

IMIQUIMOD, A TOLL-LIKE RECEPTOR 7 LIGAND, INHIBITS AIRWAY REMODELLING IN A MURINE MODEL OF CHRONIC ASTHMA

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SUMMARY

1. Imiquimod, a synthetic Toll-like receptor (TLR) 7 ligand, has been shown to attenuate airway inflammation and airway hyperresponsiveness (AHR) in acute murine models of allergic asthma. In the present study, we investigated the effect of imiquimod on allergen-induced airway remodelling in chronic experimental asthma.

2. Ovalbumin (OVA)-sensitized mice were chronically challenged with aerosolized OVA for 8 weeks. Some mice were exposed to an aerosol of 0.15% imiquimod daily during the period of OVA challenge. Twenty-four hours after the last OVA challenge, mice were evaluated for the development of airway inflammation, AHR and airway remodelling. The levels of total serum IgE and Th2 cytokines (interleukin (IL)-4, IL-5 and IL-13) in bronchoalveolar lavage fluid (BALF) and the expression of transforming growth factor (TGF)- β 1 protein in lungs were measured by ELISA and immunohistochemistry, respectively.

3. The results demonstrated that imiquimod significantly inhibited chronic inflammation, persistent AHR and airway remodelling in chronic experimental asthma. In addition, imiquimod reduced levels of total serum IgE and BALF Th2 cytokines and diminished expression of TGF- β 1 in remodelled airways.

4. In summary, the results of the present study indicate that imiquimod may attenuate the progression of airway inflammation and remodelling, providing potential in the treatment of asthma.

Key words: airway remodelling, asthma, imiquimod, Toll-like receptor.

INTRODUCTION

Bronchial asthma is an allergic inflammatory disease characteristic of chronic airway inflammation, airway hyperresponsiveness (AHR) and airway remodelling.¹ Airway remodelling manifests structural and morphometric changes, such as subepithelial fibrosis, mucous gland hyperplasia, myofibroblast and smooth muscle proliferation and vascular changes.^{2,3} It is generally thought that airway remodelling is derived from chronic repetitive injury to the airway wall following airway inflammation, playing an important role in the pathophysiology of AHR.⁴

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As a family of pattern recognition receptors, Toll-like receptors (TLRs) play a crucial role in both innate and adaptive immunity.^{5,6} Resiquimod (a TLR7/8 ligand) has recently been reported to decrease airway mucus expression and smooth muscle hypertrophy/hyperplasia in a rat model of chronic asthma.⁷ Imiquimod, a synthetic TLR7 ligand, also belongs to the chemical class of imidazoquinolinamines. We have demonstrated previously that imiquimod attenuates acute inflammation and AHR in allergen-induced murine models of asthma.^{8,9} However, it is still unknown whether imiquimod interferes with the structural changes typical of chronic airway disease. In the present study, we investigated the effects of imiquimod on airway remodelling in a murine model of chronic asthma.

METHODS

Antigen sensitization, challenge and treatment

Eighteen female BALB/c mice (6–8 weeks old) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Mice were randomly divided into three groups: (i) a control group; (ii) an ovalbumin (OVA)-challenged group; and (iii) an imiquimod-treated group. Imiquimod was supplied by the Clinical Pharmacological Department of Nanjing Medical University, Nanjing, China. On Days 0 and 14, mice in the OVA and imiquimod groups were immunized by i.p. injections of 100 μ g chicken egg ovalbumin (Grade V; Sigma, St Louis, MO, USA) emulsified in 1 mg aluminium hydroxide in a total volume of 0.2 mL. For aerosolized challenges, mice were placed in a plexiglas box (29 $\times$ 22 $\times$ 18 cm) and were exposed to aerosolized OVA using an ultrasonic nebulizer (NE-U11B; Omuron, Tokyo, Japan). From Day 21, mice were exposed to an aerosol of 1% OVA for 30 min/day, 3 days/week for 8 weeks. Mice in the imiquimod group were also exposed to an aerosol of 0.15% imiquimod for 30 min daily for 8 consecutive weeks following each airway challenge. Mice in the control group received the same schedule for sensitization and challenge with an equivalent amount of 0.9% sterile saline instead of OVA.

