

Locally instilled tumor necrosis factor- α antisense oligonucleotide inhibits allergic inflammation via the induction of Tregs

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Abstract

Background Anti-tumor necrosis factor (TNF)- α therapeutics has the potential to alleviate allergic inflammation. However, in previous studies, the systemic administration of anti-TNF- α agents was frequently accompanied by many adverse effects, such as infection, immunogenicity and malignancy. Efforts are made in the present study to evaluate whether or not local administration of TNF- α antisense oligonucleotide would inhibit allergic airway inflammation and influence systemic immune responses in an ovalbumin-induced asthmatic murine model.

Methods The treatment effects of TNF- α antisense oligonucleotide on mice, as well as the alternative proportion of regulatory T cells and T_H2 cells, were examined and compared with untreated mice.

Results Local administration of TNF- α antisense oligonucleotide resulted in significantly inhibited TNF- α expression, remarkably decreased inflammatory cell infiltration and dramatically reduced mucus hypersecretion. These treatment effects were associated with induced $CD4^+CD25^+Foxp3^+$ regulatory T cells, reduced T_H2 cells and generally decreased T_H2 -type cytokines expression in bronchoalveolar lavage fluid. Systemic immunosuppression was not triggered by local antisense oligonucleotide administration because the proportion of $CD4^+CD25^+Foxp3^+$ regulatory T cells in the blood, thymus or spleen was not affected. Attenuated 4-1BBL expression was likely involved in the alternative proportion of T cells.

Conclusions These findings demonstrate that local administration of TNF- α antisense oligonucleotide contributes to anti-inflammatory action via the enhancement of regulatory T cells-mediated immune tolerance, which is not accompanied by systemic immunosuppression associated with systemically-induced regulatory T cells. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords allergic airway inflammation; antisense oligonucleotide; anti-TNF- α therapeutics; regulatory T cells; T_H2 cell-driven inflammation

Introduction

Asthma is a chronic inflammatory pulmonary disease characterized by airway hyperresponsiveness, chronic eosinophilia, elevated serum immunoglobulin (Ig)E, excessive mucus production, and airway remodeling [1]. The pathogenesis of asthma is associated with the expansion and activation of type 2 helper T cells (T_H2) cells in the airway [2]. These activated T_H2 cells up-regulate the expression of a gene cluster encoding T_H2 -type cytokines interleukin

Received: 23 February 2012

Revised: 24 April 2012

Accepted: 29 April 2012

(IL)-4, IL-5, IL-9 and IL-13, which are involved in IgE synthesis, the recruitment of mast cells, and the maturation of eosinophils and basophils [3]. By these T_H2 -biased responses, immune tolerance against allergens is broken down and lung function is threatened by on-going allergic airway inflammation.

Patients with refractory asthma have obvious evidence of an up-regulated tumor necrosis factor α (TNF- α) axis [4]. In recent years, TNF- α has served as a potential therapeutic target for the treatment of asthma. There are six reported clinic trials concerned with the systemic administration of anti-TNF- α monoclonal antibody (mAb) or soluble receptor for treating asthma, although contradictory results have emerged. Two trials have confirmed the efficacy of anti-TNF- α soluble receptor Etanercept [4,5] and two trials have denied this efficacy [6,7]. In addition, a trial using anti-TNF- α mAb Infliximab resulted in a reduced number of mild exacerbations [8], whereas another trial using Golimumab led to serious adverse events, and even one death and eight malignancies occurred in the Golimumab groups [9]. From these studies, the use of anti-TNF- α therapeutics reveals its potential for the treatment of asthma, especially refractory asthma with overexpressed TNF- α , although the therapeutic method needs to be discussed and the serious adverse effects need to be dealt with.

Indeed, in the past 10 years, there has been serious concern on the adverse effects that accompany the systemic administration of anti-TNF- α mAb or soluble receptor for inhibiting pathologic inflammation systemically. Infections, immunogenicity and malignancies, as well as pneumonia and interstitial lung disease, have been reported in the therapeutic processes of various inflammatory diseases [10]. There were obvious clues that immunogenicity and comprehensive immunosuppression caused by systemic administration of protein-based anti-TNF- α agents were involved. Thus, the strategy that local administration of anti-TNF- α agent with no proteantigen-triggered immunogenicity, in which nucleic acid antisense drugs are the preferred candidate, is proposed as an alternative for treating these inflammatory diseases.

In previous studies and trials, the mechanism of anti-TNF- α therapeutics on allergic inflammation was attributed to the inhibition effect on TNF- α -mediated eosinophilic disorder, in which the attenuated chemokines, adhesion molecules and various cytokines were involved [5,11]. Although eosinophils were associated with allergic inflammation, more recent studies have indicated that the allergen-induced T_H2 responses must play more critical part in allergic inflammatory pathology [3]. This conceptual improvement resulted in a varied progression with respect to the restored immune tolerance against T_H2 responses, such as glucocorticoid-induced regulatory T cells (Tregs)-mediated immune tolerance [12]. However, there was little concern about the influence of anti-TNF- α therapeutics on allergen-induced T_H2 responses, and the mechanism of anti-TNF- α therapeutics on allergen-induced T_H2 inflammation remains unclear.

In the present study, we focused on the treatment effect of local TNF- α antisense oligonucleotide (ASO) administration in an ovalbumin (OVA)-induced asthmatic murine model. ASO ISIS25302, which was designed to target the 3'-UTR of the primary RNA transcript of TNF- α , was administered intranasally. ASO was enriched in the airway epithelium and significantly inhibited TNF- α expression was found in bronchoalveolar lavage fluid (BALF) and the airway epithelium. Accompanied by inhibited TNF- α expression, the infiltrates of eosinophils and mast cells were remarkably attenuated, and mucus production was apparently reduced. For the mechanism study, we found that inhibited TNF- α expression caused by instilled TNF- α ASO contributed to a dramatically increased proportion of $CD4^+CD25^+Foxp3^+$ Tregs in BALF. In parallel, the proportion of $CD3^+CD8^+IL-4^+$ T_H2 cells and the levels of T_H2 -type cytokines IL-4, IL-5, IL-13 in BALF were remarkably decreased. All these effects were not concerned with any significant difference in the proportion of $CD4^+CD25^+Foxp3^+$ Tregs in the blood, thymus and spleen. We also found that co-stimulatory molecule ligand 4-1BBL in trachea tissue and lung tissue was restricted by the inhibited TNF- α expression, which was likely involved in the alternative proportion of T cells. This mechanism of anti-TNF- α therapeutics probably provides hints with respect to a new therapeutic target of allergic inflammation.

Materials and methods

Mice

Female BALB/c aged 6–8 weeks of age was obtained from Laboratory Animal Center of Nanjing University (Nanjing, China). All animals were housed in a SPF facility and received human care in accordance with Chinese legal requirements.

Antisense oligodeoxynucleotide

The phosphorothioate-modified antisense oligonucleotide used in the present study, ISIS 25302, which was designed to target the 3'-UTR of the primary RNA transcript of TNF- α , was synthesized by Genset (La Jolla, CA, USA).

