdSer) on apoptotic cells, thereby inducing asthma (Encinas et al. 2005; DeKruyff et al. 2010). Fine particulate matter (PM_{2.5}) exacerbates airway inflammation and AHR in patients with asthma. In addition, exacerbated AHR associated with allergic asthma induced by PM_{2.5} is related to increased apoptosis and TIM-1 activation (Zhao et al. 2018). Several groups have focused on recombinant mouse TIM-1 monoclonal antibodies (mAbs) by targeting TIM-1 to determine the function of TIM-1 in Th2-associated diseases (Xiao et al. 2015; Hosseini et al. 2018). In an OVA-induced model of asthma, anti-TIM-1 mAbs have been demonstrated to dramatically block inflammatory cell infiltration and reduce Th2 cytokine production (Encinas et al. 2005). The relationship between TIM-1 and asthma implies that targeting TIM-1 with recombinant anti-TIM-1 mAbs may reduce mucous cell metaplasia.

Therefore, the present study investigated the effect of intranasal administration of anti-TIM-1 on airway inflammation and AHR in OVA-immunized mouse models of asthma. In addition, the effects of anti-TIM-1 stimulation on the expression levels of signal transducer and activator of transcription (STAT)1, STAT6, T-box transcription factor (T-bet), B-cell lymphoma gene 2 (BCL2) and GATA binding protein-3 (GATA3) were studied. The data revealed that the effect of anti-TIM-1 was dependent on STAT1 and STAT6 pathways.

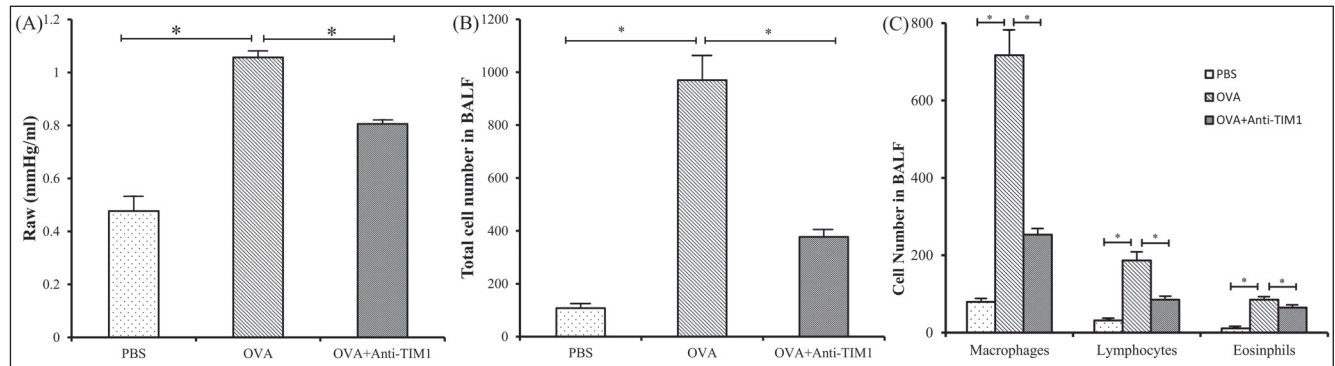


Fig. 1: Intranasal administration of Anti-TIM1 alleviates airway inflammation and AHR in the mice model of asthma. (A) Raw was measured in different groups. (B) The total cell number in the BALF samples. (C) The differential cell number in the BALF samples. Raw, Airway resistance; BALF, bronchoalveolar lavage fluid; Anti-TIM1, Tim1 antibody; OVA, ovalbumin. The data are expressed as the mean $\pm$ standard error of the mean of three independent experiments. * $P < 0.05$ indicates significant differences.