Evaluation of AHR

Airway responsiveness to acetylcholine chloride (ACh) was measured with an AniRes 2005 animal lung function analysis system (SYNOL High-Tech, Beijing, China). Mice were anaesthetized by i.p. injection of pentobarbital sodium (70 mg/kg) 24 h after the last OVA challenge. A plastic tube, 2 mm internal diameter, was inserted into the trachea via a tracheotomy for mechanical ventilation and a 27 gauge needle was inserted into the caudalis vein for drug administration. Mice were then placed in a whole-body plethysmography chamber and ventilated mechanically at a rate of 90 breaths/min with a tidal volume of 6 mL/kg. After establishment of stable airway pressure recording, ACh was administered i.v. with a microinfusion pump at a rate of 36 mL/h in progressively increasing doses (10, 30, 90 and 270 μ g/kg). After the administration of each dose, data were collected continuously from 5 s to 1 min and maximum values of lung resistance (RL) were taken to express changes in murine airway function.^{9–11}

Analysis of bronchoalveolar lavage fluid and serum

After measurement of AHR, mice were bled by retro-orbital puncture using heparinized capillary tubes. Blood samples were centrifuged at 1000 *g* for 10 min at 4°C and plasma was stored at –70°C until analysis. After removal of blood, airway lumina were washed three times with 0.4 mL saline. Bronchoalveolar lavage fluid (BALF) was centrifuged (1000 *g* for 10 min at 4°C) and the total number of BALF cells was counted with a haemocytometer. Differential cell counts were performed using cytopspin techniques and Wright's staining by counting at least 200 cells. Commercially available ELISA kits were used to determine interleukin (IL)-4 (Jingmei Biotech, Shanghai, China), IL-5 and IL-13 (R&D Systems, Minneapolis, MN, USA) levels in BALF and total serum IgE (BPB Biomedicals, Franklin, MA, USA). The limits of detection were 4 pg/mL for IL-5 and IL-13, 7 pg/mL for IL-4 and 1 ng/mL for total serum IgE.

Histological examination

After mice had been killed, the left lung was immersed in 10% formaldehyde overnight and then embedded in paraffin, sectioned (5 µm) and either stained with haematoxylin–eosin, periodic acid–Schiff (PAS) and Masson's trichrome stain or processed for immunohistochemistry. Histological analyses were performed by pathologists blinded to the treatment groups. The area of peribronchial trichrome staining in a paraffin-embedded lung was outlined and quantified with a light microscope attached to an image-analysis system (Image-Pro Plus; Media Cybernetics, Minneapolis, MN, USA). Results are expressed as the area of trichrome staining per µm length of basement membrane of bronchioles with an internal diameter of 150–200 µm by counting at least 10 bronchioles in each slide. To quantify the level of mucus expression in airways, the number of PAS-negative and PAS-positive epithelial cells (goblet cells) in individual bronchioles was counted, as described previously.¹² The percentage of PAS-positive cells per bronchiole was calculated based on the number of PAS-positive epithelial cells per bronchus divided by the total number of epithelial cells in each bronchiole.

Immunohistochemistry for α -smooth muscle actin and transforming growth factor- β 1

Sections were deparaffinized in xylene and rehydrated in graduated alcohol solutions.

Endogenous peroxidase activity was blocked by incubation of sections with 3% H₂O₂. To prevent non-specific absorption of immunoglobulin, specimens were flooded with 5% normal goat serum. Thereafter, specimens were incubated for 90 min with mouse anti-human α -smooth muscle actin (SMA) monoclonal antibody (DAKO, Glostrup, Denmark) at a dilution of 1 : 50 and rabbit anti-mouse transforming growth factor (TGF)- β 1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1 : 50. Substitution of each primary antibody with normal mouse or rabbit IgG was used separately as a negative control. Slides were incubated overnight at 4°C, rinsed three times with phosphate-buffered saline (PBS) and then incubated with peroxidase-labelled goat anti-mouse IgG (Envision system; DAKO) or goat anti-rabbit IgG (Envision system; DAKO) for 30 min at 37°C and then washed again with PBS followed by staining with diaminobenzidine (DAB; Santa Cruz Biotechnology). Sections were counterstained with haematoxylin, dehydrated and observed under a light microscope. Data are expressed as the area of α -SMA immunostaining per µm length of basement membrane of bronchioles with an internal diameter of 150–200 µm. The number of peribronchial cells expressing TGF- β 1 was evaluated as described previously.^{13,14}

Measurement of hydroxyproline content

Total collagen content of the right lungs was determined by hydroxyproline assay. The hydroxyproline content was detected using a commercially available hydroxyproline detection kit (Nanjing Jiancheng Biotechnology, Nanjing, China). Briefly, frozen lung tissues were homogenized by a

Polytron tissue homogenizer in saline containing 0.1 mol/L phenylmethylsulphonyl fluoride. The homogenized sample was hydrolysed in 6 mol/L HCl and the hydroxyproline concentration was determined as described previously.¹⁵