The sequence of ISIS25302 is: 5'-AACCCATCGGCTGG-CACCAC-3'. As a scrambled control oligonucleotide (SCO), ISIS 18154 (5'-TCAAGCAGTGCCACCGATCC-3') was used. Rhodamine was modified onto the TNF- α ASO by Invitrogen (Carlsbad, CA, USA).

Other methods

The methods for OVA sensitization, airway challenge, ASO distribution, measurement of cytokines in BALF, flow cytometry for the proportion of $CD4^+CD25^+Foxp3^+$ Tregs and $CD3^+CD8^+IL-4^+$ T_H2 cells, histology, immunohistochemistry for TNF- α , reverse transcriptase-polymerase chain reaction (RT-PCR) assay for TNF- α and 4-1BBL, western blot analysis for 4-1BBL, and immunofluorescent analysis for 4-BBL, are described in the Supporting information.

Statistical analysis

Results are expressed as the mean $\pm$ SEM. Differences between groups were analyzed by the Kruskal–Wallis analysis of variance test. $p < 0.05$ was considered statistically significant.

Results

Local TNF- α ASO administration inhibits TNF- α expression in BALF, trachea tissue and lung tissue

Figure 1A shows the schematic timeline of the experiments. Sensitized Balb/c mice were challenged with aerosolized

OVA and intranasally administered with phosphate-buffered saline, TNF- α SCO, TNF- α ASO and rhodamine-labeled TNF- α ASO, respectively. Administration of rhodamine-labeled TNF- α ASO in an instillation manner resulted in the enrichment of ASO in the airway epithelium of trachea tissue and lung tissue (Figure 1B). Twenty-four hours after the last instilled treatment, the concentration of TNF- α protein in BALF was examined using an enzyme-linked immunosorbent assay (ELISA). A significant increase in TNF- α expression was observed in the OVA-challenged mice, whereas the concentration of TNF- α was reduced by 69.1% in the TNF- α ASO treated mice compared to OVA challenged mice (Figure 1C). We also examined TNF- α mRNA expression and TNF- α protein distribution in trachea tissue and lung tissue. Using an RT-PCR assay, the level of TNF- α mRNA was found to be increased in both trachea and lung tissue of the OVA-challenged mice. After mice were treated with TNF- α ASO,

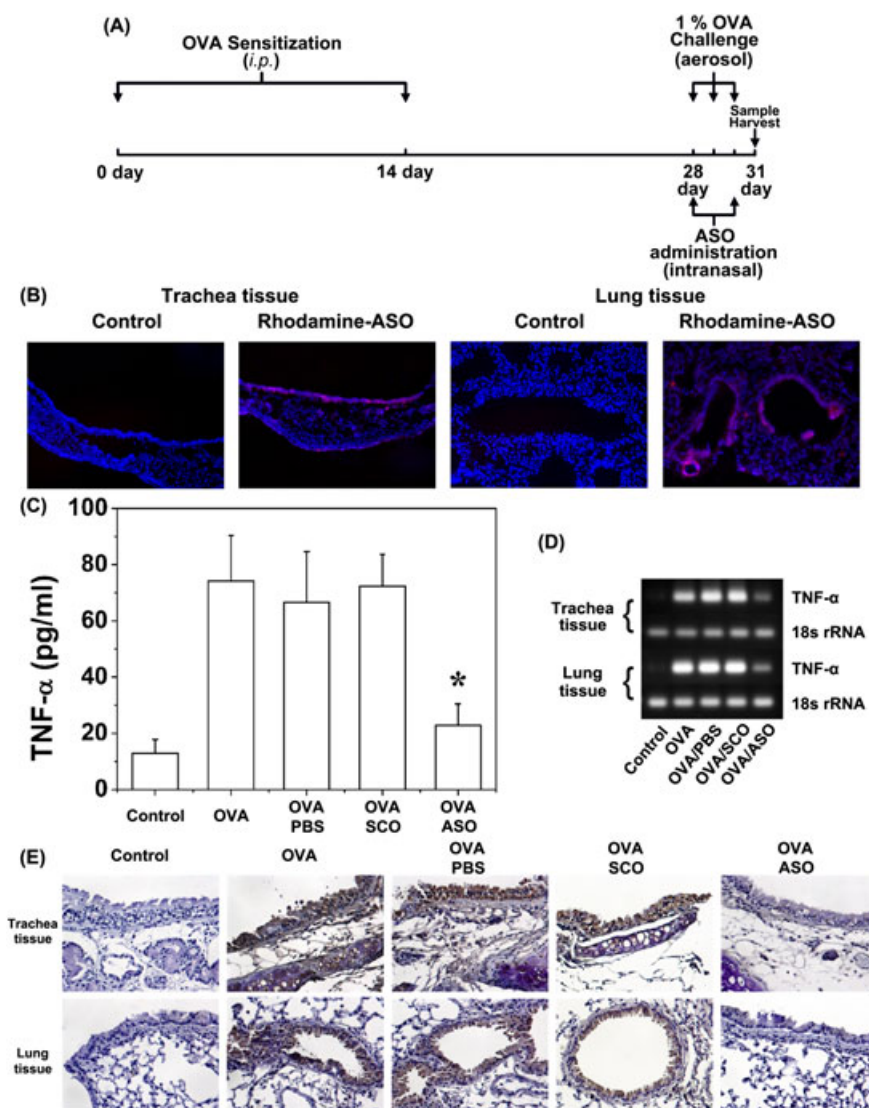


Figure 1. Instillation of TNF- α ASO inhibits OVA-induced TNF- α expression. (A) Schematic timeline to establish OVA-induced allergic airway inflammation and to administer TNF- α ASO intranasally. (B) Distribution of rhodamine-labeled TNF- α ASO ($\times 200$ magnification). Red for rhodamine-labeled TNF- α ASO; blue for 4,6-diamidino-2-phenylindole (DAPI) nuclear staining. (C) Concentration of TNF- α in BALF ($n = 8$ for each group, with the experiments carried out in triplicate). (D) RT-PCR analysis of TNF- α mRNA production. (E) Immunohistochemistry for TNF- α expression ($\times 400$ magnification). * $p < 0.05$ versus OVA-challenged mice.

TNF- α mRNA expression in the two tissues was apparently decreased (Figure 1D). By immunohistochemistry, the airway epithelium of trachea tissue and lung tissue was detected as the main source of TNF- α expression in the OVA-challenged mice. By contrast, TNF- α expression in the airway epithelium was substantially reduced by TNF- α ASO treatment (Figure 1E).