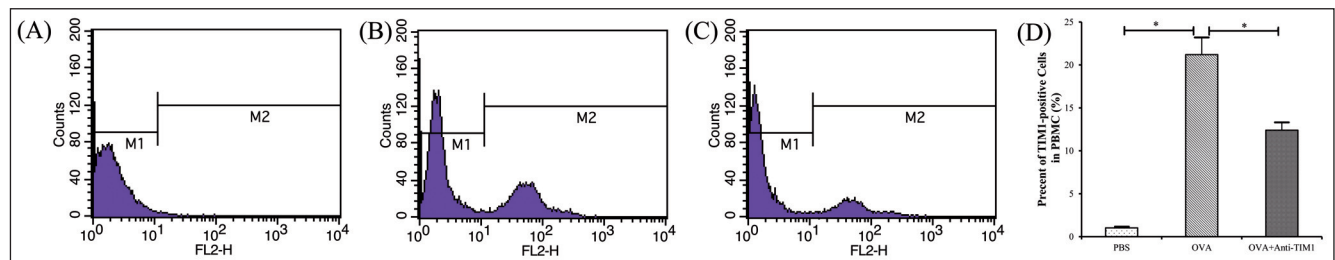


Fig. 2: Flow cytometric analysis of the percentage of TIM-1-positive cells in PBMCs. (A) The percentage of TIM-1-positive cells in PBS group. (B) The percentage of TIM-1-positive cells in OVA group. (C) The percentage of TIM-1-positive cells in OVA+TIM1 group. (D) Significantly reduced percentage of TIM-1-positive cells was observed in Anti-TIM1-treated mice compared with OVA-exposed mice. M1 represents the proportion of TIM-1 negative cells. M2 represents the proportion of TIM-1 positive cells. The data are expressed as the mean $\pm$ standard error of the mean of three independent experiments. Anti-TIM1, Tim1 antibody; OVA, ovalbumin. * $P < 0.05$ indicates significant differences.

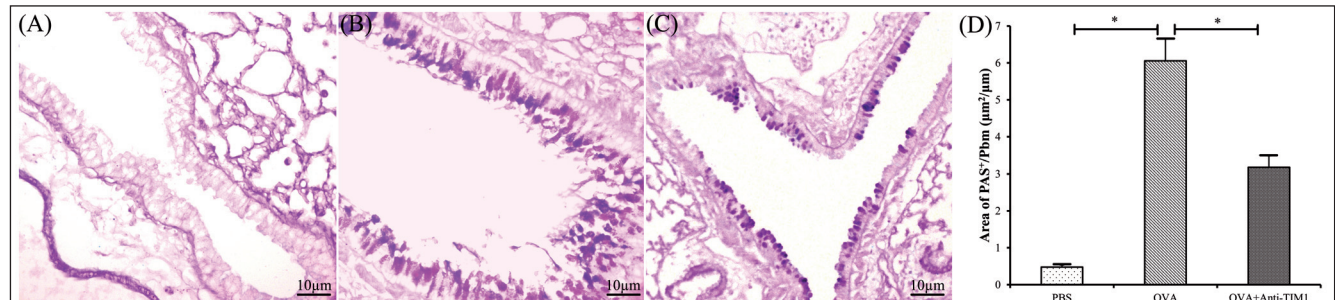


Fig. 3: Intranasal administration of Anti-TIM1 prevents OVA-induced airway remodeling in the mice model of asthma. (A) Representative photomicrographs of PAS-stained lung sections from PBS group (x200 magnification). (B) Representative photomicrographs of PAS-stained lung sections from OVA group (x200 magnification). (C) Representative photomicrographs of PAS-stained lung sections from OVA+TIM1 group (x200 magnification). (D) Significantly reduced airway mucus production was observed in Anti-TIM1-treated mice compared with OVA-exposed mice. The data are expressed as the mean $\pm$ standard error of the mean of three independent experiments. Anti-TIM1, Tim1 antibody; OVA, ovalbumin; PAS, periodic-acid Schiff; Pbm, perimeter of basement membrane. * $P < 0.05$ indicates significant differences.

2. Investigation and results

2.1. Intranasal administration of anti-TIM-1 alleviates allergic airway inflammation and AHR in mice model of asthma

In OVA-challenged mice, airway responsiveness to OVA was significantly enhanced compared with that PBS-challenged mice (Fig. 1A). In the OVA+Anti-TIM1 group, anti-TIM1 reversed OVA-induced AHR as reflected by a significant reduction in airway resistance in lung compliance compared with the OVA group (Fig. 1A). The total cell number from BALF samples in the PBS group was significantly lower compared with the OVA group. Moreover, the total cell number in OVA+anti-TIM1 group significantly decreased compared with the OVA group (Fig. 1B). Furthermore, the numbers of other inflammatory cells from BALF in the OVA+Anti-TIM1 group, such as macrophagocytes, lymphocytes and eosinophils, were also significantly reduced compared with the OVA group (Fig. 1C).

2.2. Intranasal administration of anti-TIM1 reduces TIM1 positive cell in PBMCs and suppresses mucous cell hyperplasia in mice model of asthma

The OVA group exhibited significantly higher levels of TIM1 positive cells while significantly lower levels of the TIM1 positive cells in PBMCs were observed compared with in the PBS group (Fig. 2). Compared with the OVA group, intranasal administration of anti-TIM1 led to a significant reduction of the TIM1 positive cells in PBMCs (Fig. 2).

To investigate whether anti-TIM1 plays a role in the development of mucous production, the mRNA expression levels of MUC5AC and airway epithelium were used to evaluate anti-TIM1 levels in mice model of asthma. The mRNA expression levels of MUC5AC were increased in the OVA group compared with the PBS group, whereas these were reduced in the OVA+Anti-TIM1 group compared with the OVA group (Fig. 5F). The OVA+Anti-TIM1 group exhibited significantly reduced mucous cell hyperplasia compared with the corresponding indices noted in the OVA group (Fig.3).