Statistical analysis

Data are expressed as the mean $\pm$ SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) and least significant difference (LSD) test with spss software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Imiquimod decreases AHR in chronic experimental asthma

To examine the effect of imiquimod on airway hyperreactivity, we measured airway resistance in anaesthetized mice using invasive whole-body plethysmography. There were no significant differences in baseline airway resistance among the three groups. The airway resistance in the OVA and imiquimod groups was obviously increased in a dose-dependent manner by administration of ACh, whereas only a slight increase could be detected in the control group. The airway resistance generated by administration of ACh at doses from 30 to 270 µg/kg increased significantly in the OVA and imiquimod groups compared with the control group. The OVA group had significantly greater airway resistance than the control group ($P < 0.01$; Fig. 1a). Treatment with imiquimod led to a sharp decrease in airway resistance compared with the OVA group ($P < 0.05$; Fig. 1a).

Imiquimod attenuates chronic airway inflammation

Apart from macrophages, very few inflammatory cells were detected in the saline-sensitized and -challenged control mice. Sensitization and challenge with OVA resulted in a marked increase in the number of eosinophils in BALF. Treatment with imiquimod significantly decreased the number of eosinophils and total inflammatory cells in BALF ($P < 0.05$; Fig. 1b). The anti-inflammatory effects of imiquimod were further demonstrated following histological examination of haematoxylin–eosin stained sections. A marked influx of inflammatory cells into the airway and around the blood vessels was observed in OVA-sensitized/challenged mice, but not in the saline-treated control mice. Mice treated with imiquimod showed a marked reduction in inflammatory cell infiltration within the peribronchiolar and perivascular regions (Fig. 2a–c).

Imiquimod reduces levels of Th2 cytokines in BALF and total serum IgE

Levels of the Th2 cytokines IL-4, IL-5 and IL-13 in BALF were significantly increased in OVA-sensitized/challenged mice compared with the control group ($P < 0.01$; Table 1). Daily administration of imiquimod reduced the levels of these Th2 cytokine in BALF compared with the OVA group ($P < 0.05$; Table 1). Total serum IgE levels were significantly enhanced in the OVA group compared with the control group ($P < 0.01$; Table 1). Treatment with imiquimod reduced total serum IgE levels to near baseline compared with the OVA group ($P < 0.05$; Table 1).