Local TNF- α ASO administration inhibits OVA-induced airway inflammatory cell infiltration

OVA challenge induced the infiltration of allergic inflammatory cells into the peribronchiolar and perivascular connective tissues, whereas local TNF- α ASO administration attenuated the recruitment of these inflammatory cells [Figure 2A, hematoxylin and eosin (H&E) row]. OVA

challenge also resulted in a significant increase of mucus production that was secreted by goblet cells in the airway epithelium, whereas mucus production was apparently reduced in TNF- α ASO treated mice [Figure 2A, periodic acid-Schiff (PAS) row]. The average ratio of PAS positive cells to total epithelial cells in TNF- α ASO treated mice was 8.1%, which was significantly less than that of 68.8% in OVA challenged mice (Figure 2B). By Congo red and toluidine blue staining, it was observed that the OVA-induced infiltration of eosinophils into lung tissue and mast cells in the subepithelial of trachea tissue was enhanced. After mice were treated with TNF- α ASO, the numbers of infiltrated eosinophils (Figure 2A, Congo red row) and mast cells (Figure 2A, toluidine blue row) were prominently reduced. By calculation, the average number of eosinophils per single scope significantly decreased from 16 to 7 (Figure 2C), and that of mast cells also significantly decreased from 10 to 1 (Figure 2D).

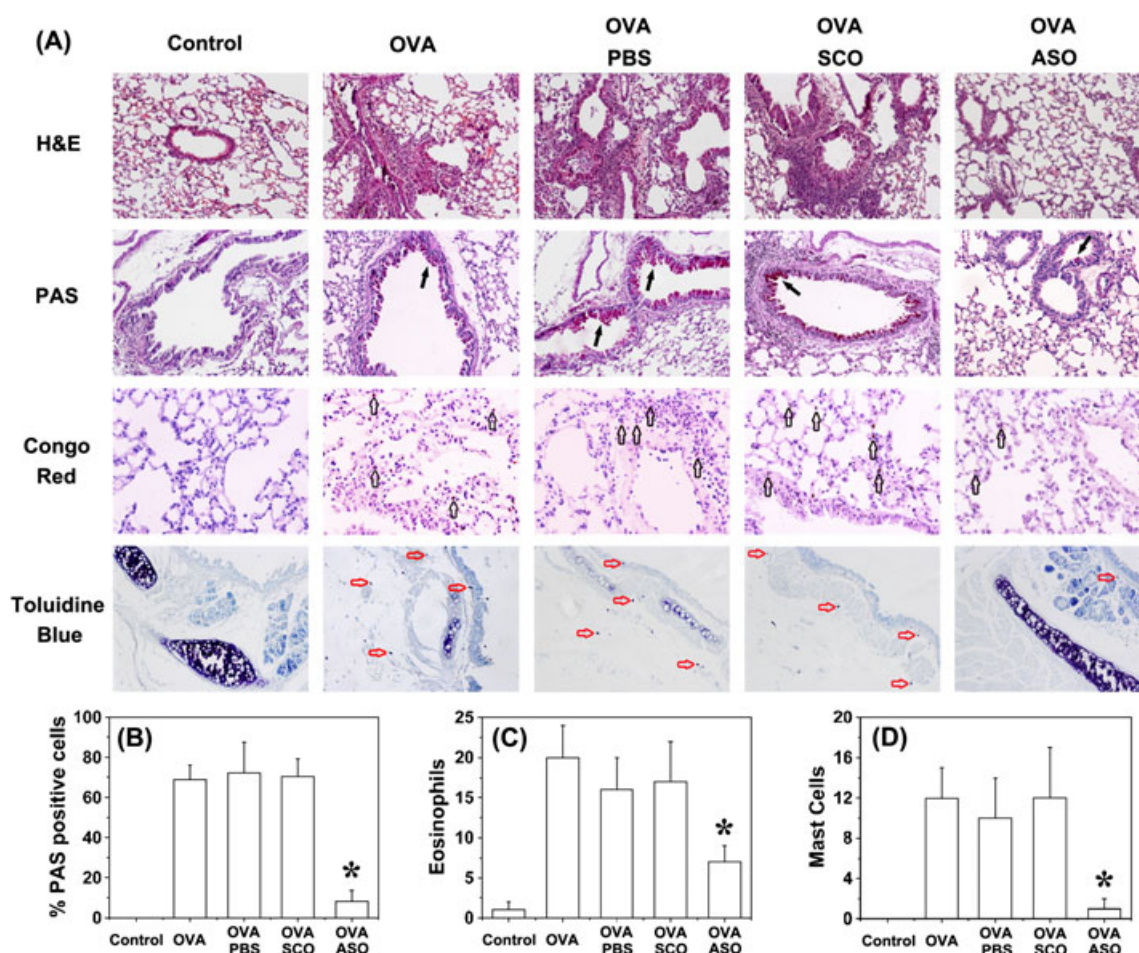


Figure 2. Local TNF- α ASO administration inhibits OVA-induced mucus hypersecretion and infiltrates of eosinophils and mast cells. (A) Histology of lungs or tracheas from TNF- α ASO treated mice. H&E staining indicates peribronchial infiltrates of inflammatory cells ($\times 200$ magnification). PAS staining showing mucus production (some indicated by black arrows) in the lung airway epithelium ($\times 200$ magnification). Congo red staining showing eosinophil infiltration (some indicated by open arrows) into lung airways ($\times 400$ magnification). Toluidine blue staining showing mast cell infiltration (some indicated by red open arrows) into the subepithelial of trachea tissues ($\times 200$ magnification). (B) The average ratio of PAS positive cells to total epithelial cells. (C) The average number of eosinophils per single scope at a magnification of $\times 400$ in Congo red staining lung sections. (D) The average number of mast cells per single scope at a magnification of $\times 200$ in toluidine blue staining trachea sections ($n = 10$ for each group, with the experiments carried out in triplicate). * $p < 0.05$ versus OVA-challenged mice.

The levels of T_H2 -type cytokines and OVA-induced eosinophil recruitment in BALF (but not the level of T_H1 -type cytokines and the number of lymphocytes) are inhibited by local TNF- α ASO administration

BALF was collected 24 h after the last instilled treatment. Subsequently, total and differential cells were counted. OVA challenge significantly increased total cell, eosinophil and lymphocyte counts in BALF (Figure 3A). After local TNF- α ASO administration, the average number of total cells in BALF significantly decreased from 26.3×10^4 to 6.2×10^4 per ml and that of eosinophils significantly decreased from 17.1×10^4 to 8.8×10^4 per ml (Figure 3A). However, the number of lymphocytes in BALF was not affected by TNF- α ASO instillation (Figure 3A).

The levels of T_H2 -type cytokines in BALF were measured using ELISA. OVA challenge remarkably induced the release of IL-4, IL-5 and IL-13 into BALF (Figure 3B). Treatment of OVA-challenged mice with TNF- α ASO

significantly reduced the concentration of IL-4 from 56.8 pg/ml to 16.1 pg/ml, as well as the concentration of IL-5 from 80.1 pg/ml to 30.2 pg/ml and that of IL-13 from 145.6 pg/ml to 31.2 pg/ml (Figure 3B). We also examined the levels of T_H1 -biased pro-inflammatory cytokine IL-12 and T_H1 -type cytokine IFN- γ in BALF. Instilled TNF- α ASO did not result in significant differences of IL-12 and IFN- γ (Figure 3C). Moreover, the concentration of TGF- β in BALF was also not affected by TNF- α ASO treatment (Figure 3C). The level of IL-10 in BALF was increased from 22.6 pg/ml to 272.6 pg/ml when OVA challenge was conducted, and decreased to 117.9 pg/ml after TNF- α ASO was instilled (Figure 3C).