2.3. Intranasal administration of anti-TIM1 suppresses production of IL-4, IL-5 and IL-13, whereas it induces IFN- γ production in mice model of asthma

To determine whether anti-TIM1 ameliorates inflammation and whether AHR can reflect altered cytokine profiles, the concentration levels of the latter markers were examined in both BALF and serum samples derived from mice. The OVA group exhibited significantly higher levels of IL-4, IL-5, and IL-13, while significantly lower levels of the IFN- γ were observed compared with in the PBS group (Fig. 4). Compared with the OVA group, intranasal administration of anti-TIM1 led to a significant reduction of IL-4, IL-5, and IL-13, and a significant induction of IFN- γ (Fig. 4).

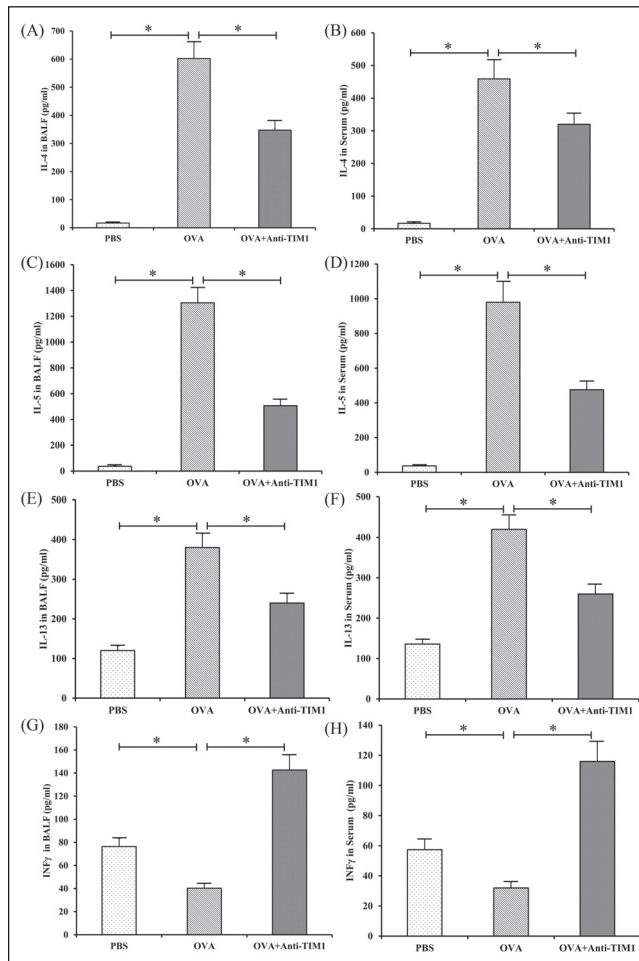


Fig. 4: The concentration levels of IL-4, IL-5, IL-13 and IFN- γ in serum and BALF samples were measured by ELISA. The concentration levels of (A) IL-4, (C) IL-5, (E) IL-13 and (G) IFN- γ in BALF. The concentration levels of (B) IL-4, (D) IL-5, (F) IL-13 and (H) IFN- γ in serum. The data are expressed as the mean $\pm$ standard error of the mean of three independent experiments. IL, interleukin; BALF, bronchoalveolar lavage fluid; Anti-TIM1, Tim1 antibody; OVA, ovalbumin; IFN, interferon. *P < 0.05 indicates significant differences.

2.4. Intranasal administration of anti-TIM1 in mice activates the STAT1 and STAT6 pathways

The involvement of the possible signaling pathways that include anti-TIM1 was assessed. Both mRNA and protein levels of STAT1, STAT6, T-bet, BCL2, and GATA3 were detected in lung tissues using RT-qPCR and western blotting analyses. Compared with the OVA group, the mRNA and protein expression levels of T-bet were significantly upregulated in the OVA+Anti-TIM1 group (Fig. 5A and 6B). In addition, the mRNA and protein expression levels of BCL2 and GATA3 were increased in the OVA group compared with the PBS group, whereas these were reduced in the OVA+Anti-TIM1 group compared with the

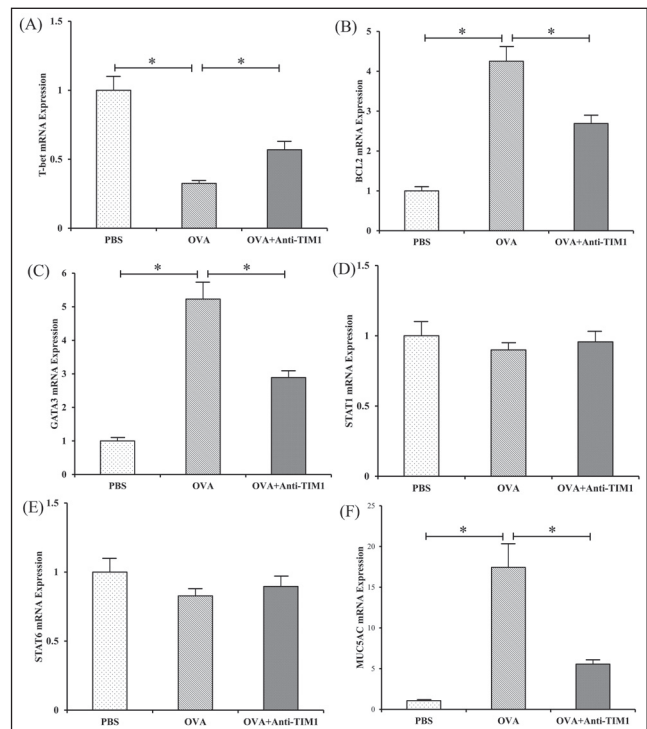


Fig. 5: RT-qPCR analysis of intranasal administration of Anti-TIM1 in the mice model of asthma. The expression of (A) T-bet, (B) BCL2, (C) GATA3, (D) STAT1, (E) STAT6 and (F) MUC5AC mRNA in mouse lung tissues were evaluated by RT-qPCR. The graphs display a summary of two to three independent experiments. Data are expressed as the mean $\pm$ standard error of the mean. Anti-TIM1, Tim1 antibody; OVA, ovalbumin; STAT, signal transducer and activator of transcription; BCL2, B-cell lymphoma gene 2; T-bet, T-box transcription factor; GATA3, GATA binding protein-3; MUC5AC, Mucin 5AC; RT-qPCR, reverse transcription-quantitative PCR. Data are presented as means $\pm$ SE. *P < 0.05 indicates significant differences.

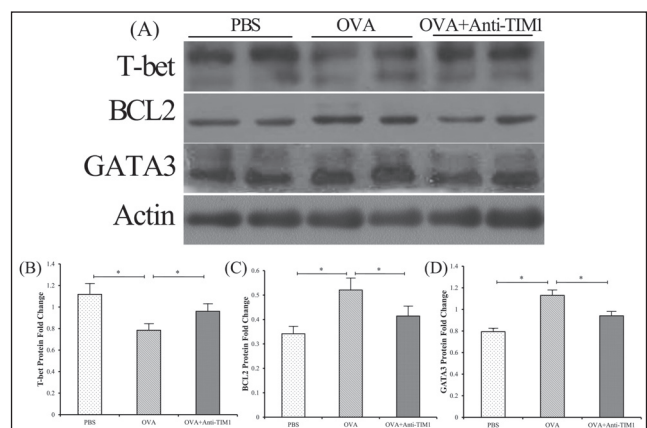


Fig. 6: Western blot analysis of intranasal administration of Anti-TIM1 in the mice model of asthma. (A) Western blots of T-bet, BCL2 and GATA3 in mouse lung tissues. Corresponding densitometric analyses of the protein bands of (B) T-bet/Actin, (C) BCL2/Actin, (D) GATA3/Actin. The graphs display a summary of two to three independent experiments. Data are expressed as the mean $\pm$ standard error of the mean. Anti-TIM1, Tim1 antibody; OVA, ovalbumin; BCL2, B-cell lymphoma gene 2; T-bet, T-box transcription factor; GATA3, GATA binding protein-3. Data are presented as means $\pm$ SE. *P < 0.05 indicates significant differences.

OVA group (Figs. 5B, 5C, 6C, and 6D). Compared with the OVA group, the mRNA expression levels of STAT1 and STAT6 exhibited no significant changes in the OVA+Anti-TIM1 group (Fig. 5D, 5E, and 6A). The phosphorylated levels of STAT1 and STAT6 proteins were reduced in the OVA group, whereas these were significantly upregulated in the OVA + Anti-TIM1 group (Fig. 7A, 7B, and 7C).