Local TNF- α ASO administration decreases the ratio of OVA-induced $CD3^+CD8^-IL-4^+ T_H2$ cells in BALF

T_H2 cells in BALF, which were capable of secreting IL-4, IL-5 and IL-13, were analyzed by flow cytometry. The ratio of $CD8^-IL-4^+$ cells divided by total $CD3^+$ cells

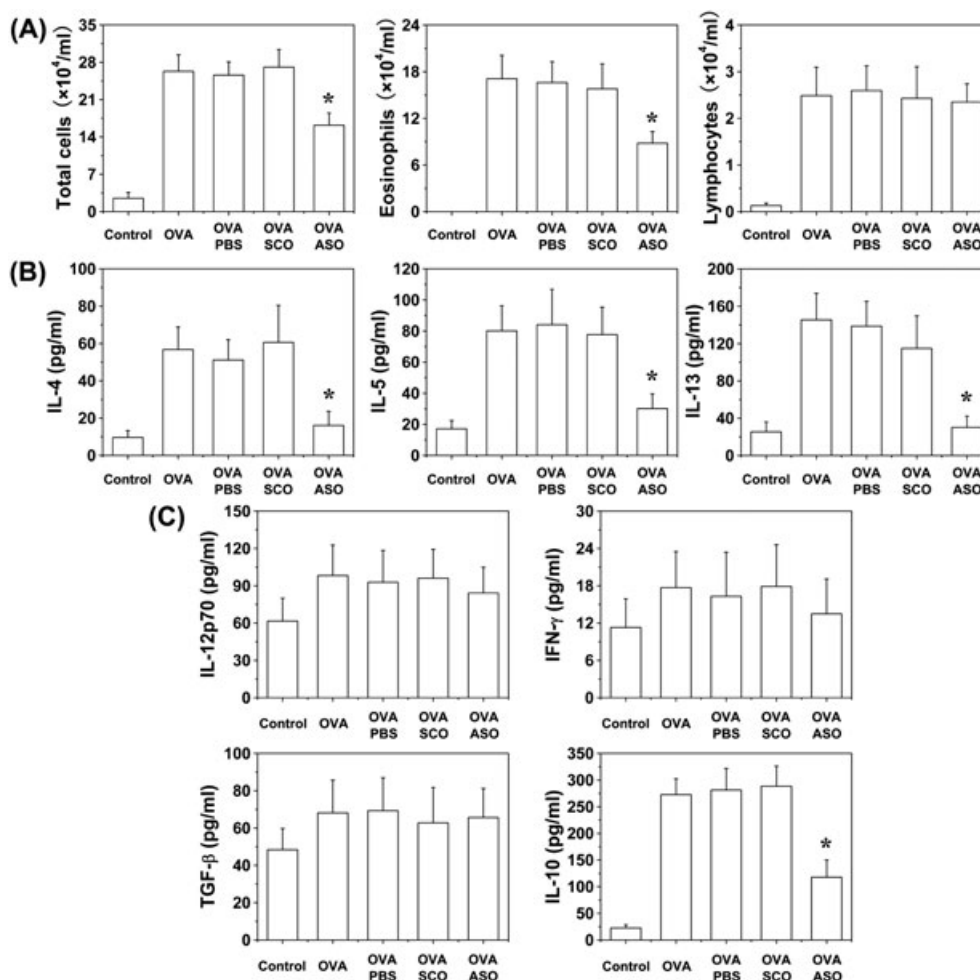


Figure 3. Local TNF- α ASO administration inhibits OVA-induced eosinophil infiltration, T_H2 -type cytokine production and IL-10 in BALF, whereas the number of infiltrated lymphocytes, the levels of T_H1 -biased cytokines and TGF- β in BALF are not affected. (A) The numbers of total cells, eosinophils and lymphocytes in BALF. (B) The levels of T_H2 -type cytokines IL-4, IL-5 and IL-13 in BALF. (C) The levels of T_H1 -biased cytokines IL-12 and IFN- γ , as well as the levels of TGF- β and IL-10, in BALF ($n = 8$ for each group, with the experiments carried out in triplicate). * $p < 0.05$ versus OVA-challenged mice.

was measured. It was determined that OVA challenge increased the ratio of CD8⁺IL-4⁺ cells from 0.75% to 3.43% (Figure 4A). In parallel, the absolute number of CD3⁺CD8⁺IL-4⁺ cells per ml BALF significantly increased from approximately 150 to over 1800 (Figure 4B). The instillation of TNF- α ASO to the OVA-challenged mice led to remarkable decrease in the ratio of CD8⁺IL-4⁺ cells from 3.43% to 1.25% (Figure 4A) and the number of CD3⁺CD8⁺IL-4⁺ cells per ml BALF from over 1800 to approximately 800 (Figure 4B).

The proportion of CD4⁺CD25⁺Foxp3⁺ Tregs in BALF was significantly increased by local TNF- α ASO administration

Tregs in BALF, which was implicated in the suppression of T_H2 responses to allergen challenge and the maintenance

of peripheral immune tolerance, was analyzed by flow cytometry. CD4⁺ cells were gated and the proportion of CD25⁺Foxp3⁺ cells was determined. OVA challenge gave rise to an increased ratio of CD25⁺Foxp3⁺ cells from 2.28% to 7.05% (Figure 5A). When calculated as the absolute number, CD4⁺CD25⁺Foxp3⁺ Tregs that were induced by OVA rose from approximately 60–722 per ml BALF (Figure 5B). Treatment of OVA-challenged mice with TNF- α ASO resulted in a significantly increased ratio of CD25⁺Foxp3⁺ cells from 7.05% to 15.52% (Figure 5A) and the absolute number of CD4⁺CD25⁺Foxp3⁺ Tregs per ml BALF was also increased from 722 to approximately 1500 (Figure 5B).

We also measured the ratio of CD25⁺Foxp3⁺ cells to total CD4⁺ cells in the blood, thymus and spleen. Local TNF- α ASO administration in an intranasal instillation manner led to a slight increase in the proportion of CD4⁺CD25⁺Foxp3⁺ Tregs in blood (Figure 5C), as well as a slight decrease in the proportion of CD4⁺CD25⁺Foxp3⁺ Tregs in the thymus (Figure 5D) and spleen (Figure 5E).