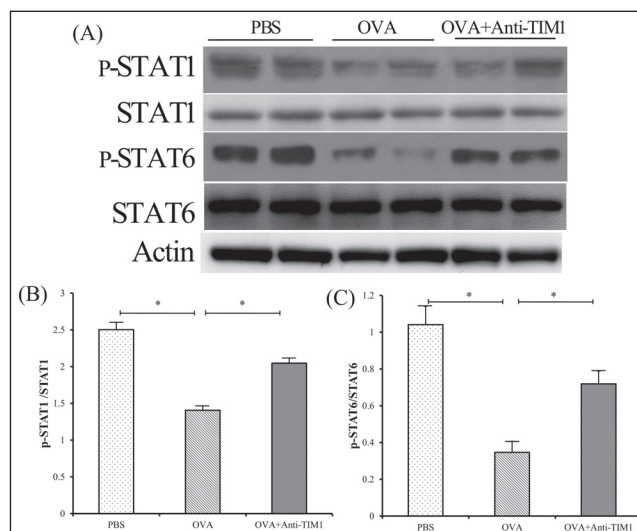


Fig. 7: Western blot analysis of the protein expression levels of p-STAT1 and p-STAT3 in mouse lung tissues. (A) Western blots of p-STAT1, STAT1, p-STAT6 and STAT6 in mouse lung tissues. Corresponding densitometric analyses of the protein bands of (B) p-STAT1/ STAT1 and (C) p-STAT6/ STAT6. The graphs display a summary of two to three independent experiments. Data are expressed as the mean $\pm$ standard error of the mean. Anti-TIM1, Tim1 antibody; OVA, ovalbumin; STAT, signal transducer and activator of transcription; p, phosphorylated. Data are presented as means $\pm$ SE. * $P < 0.05$ indicates significant differences.

3. Discussion

Asthma is typically characterized by chronic airway inflammation and associated with AHR. Asthma exacerbations usually occur in response to exposure to an external agent (e.g., viral infection of the upper respiratory tract, pollen, and air pollutants) (Xu et al. 2020). TIM-1 has been identified as an important susceptibility gene for asthma (Pouladian et al. 2017; Xie et al. 2017; Chiou et al. 2018; Niu et al. 2018). Previous studies have found that the inhibition of TIM-1 signaling can reduce the level of T cell infiltration into allergic skin tissues and tissues associated with autoimmune diseases (Angiari et al. 2014; Brunton et al. 2019). In a previous study, the injection of intraperitoneal anti-TIM-1 antibodies into a mouse model of allergen-induced asthma was found to reduce the level of Th2 cytokines and inhibited inflammatory cell infiltration in the airway (Encinas et al. 2005). AHR is one of the key features characteristic of asthma and one of the main factors responsible for the pathogenesis of this disease (O'Byrne and Inman 2003). AHR is characterized by increased airway resistance and decreased airway compliance (Kaczka et al. 1999). The results of the present study highlighted that intranasal administration of Anti-TIM1 could reduce inflammatory cell numbers in BALF samples and reverse methacholine-induced AHR. This indicated that Anti-TIM1 could effectively attenuate airway inflammation and AHR in experimental asthma, which was consistent with previous studies that showed that anti-TIM-1 modulates TIM-1 activity in response to allergen challenge in models of asthma (Hosseini et al. 2018).

Tim-1 is a transmembrane protein expressed on activated lymphocytes and appears to have a role as a co-stimulatory receptor in T cells. TIM-1 is a receptor for phosphatidylserine (PtdSer). TIM-1 recognition of PtdSer induces the constitutive expression of TIM-1 on NKT cells and results in AHR (Lee et al. 2010). In addition, a TIM-1 deficiency has been shown to reduce the incidence of allergic asthma in a mouse model (Kim et al. 2013). It has also been reported that agonistic TIM-1 mAbs (clone 3B3 and clone 1H8.2) augment T cell-mediated immune responses, whereas an antagonistic antibody inhibits the immune response through regulatory B cells (Ding et al. 2011). In this study, the percentage of TIM-1-positive cells was significantly downregulated in the OVA+Anti-TIM1 group compared with those noted in the OVA group. These findings suggested that TIM1 plays a role in allergic asthma, which is in accordance with previously reported data.

Mucous cell metaplasia in the airway epithelium is a pathogenic hallmark of asthma. Mucous metaplasia and hypersecretion can result in airway obstruction and significantly contribute to asthma-associated morbidity and mortality. In both autopsy biopsies of patients with fatal asthma and lung biopsies of individuals with severe asthma, mucous plugs and cellular debris have been observed in the small- and medium-sized airways (Kuyper et al. 2003). Furthermore, mucous cells located in the airway epithelium at the time of autopsy were 30-fold higher in patients with fatal asthma compared to those with asthma who died of other causes. Although airway mucous plugging is a primary cause of asthma-related death, the molecular mechanisms underlying mucous metaplasia and mucous hyper-secretion remain unclear. Mucous hyper-secretion involves increased mucin gene expression. Among the identified mucin genes, secreted mucin MUC5AC appears to be the most highly expressed in asthmatic epithelial cells, with several studies reporting an increase in MUC5AC and mucin 5B (MUC5B) expression in the human airway epithelium (Rose and Voynow 2006; Kettle et al. 2010; Monzon et al. 2011; Kwak et al. 2018). In this study, the H&E staining results indicate that the OVA-challenged mice treated with anti-TIM-1 exhibited a significant decrease in mucous cells in the bronchial epithelium. The mRNA expression of MUC5B was significantly downregulated in the OVA+Anti-TIM1 group compared with those in the OVA group. The understanding the functions of TIM-1 in contributing to asthma susceptibility and severity is of great importance to both asthma treatment and prevention.

TIM-1 can promote T cell proliferation and differentiation by binding to different agonistic ligands (Meyers et al. 2005; de Souza et al. 2008; Kim et al. 2010). TIM-1 molecules bound with agonistic TIM-1 mAbs (Xiao et al. 2007) or other agonistic ligands can produce a strong costimulatory signal for the activation of T cells, thereby promoting T cell differentiation and proliferation *in vivo*, activating cytokine production, and enhancing the antigen-induced immune response of T cells (Sizing et al. 2007; Schweigert et al. 2014). TIM-3 and TIM-1 expression on the surface of mouse mast cells promotes the secretion of IL-13, IL-5 and IL-4, indicating that mast cells also regulate immune function through members of the TIM family (Nakae et al. 2007). In CD4⁺ T cells, inhibiting TIM-1 signaling in CD4⁺ T cells can reduce the level of white blood cells and the production of inflammatory mediators, which can reduce the level of tissue damage caused by excessive inflammatory reactions (Rong et al. 2011; Balasubramanian et al. 2012). Previous studies showed that certain cytokines, such as IL-4, IL-5 and IL-13, play important roles in the initiation of airway inflammation, AHR and in variable airflow obstruction (Wills-Karp 1999; Jacobsen et al. 2015). In a model of acute renal injury induced by cisplatin, blocking TIM-1 signaling can significantly reduce the number of CD8⁺ T cells and inhibit IFN- γ secretion, indicating that TIM-1 costimulatory signaling can enhance the effect of CD8⁺ T cells (Nozaki et al. 2011). IFN- γ plays a role in the suppression of AHR and eosinophil lung infiltration, indicating that anti-TIM1 may have regulatory effects on asthmatic immune responses and promote the development of Th1 (Tumes et al. 2017). Our current study demonstrates that the serum and BALF concentration levels of the Th1 cytokines (IFN- γ) increased, whereas levels of Th2 cytokines (IL-4, IL-5, and IL-13) decreased in the OVA+anti-TIM1 group compared with the OVA group, which suggested that enhanced production of Th2 cytokines and/or decreased production of Th1 cytokines may contribute to the effects of anti-TIM1 on allergic asthma in this experimental model. Asthma is a chronic inflammatory disorder in which Th2, Th1 and suppressive T cells (Tregs) play a role. The transcription factors T-bet and GATA3 are important reciprocal determinants of Th1 and Th2 T helper cell differentiation (Robinson and Lloyd 2002). T-bet is important for Th1 and GATA-3 for Th2 differentiation from naïve T cells. Moreover, T-bet can directly interact with GATA-3, which interferes with the binding of GATA-3 to its target DNA, and causes inhibition of Th2 differentiation (Hwang et al. 2005). In the current study, it was shown that GATA3 was suppressed and the T-bet was increased in the OVA+anti-TIM1 group, whereas the opposite

effects were noted in the OVA group. These results indicated that the anti-TIM1 may affect the Th1 and Th2 T helper cell differentiation in the OVA-induced mice model of asthma. Epithelial lesion in adult asthma is due to the reduced epithelial cell proliferation and increased apoptosis (Trautmann et al. 2005). BCL2 affected apoptosis of the airway epithelium in asthma by cell death and survival (Holgate 2011; Hata et al. 2015). Our results demonstrate that anti-TIM1 suppress BCL2 expression and reduce the level of BCL2 in asthmatic mice, which indicated that anti-TIM1 could participate in apoptosis by modulate BCL2 expression.

STAT signaling pathways are candidates for influencing inflammatory disease, including asthma. The STAT1 pathway is important for the induction of Th1 differentiation and the inhibition of Th2 differentiation (Lu et al. 2020). STAT6 also plays a critical role in Th2 cell differentiation and in allergic lung inflammation (Yang et al. 2020). Previous studies showed that STAT6 and STAT1 might be crucial signal transducers in the development of BSM hyperresponsiveness by antigen exposure (Chiba et al. 2010, 2011). In the present study, the results showed that the STAT1 and STAT6 pathways were suppressed in the OVA group and activated in the OVA+ anti-TIM1 group. This evidence demonstrated that anti-TIM1 upregulated the phosphorylation of STAT1 and STAT6 proteins and restored the STAT1 and STAT6 signaling pathways in the asthma mouse model used.