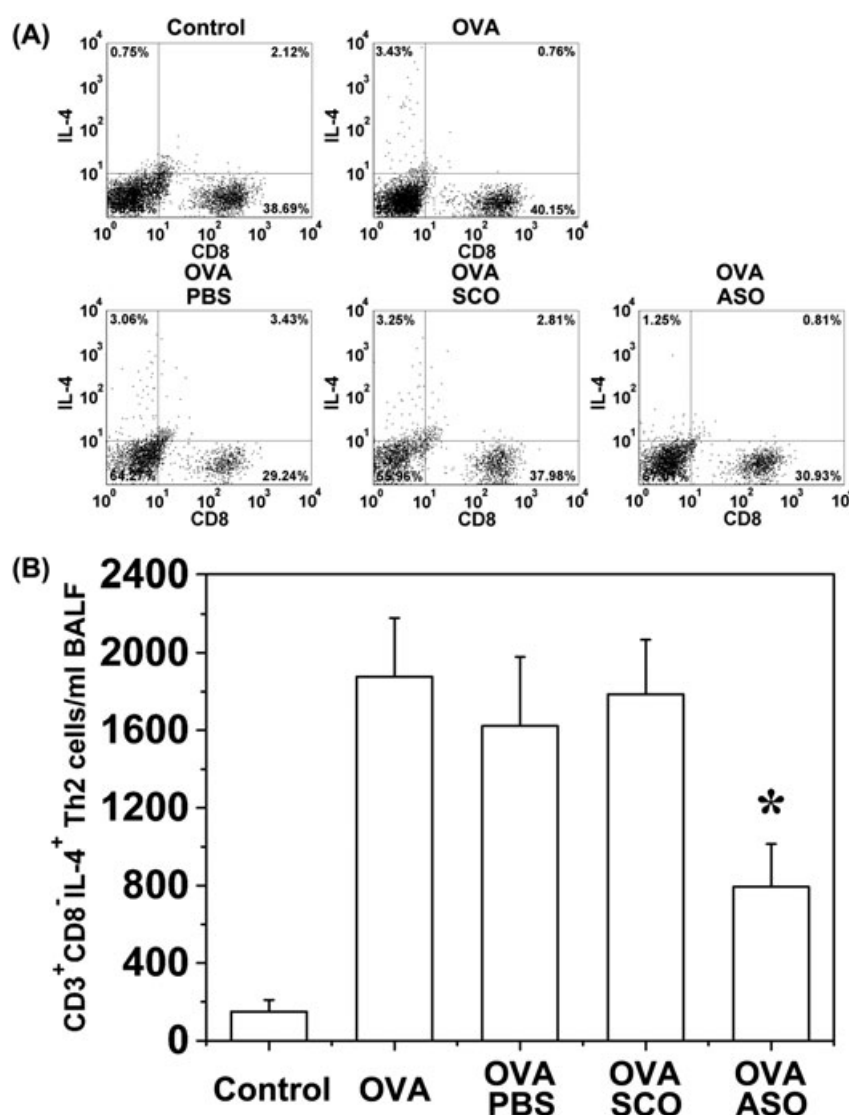


Figure 4. Local TNF- α ASO administration inhibits OVA-induced CD3⁺CD8⁺IL-4⁺ T_H2 cells in BALF. (A) Flow cytometry analysis of CD3⁺CD8⁺IL-4⁺ T_H2 cells in BALF. (B) The absolute number of CD3⁺CD8⁺IL-4⁺ T_H2 cells in BALF ($n = 24$ for each group, each group contains six samples and each sample is comprised of four combined cell pellet from BALF to ensure the cell numbers exceed 2×10^6 in total). The experiments were carried out in triplicate. * $p < 0.05$ versus OVA-challenged mice.

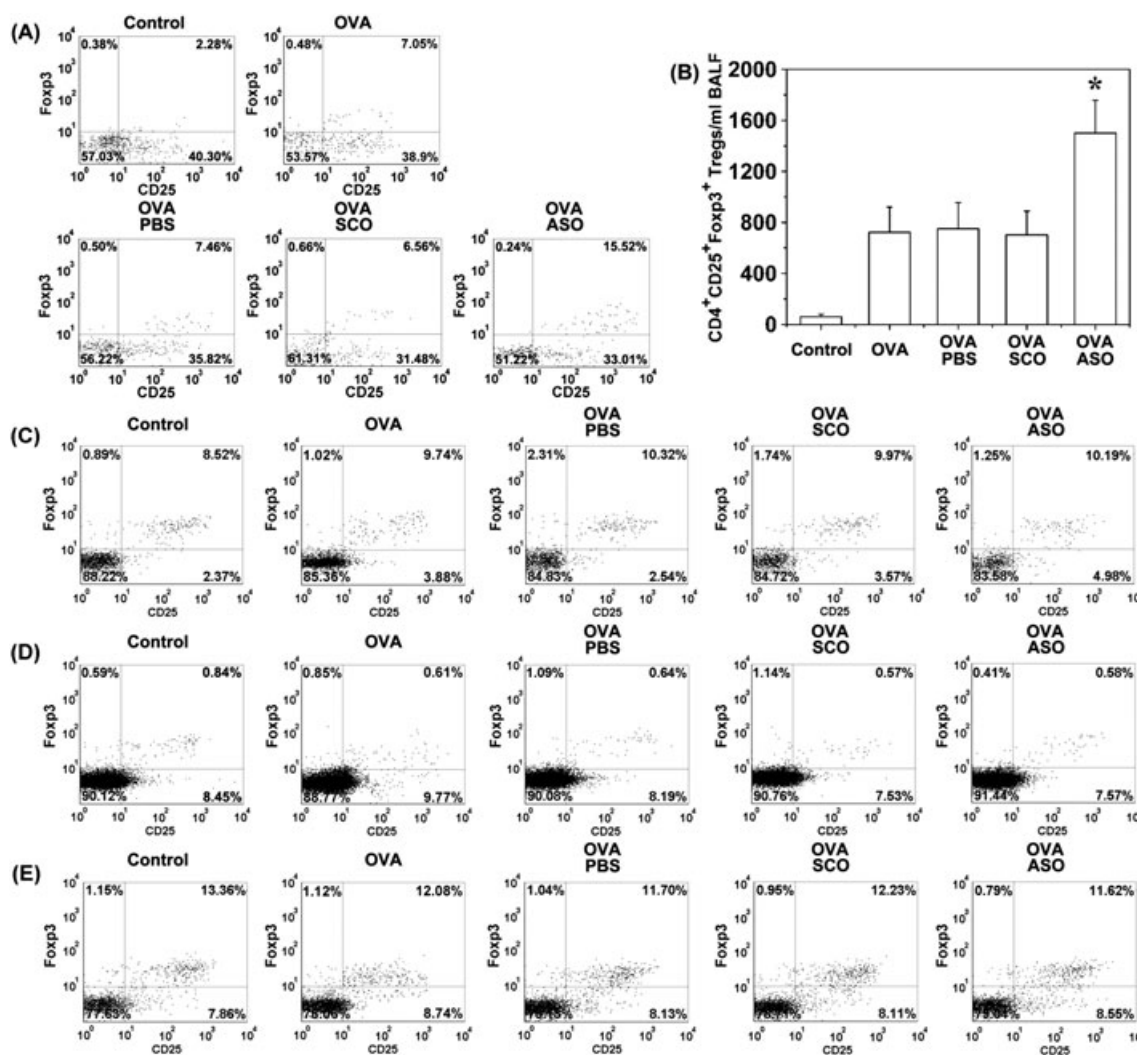


Figure 5. Local TNF- α ASO administration leads to remarkably increased CD4⁺CD25⁺Foxp3⁺ Tregs in BALF. (A) Flow cytometry analysis of CD4⁺CD25⁺Foxp3⁺ Tregs in BALF. (B) The absolute number of CD4⁺CD25⁺Foxp3⁺ Tregs in BALF. (C) Flow cytometry analysis of CD4⁺CD25⁺Foxp3⁺ Tregs in blood. (D) Flow cytometry analysis of CD4⁺CD25⁺Foxp3⁺ Tregs in the thymus. (E) Flow cytometry analysis of CD4⁺CD25⁺Foxp3⁺ Tregs in the spleen ($n = 24$ for each group, each group contains six samples and each sample is comprised of four combined cell pellet from BALF to ensure the cell numbers exceed 2×10^6 in total). The experiments were carried out in triplicate. * $p < 0.05$ versus OVA-challenged mice.