In summary, the present study shows that the intranasal administration of anti-TIM1 could relieve airway inflammation and AHR by restored both the STAT1 and STAT6 pathways in experimental asthmatic mice. Additional studies are required to ensure the potential therapeutic applications of anti-TIM1 in asthma. Future studies are required to clarify the precise mechanism by which TIM-1 participates in the STAT signal pathway.

4. Experimental

4.1. Mice

A total of 24 female C57BL/6 mice were provided by the Experimental Animal Center of Second Military Medical University in Shanghai, China, and were housed in a specific pathogen-free facility and standard conditions including 12h light/dark cycle, room temperature of 22 °C, relative humidity of 60% and free access to food and water. The mice were studied at 6-8 weeks of age and age-matched to control mice. All of the animal experiments were approved by the Animal Care and Use Committee of Second Military Medical University (IACUC0035) in Shanghai.

4.2. Antigen sensitization and challenge

The mice were separated into the following three groups (n = 8 per group): PBS group, OVA group and OVA+anti-TIM-1 group. The mouse model of asthma was established according to previously described methods (Poynter et al. 2002). Briefly, in OVA and OVA+anti-TIM1 groups, active sensitization was performed by intraperitoneally injecting 20µg ovalbumin (OVA) (grade V; Sigma, St Louis, Mo) combined with 2 mg alum (Imject Alum; Pierce, Rockford, Ill) on days 1 and 8. The mice in the PBS group were treated with phosphate-buffered saline (PBS) containing 2 mg alum. Then, in OVA and OVA+anti-TIM1 groups, the mice were challenged intratracheally three times with 2.5% OVA on days 15, 16, and 17. Mice in the PBS group were intratracheally challenged three times with PBS on days 15, 16, and 17.

4.3. Anti-TIM-1 antibody treatment

Antibody treatment was performed on day 21 by administering intranasal instillations. In the PBS group, PBS-challenged mice were treated with 100 µg isotype-matched rat IgG2B antibodies (clone141945, R&D Systems) in 50 µL PBS. In the OVA group, OVA-challenged mice were treated with 100 µg isotype-matched rat IgG2B antibodies (clone141945, R&D Systems) in 50 µL PBS. In the OVA+anti-TIM1 group, the OVA-challenged mice were treated with 100 µg of anti-TIM-1 mAbs (clone 222414; R&D Systems) in 50 µL PBS. Each group included eight mice.

4.4. Pulmonary function testing

After 24 h of antibody treatment, each group was tested for pulmonary functionality using a Buxco PFT system (BioSystem XA, Wilmington, NC 28405, USA). The mice were intubated with a special tube and then connected to the PFT system. The respiratory targets were determined according to the manufacturer's protocol. Airway resistance (Raw) was the main indicator used to assess airway responsiveness.

4.5. Percentage of TIM-1-positive peripheral blood mononuclear cells

After evaluating pulmonary function, the mouse peripheral blood was sampled by removing the eyeball and collecting the blood into sodium citrate-containing tubes. Peripheral blood mononuclear cells (PBMCs) were isolated using lymphoprep density gradient separation. The percentage of TIM-1-positive PBMCs was assayed using a

FacsCalibur flow cytometer (BD Biosciences, USA), according to the manufacturer's instructions. Briefly, after washing with staining buffer, the cellular suspensions were labeled with biotinylated anti-mouse TIM-1 mAbs (eBioscience). The samples were incubated in the dark at 4 °C for 30 min, washed twice with PBS, and labeled with streptavidin-phycoerythrin (PE)-conjugated secondary antibodies (eBioscience). The cells were washed, resuspended in 0.2 mL PBS, and the percentage of TIM-1-positive PBMCs in cellular suspensions were analyzed using CellQuest software (BD Biosciences).

4.6. Analysis of bronchoalveolar lavage cells

Immediately after bleeding, a cannula was inserted into the trachea of the mice using a foregoing tube. The lungs were lavaged three times with 1.5 mL aliquots of 4 °C saline and the fluid were collected in a 1.5-mL centrifuge tube. The lavage fluid was subsequently centrifuged at 400 g for 10 min, and the supernatant was decanted and collected. The supernatant was then collected and stored at -20 °C until further use. The sediments were smeared, fixed, and stained with hematoxylin and eosin (H&E). The cellular morphology was observed under a microscope (OlympusBX51, Japan), sorted, and counted. We primarily observed the cell types (i.e., macrophages, lymphocytes, and eosinophils) by observing 10 random fields in each film under the 400 times microscope.

4.7. Enzyme-linked immunosorbent assay (ELISA)

The cytokine concentration in bronchoalveolar lavage fluid (BALF) and serum was determined using an ELISA. Briefly, after bleeding, the mice were fixed on the experimental platform. Tracheotomy and tracheal intubation were performed. Cold normal saline (1.5 mL) was injected into the lung three times slowly, and then absorbed back slowly. BALF was collected in an 1.5 mL EP tube and centrifuged for 10 min with 1029 × g. The supernatant was collected and the expression of cytokines was detected by ELISA.

The supernatant was collected, and the concentration of IL-4, IL-5, IL-13 and IFN-γ was determined in duplicate using a commercially available solid phase sandwich ELISA kit (R&D Systems), according to the manufacturer's protocol. The assays were performed in duplicate and the results are expressed as pg/mL.

4.8. Lung histological examination

The lung tissues were removed from mice following a bronchoalveolar lavage. The left lung was fixed in 10% zinc-formalin and embedded in paraffin. Approximately 5 µm-thick sections were made, and stained with Alcian blue and periodic acid-Schiff stain (AB-PAS) to detect mucous cells. PAS-stained sections were used for evaluating goblet cell hyperplasia. The bronchioles with 1.0-2.5 mm-long epithelial basement membranes were selected, and the area of goblet cells was measured as the area of PAS-positive staining using Image-Pro Plus 6.0. Subsequently, the ratio of the area of PAS-positive staining to Pbm was calculated.

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

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription was performed, and cDNA was synthesized from 2 µg of total RNA using an oligo(dT)18 primer and M-MLV reverse transcriptase (Takara, Syuzou, Shiga, Japan) for quantitative PCR. mRNA expression was determined using an ABI Prism 7300 Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Taq™ (Takara, Syuzou, Shiga, Japan). The primer sequences and the expected size of PCR products are listed in the Table. PRISM samples contained 1× SYBR Green Master Mix, 1.5 µL of 5 µM primers, and 25 ng of synthesized cDNA in a 25 µL reaction volume. The reaction system was heated to 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 56 °C for 5 s, and extension at 72 °C for 20 s. All PCR assays were performed in triplicate. A melting curve analysis was used to control amplification specificity.

Table: Primer sequences used for reverse transcription quantitative PCR

Gene	Sequence(5'-3')	Product Size(bp)
STAT1	F:CACGCTGCCTATGATGTCTCG R:CGCTTGCTTTCCGATGTTG	131
STAT6	F:GGCAGTGGTTTGATGGTGTC R:TAAAGCGGAGGAGGAAGGTC	147
T-bet	F:CTGGACCAACTGTCAACTGC R:CTCGGAACCTCCGCTTCATAAC	174
BCL2	F:CCCCTGGCATCTTCTCCTTC R:AAGAGTTTCCTCCACACCGT	250
GATA3	F:ACCACCCATTACCACTTATCC R:CGGTTCTGCCCATTCATTTTAT	250
MUC5AC	F:CAGCATCATCAACAGCGAAAC R:TAGTCACAGAACAGTGGGCAGA	216
β-Actin	F:TGTGCCCATCTACGAGGGCTAT R:TTTGATGTCACGCACGATTTC	155

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

4.10. Western blot analysis

Total proteins were extracted from mouse lung tissues and loaded on 5% SDS polyacrylamide gels. A bicinchoninic acid protein assay kit (cat. no. 23225; Pierce; Thermo Fisher Scientific, Inc.) was used to determine protein concentration. Following electrophoresis, the proteins were transferred to PVDF membranes (EMD Millipore). The membranes were blocked in 5% BSA in TBS-Tween-20 (0.15%) at 37 °C for 1 h. Subsequently, membranes were incubated with primary antibodies against STAT1, STAT6, T-bet, BCL2 and GATA3 (1:1,000 dilution; all from Abcam, Cambridge, MA, USA.), phosphorylated(p)-STAT3 (Tyr701) (1:1000) (Cell Signaling Technology, Beverly, MA, USA), p-STAT6(Tyr641) (1:1000) (Cell Signaling Technology, Beverly, MA, USA), and a monoclonal β -actin antibody used as the loading control (Sigma-Aldrich). After incubation with the primary antibodies overnight at 4 °C, the membranes were incubated with rabbit anti-mouse immunoglobulin G secondary antibody (1:5,000 dilution; Bio-Rad Laboratories, Inc.) for 1 h at 25 °C. The blots were visualized with a chemiluminescent detection system (ECL; GE Healthcare) according to the manufacturer's instructions.

4.11. Statistical analysis

Statistical analyses were performed using SPSS 16.0 Statistical Software (SPSS, Inc., Chicago, IL, USA). All data were expressed as the mean $\pm$ SD of at least three separate repeated experiments. The differences between groups were analyzed using a one-way analysis of variance or Dunnett's test. A threshold of $p < 0.05$ was considered to indicate statistical significance.

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