Co-stimulatory molecule ligand 4-1BBL was inhibited by local TNF- α ASO administration

4-1BBL, one of the co-stimulatory molecule ligands that was involved in T cell co-stimulation, was examined using the RT-PCR assay, western blot analysis and immunofluorescent analysis. 4-1BBL mRNA expression and protein production were up-regulated in both trachea and lung tissue from OVA-challenged mice, and local TNF- α ASO administration effectively attenuated the OVA-induced up-regulation of 4-1BBL with respect to both mRNA and protein levels (Figures 6A and 6B). By immunofluorescent analysis, increased 4-1BBL expression was investigated in the airway epithelium of lung tissue after OVA challenge was conducted. After TNF- α ASO was instilled, 4-1BBL expression was apparently reduced (Figure 6C). By calculation, local TNF- α ASO administration contributed to the

significantly decreased ratio of 4-1BBL positive cells to total airway epithelial cells from 85.5% to 21.9% (Figure 6D).

Discussion

Compared to patients with mild-to-moderate asthma, patients with refractory asthma have obvious evidence of an up-regulated TNF- α axis, and TNF- α therefore has received much attention recently in refractory asthma [4]. These patients comprise 5–10% of asthma patients [13] and are particularly resistant to conventional therapy [11]. Thus, targeting TNF- α was considered as a new therapeutic approach for asthma. Systemic administration of anti-TNF- α mAb or soluble receptor has been explored and evaluated. Two reported trials have confirmed the efficacy of anti-TNF- α soluble receptor Etanercept [4,5] and two trials have denied this efficacy [6,7]. In addition, treatment with anti-TNF- α mAb Infliximab resulted in a reduced number of mild

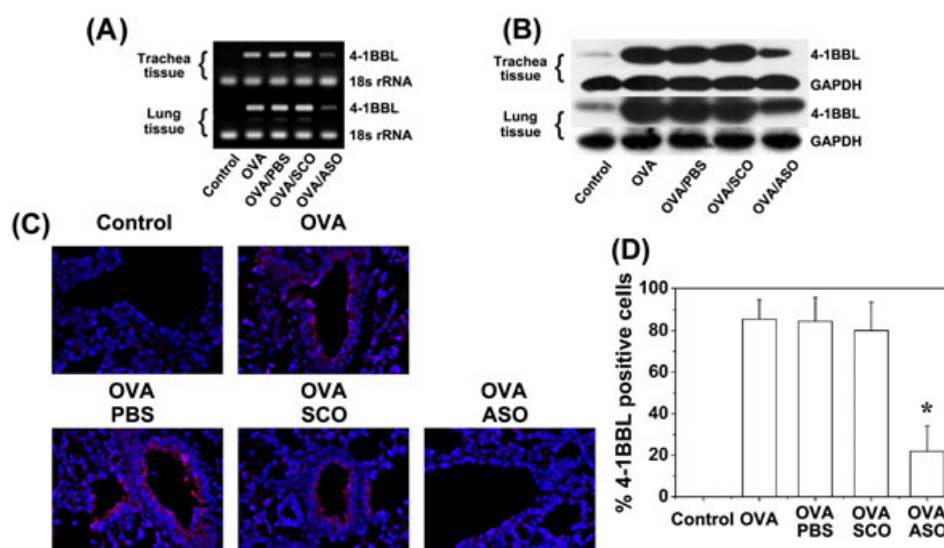


Figure 6. 4-1BBL expression in the trachea tissue and the lung tissue is inhibited by instilled TNF- α ASO. (A) RT-PCR analysis of 4-1BBL mRNA production. (B) Western blot of 4-1BBL protein expression ($n = 8$ for each group, with the experiments carried out in triplicate). (C) Immunofluorescent analysis of 4-1BBL in the lung tissue ($\times 400$ magnification). Red for 4-1BBL; blue for DAPI nuclear staining. (D) The average ratio of 4-1BBL positive cells to total airway epithelial cells ($n = 8$ for each group, with the experiments carried out in triplicate). * $p < 0.05$ versus OVA-challenged mice.

exacerbations [8], whereas treatment with Golimumab led to serious adverse events, and even one death and eight malignancies occurred in the Golimumab groups [9].

As a result of the immunosuppressive properties of anti-TNF- α agents, treatment for various inflammatory diseases by the systemic administration of anti-TNF- α mAb or soluble receptor was associated with many aspects of adverse effects, such as infection, immunogenicity and malignancy, as well as pneumonia and interstitial lung disease [10]. The efficacy of those anti-TNF- α agents is always accompanied by risks related to systemic immunosuppression. This problem led us to try a new strategy in which local administration of anti-TNF- α agent with no proteantigen-triggered immunogenicity is explored as an alternative for treating T cell-driven allergic airway inflammation. Nucleic acid antisense drugs are one of the preferred candidates. In a previous study, local ASO administration against IL-5, stem cell factor and p38 α mitogen-activated protein kinase, as well as the common β chain of IL-3, IL-5 and granulocyte macrophage colony-stimulating factor, resulted in alleviated allergic airway inflammation in the murine models [14]. Moreover, the efficacy of TPI ASM8 against CCR3 and IL-5 receptors, as well as AIR 645 against IL-4 receptor α on asthma, is currently undergoing clinic trials [15]. In a safety assessment of inhaled TPI ASM8 ASO in monkeys, ASO was found to be safe and well tolerated, with an absence of a generalized inflammatory response, very limited changes in the respiratory tract and very limited systemic exposure [16].

In recent work, a potent and highly selective TNF- α ASO, which represented a favorable therapeutic effect on the protection of mice from TNBS-induced colitis and CD4⁺CD45RB^{hi} T cells-mediated colitis in a previous study [17], was intranasally administered and the treatment effects were evaluated using an OVA-induced asthmatic murine model. Our present findings demonstrate that local TNF- α

ASO administration inhibited TNF- α expression significantly, which was associated with remarkably alleviated T_H2-biased responses. A series of studies have led to the conclusion that the immune balance inclined to a pro-inflammatory T_H2 response is a central feature in allergic inflammation [18]. It has been indicated that up-regulated T_H2 cytokines IL-4 and IL-5 account for eosinophil infiltration into airways and eosinophil activation [19–21]. Moreover, IL-5 and IL-13 are linked to mucus hypersecretion in the T_H2-responsive airway epithelium [22,23]. Therefore, approaches aiming to inhibit T_H2 cells and T_H2 responses play a vital role in the treatment of allergic airway inflammation. Although T_H1-biased cytokines are of great importance in the reciprocally inhibitory functions of T_H1 and T_H2 cells [24,25], our data show that the levels of IL-12 and IFN- γ in BALF were unchanged. This result demonstrates that T_H1-biased cytokines were most probably not involved in the inhibited T_H2 responses by local TNF- α ASO administration. Indeed, there are several reports concerned with exacerbated T_H2 inflammation by the enhancement of T_H1 responses [26–28] suggesting a synergistic effect of the T_H1 response with T_H2 inflammation in asthma.

Besides T_H1 responses, Tregs served as another modulator that plays a critical role in inhibiting T_H2-biased responses to allergen challenge and maintaining immune tolerance [29,30]. Approaches for inducing Treg-mediated tolerance were therefore investigated in previous studies. In a murine model, adoptive transfer of CD4⁺CD25⁺ Tregs before allergen challenge of sensitized mice led to inhibited differentiation of naïve CD4⁺ T cells into T_H2 cells [31]. In another study, oral administration of OVA resulted in the generation of peripheral antigen-specific CD4⁺CD25⁺Foxp3⁺CD45RB^{low} Tregs that were responsible for inhibited lung inflammation [32]. Moreover, several studies have demonstrated that retinoic acid [33] and glucocorticoids [12] could benefit

immune tolerance to allergic stimulation by the induction of CD4⁺CD25⁺Foxp3⁺ Tregs. Thus, strategies aiming to shift the balance towards Treg-mediated tolerance held great promise for the treatment and prevention of allergic airway inflammation. Given the suppressive potency of Tregs on T_H2 responses, we examined the proportion of CD4⁺CD25⁺Foxp3⁺ Tregs in BALF after local anti-TNF- α treatment. Anti-TNF- α agents were found to participate in the induction of thymic or splenic Tregs when systemic administration of these agents is performed. In neonatal NOD mice, anti-TNF- α treatment was associated with the induction of peripheral CD4⁺CD25⁺ Tregs [34]. The consistent result was achieved in a murine histoplasmosis model with intraperitoneally injected antagonistic mAb against TNF- α [35]. These studies implied the potential relationship between anti-TNF- α treatment and thymic or splenic Tregs. Nevertheless, the mechanism of anti-TNF- α therapeutics on allergen-induced asthmatic inflammation remains unclear.

In the present study, we found that inhibited TNF- α expression caused by local ASO administration contributed to a significantly increased proportion of CD4⁺CD25⁺Foxp3⁺ Tregs in BALF, which was parallel to the remarkably decreased proportion of CD3⁺CD8⁺IL-4⁺ T_H2 cells in BALF. Our data further demonstrated that these induced CD4⁺CD25⁺Foxp3⁺ Tregs were not related to TGF- β or IL-10 secretion in BALF, and they were also not matured in the thymus or spleen because the proportion of CD4⁺CD25⁺Foxp3⁺ Tregs in the blood, thymus and spleen was not affected by local ASO administration. CD4⁺CD25⁺Foxp3⁺ Tregs can develop either in the thymus from CD4⁺ T cells and then migrate, or in the periphery from local naïve CD4⁺ T cells via encountering local conditions [36]. Previous studies have indicated that co-stimulatory signaling driven by the interaction between co-stimulatory molecules on the surface of T cells and their ligands serves as one of the local conditions determining the mode of T cell development in the periphery [37]. In the present study, local TNF- α ASO administration resulted in an altered expression of 4-1BBL in the airway epithelium. 4-1BBL is one of co-stimulatory molecule ligands. In many previous reports, the reported findings regarding the effect of 4-1BBL and 4-1BB signaling on T cells were contradictory. Addition of agonistic anti-4-1BB antibody led to remarkably impaired CD4⁺CD25⁺ Treg-mediated tolerance [38] and suppression of effector T cells [39], whereas another study suggested that the 4-1BB signal was strongly co-stimulatory for CD4⁺CD25⁺ T cells both *in vitro* and *in vivo* [40]. In

addition, disruption of 4-1BB/4-1BBL signaling by anti-4-1BBL mAb prevented the destruction of corneal tissue by T cells [41]. Our results support that the idea that local TNF- α ASO administration inhibits 4-1BBL expression in the airway epithelium, which might take part in the generation of CD4⁺CD25⁺Foxp3⁺ Tregs and the impairment of T_H2 cells.

Given that systemically enhanced CD4⁺CD25⁺ Tregs resulting from systemic anti-TNF- α administration was the main cause of comprehensive immunosuppression, which was also associated with an increased incidence of infection [35] and many other adverse effects including increased risk of malignancy [9], we investigated the treatment effects of local TNF- α ASO administration on OVA-induced airway inflammation. Inhibited TNF- α expression caused by instilled TNF- α ASO resulted in the comprehensive inhibition of T_H2-biased inflammation via the induction of CD4⁺CD25⁺Foxp3⁺ Tregs in BALF, which was not accompanied by systemic incitation of Tregs in the blood, thymus and spleen. Our results imply the potential of a new therapeutic strategy in which local anti-TNF- α treatment benefits the reversion of T_H2-driven inflammatory diseases with no comprehensive immunosuppression caused by systemic anti-TNF- α treatment. An aerosol formulation of TNF- α ASO enabling efficient and convenient local treatment against allergic lung inflammation will be studied.

Acknowledgements

The present study was supported by National Natural Science Foundation of China (30771036, BK2007144 and 31071232), National Science Fund for Distinguished Young Scholars (81025019), Key Program for Science and Technology Research from Chinese Ministry of Education (01005), the Chinese National Programs for High Technology Research and Development (863 Program 2006AA02Z177), National Key Pharmaceutical Program (2009ZX09503-028), Natural Science Fund for Colleges and Universities in Jiangsu Province (BK 2009237), and China Postdoctoral Science Foundation 20080431077 and 200801364. The authors declare that there are no conflicts of interest.

Supporting information

Doc S1. The methods for OVA sensitization, airway challenge, ASO distribution, measurement of cytokines in BALF, flow cytometry analysis, histology, immunohistochemistry, reverse transcriptase-polymerase chain reaction assay, western blot analysis, and immunofluorescent analysis.

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Supporting information

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Methods

Sensitization and airway challenge

Mice were immunized by intraperitoneal injection of 20 µg OVA (Sigma-Aldrich, St Louis, Missouri, USA) emulsified in 2.25 mg alum (Pierce, Rockford, IL, USA) in a total volume of 100 µl saline on the 0 day and the 14th day. After sensitization, mice were challenged via airways with 1 % OVA in saline for 30 min on the 28th, 29th and 30th day by ultrasonic nebulization. ASO and SCO were dissolved in PBS at a concentration of 5 mg/ml. The intranasal instillation of 100 µg ASO, 100 µg SCO or 20 µl PBS alone was performed on the 28th and 30th day, which was conducted 4 h after aerosol challenge. All of mice were assessed 24 h after the last instilled treatment. On the 28th day, each of three OVA-challenged mice was intranasally administered with 100 µg rhodamine-labelled ASO in 20 µl PBS, which was performed just simultaneously as the ASO instillation. After 3 hours, the distribution of rhodamine-labelled ASO in the trachea and the lung tissue was investigated on the frozen sections. Briefly, the trachea and the lung tissue were removed and embedded in OCT. Frozen sections of 5 µm were mounted on polylysine-coated glass slides and fixed in 4 % paraformaldehyde for 15 min and then washed twice. The nuclei of fixed tissue were stained with DAPI (Sigma-Aldrich, St Louis, Missouri, USA) for 6 min and washed twice. All of frozen sections were photographed under fluorescent microscopy (TE2000-U, Nikon, Japan).

Measurement of cytokines in BALF

Lungs were lavaged twice via tracheal tube with 1 ml RPMI 1640. The collected BALF was centrifuged by 300 g for 5 min at 4 °C. The supernatant was transferred into a new eppendorf tube, while the cell pellet was resuspended in RPMI 1640 and waited for flow cytometry analysis or differential cell count using a modified Wright stain [1]. By Elisa kits (eBioscience, San Diego, CA, USA), the concentration of TNF- α , IL-4, IL-5, IL-10, IL-12, IL-13, IFN- γ and TGF- β in the BALF was determined.

Flow cytometry

The frequency of CD4⁺CD25⁺Foxp3⁺ Tregs and CD3⁺CD8⁻IL-4⁺ T_H2 cells was measured by flow cytometry, using the protocols provided by MultiSciences Biotech, Hangzhou, China. In this section, each group contained 24 mice. Every 4 samples of cell pellet from BALF in the same group were combined to ensure the cell number exceeding 2×10^6 in total. 6 combined cell suspension in each group was then obtained. Every cell suspension was divided equally into two parts, one for Treg measurement and the other for T_H2 measurement. The statistics within each group was calculated from the results of the 6 combined samples.

For CD4⁺CD25⁺Foxp3⁺ Tregs, 1×10^6 lavaged cells, or thymic cells, or splenic

cells, or blood cells, were suspended in 100 μ l 1 % BSA. After Fc block, 0.125 μ g FITC-labeled anti-mouse CD4 Ab and 0.06 μ g APC-labeled anti-mouse CD8 Ab were added. The cells were washed, centrifuged, and then resuspended in 1 ml Fixation/Permeabilization working solution. 0.5 μ g PE-labeled anti-mouse Foxp3 Ab was added. After washed and resuspended, the cells were analyzed on a flow cytometer.

For CD3⁺CD8⁺IL-4⁺ T_H2 cells, 1×10^6 lavaged cells in 200 μ l RPMI 1640 were incubated with 10 μ l phorbol 12-myristate 13-acetate (1 μ g/ml, Enzo Life Sciences, Farmingdale, NY, USA) and 8 μ l Ionomycin (50 μ g/ml, Enzo Life Sciences, Farmingdale, NY, USA) for 3 h at 37 °C, followed by addition of 4 μ l Brefeldin A (0.1 mg/ml, Enzo Life Sciences, Farmingdale, NY, USA) and incubation for 1 h at 37 °C. The stimulated cells were then incubated with FITC-labeled anti-mouse CD3 Ab and APC-labeled anti-mouse CD8 Ab. After 100 μ l Fix&Perm Reagent (Invitrogen, Carlsbad, CA, USA) A was added, the cells were recovered by centrifuge, then resuspended in a mixture of 100 μ l Fix&Perm Reagent B and PE-labeled anti-mouse IL-4 Ab. Each of cell sample was washed and resuspended, then analyzed by flow cytometry.

Histology and immunohistochemistry

After lavage, tracheas and lungs of 12 mice in each group were removed and fixed in 10 % formaldehyde and embedded in paraffin. Sections of 5 μ m were mounted for

H&E, PAS, Congo red and toluidine blue stains.

For immunohistochemistry, sections of 5 μm were mounted on polylysine-coated glass slides. The slides were blocked in 5 % BSA and then incubated with 10 $\mu\text{g}/\text{ml}$ anti-mouse TNF- α monoclonal Ab (Abcam, Cambridge, UK). After washed in PBS, the slides were incubated with biotinylated secondary Ab (Abcam, Cambridge, UK), then reacted with Streptavidin-Biotin Complex (Boster, Wuhan, China). Diaminobenzidine (Boster, Wuhan, China) as the chromogen was then added. All of the slides were examined by microscopy.

RT-PCR measurement

Total RNA from trachea tissue or lung tissue was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 1 μg RNA was reversely transcribed into cDNA using AMV method (TaKaRa, Japan). PCR was performed using Premix Taq Version 2.0 kit (TaKaRa, Japan). The program was initially 5 min at 94 $^{\circ}\text{C}$, followed by 35 cycles of 30 seconds at 94 $^{\circ}\text{C}$, 30 seconds at 60 $^{\circ}\text{C}$ and 1 min at 72 $^{\circ}\text{C}$. The primer sequences were as follows:

Mouse TNF- α sense, 5'-CGCGACGTGGAAGTGGCAGAA-3',

Mouse TNF- α antisense, 5'-CTCCTCCACTTGGTGGTTTGCTACG-3'.

The product length of mouse TNF- α was 288.

Mouse 4-1BBL sense, 5'-GTGGCCGCCTGCACTGAACT-3',

Mouse 4-1BBL antisense, 5'-ACAGGAGAGCCCTGTTGTGTAGTGT-3'.

The product length of mouse 4-1BBL was 234.

Mouse 18s rRNA sense, 5'-AGGGGAGAGCGGGTAAGAGA-3',

Mouse 18s rRNA antisense, 5'-GGACAGGACTAGGCGGAACA-3'.

The product length of mouse 18s rRNA was 241.

Electrophoresis of products was performed using 2 % agarose.

Western blot analysis

Western blot analysis for 4-1BBL was conducted in the trachea tissue and the lung tissue freshly isolated from mice in each group. A total of 100 µg protein was separated by 10 % SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was probed with anti-mouse 4-1BBL Abs (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by HRP-conjugated secondary Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then visualized.

Immunofluorescent analysis

Paraffin-embedded lung tissue sections of 5 µm were mounted on polylysine-coated glass slides and immersed in 3 % H₂O₂ for 10 min and washed. Heat-induced epitope retrieval was conducted by boiling the sections. 5 % BSA was added and incubated for 30 min at room temperature. All of the sections were

incubated with goat anti-mouse 4-1BBL Abs overnight at 4 °C and washed, followed by incubation with tetramethyl rhodamine isothiocyanate-conjugated secondary Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 37 °C for 30 min. The nuclei of tissue were stained with DAPI for 6 min and washed twice. All of sections were photographed under fluorescent microscopy.

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