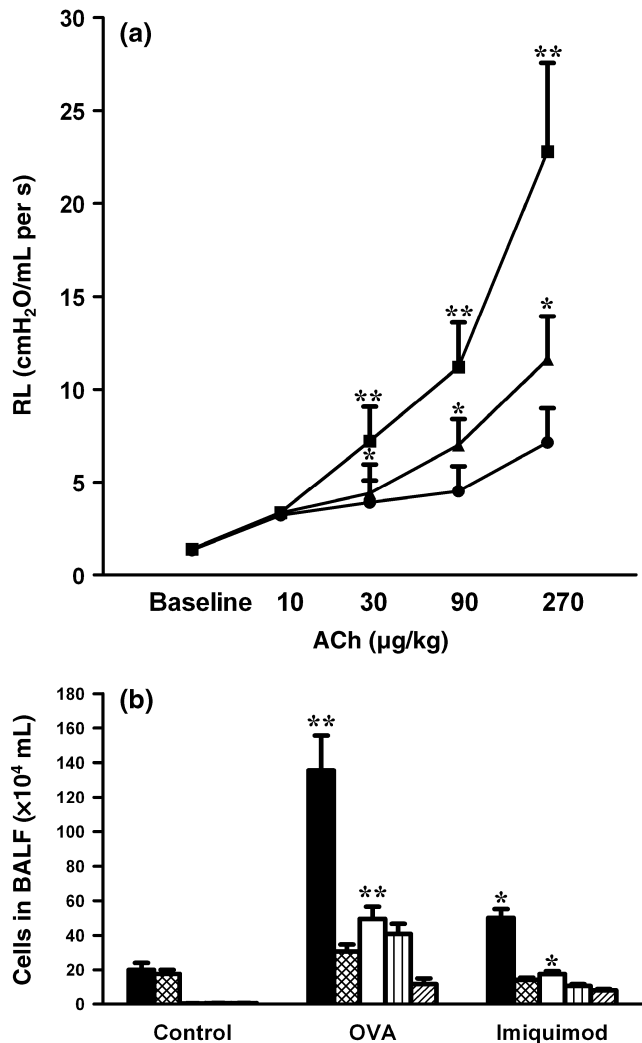


Fig. 1 Effects of imiquimod on (a) the development of airway hyperresponsiveness (AHR) and (b) inflammatory cell accumulation in bronchoalveolar lavage fluid (BALF) in a murine model of chronic asthma. (a) Imiquimod (▲) decreased AHR compared with ovalbumin (OVA)-sensitized and -challenged mice (■). (●), control. (b) Imiquimod decreased accumulation of total cells (■) in general, as well as macrophages (▨), eosinophils (□), lymphocytes (▤) and neutrophils (▧) specifically, compared with the OVA group. Data are the mean±SEM ($n = 6$ per group). * $P < 0.05$ compared with the OVA group; ** $P < 0.01$ compared with the control group.

Imiquimod inhibits airway remodelling in chronic experimental asthma

Lung sections were stained with PAS to identify mucus-containing cells in the airway epithelium (Fig. 2d–f). A significant increase in the number of PAS-positive epithelial cells was found in the OVA group compared with the negative control group ($P < 0.01$; Fig. 2g). Treatment with imiquimod reduced the number of PAS-positive cells ($P < 0.05$; Fig. 2g).

Masson's trichrome-positive peribronchiolar collagen layer (Fig. 3a–c) and areas of the α -SMA-stained smooth muscle layer (Fig. 3d–f) in OVA-sensitized and -challenged mice were also increased. Masson's trichrome staining revealed dense collagen

Table 1 Levels of interleukin (IL)-4, IL-5 and IL-13 in bronchoalveolar lavage fluid and total serum IgE

Group	BALF (pg/mL)			Total IgE in serum (ng/mL)
	IL-4	IL-5	IL-13	
Control	22.0 ± 4.3	10.4 ± 2.5	11.0 ± 2.8	120.8 ± 33.2
OVA	124 ± 29 [†]	36.8 ± 5.1 [†]	45.4 ± 7.0 [†]	657 ± 73 [†]
Imiquimod	47.0 ± 6.7*	21.3 ± 4.3*	21.4 ± 4.0*	281 ± 66*

Data are the mean±SEM ($n = 6$ per group). * $P < 0.05$ compared with the ovalbumin (OVA)-sensitized and -challenged group; [†] $P < 0.01$ compared with the control group.

Treatment with imiquimod reduced levels of the Th2-type cytokines IL-4, IL-5 and IL-13 in bronchoalveolar lavage fluid (BALF) and total serum IgE in a murine model of chronic asthma.

deposition/fibrosis throughout the interstitium in airways and blood vessels of tissues from the OVA-sensitized and -challenged group. The mean area of airway fibrosis in the OVA group was significantly enhanced compared with the negative control group ($P < 0.01$; Fig. 3g). Administration of imiquimod caused a marked reduction in collagen deposition compared with the OVA group ($P < 0.05$; Fig. 3g). The area of the α -SMA-stained smooth muscle layer in OVA-sensitized and -challenged mice was significantly greater than that in the saline-treated control ($P < 0.01$; Fig. 3h). Administration of imiquimod decreased the α -SMA immunostained area compared with the OVA group ($P < 0.05$; Fig. 3h).

Total lung hydroxyproline content in the OVA group was significantly greater than that in the control group ($P < 0.01$; Fig. 3i). In contrast, treatment with imiquimod significantly reduced total lung hydroxyproline content compared with the OVA group ($P < 0.05$; Fig. 3i).

Imiquimod ameliorates the expression of TGF- β 1 protein

There was little constitutive staining for TGF- β 1 in lungs of control mice. In contrast, immunostaining for TGF- β 1 was markedly elevated in lungs of mice in the OVA group (Fig. 4a–d). Strong cytoplasmic staining was mainly localized to bronchial epithelial cells. Treatment with imiquimod decreased TGF- β 1 expression compared with the OVA group ($P < 0.05$, Fig. 4e).

DISCUSSION

Tissue remodelling or structural changes are major features of chronic inflammatory disorders, including asthma. Murine models of acute asthma are mainly indicative of the initiating phase, whereas those of chronic asthma are particularly similar to human asthma owing to airway remodelling and subsequent loss of lung function. In the present study, we demonstrated an inhibitory effect of imiquimod on the airway remodelling induced by repetitive airway allergen challenge in mice.

It is now widely accepted that chronic airway inflammation plays a key role in asthma and that infiltration of the airways by inflammatory cells contributes to the remodelling response.¹⁶ In the present study, administration of imiquimod decreased eosinophilia and total inflammatory cells in BALF during chronic allergen challenge. In

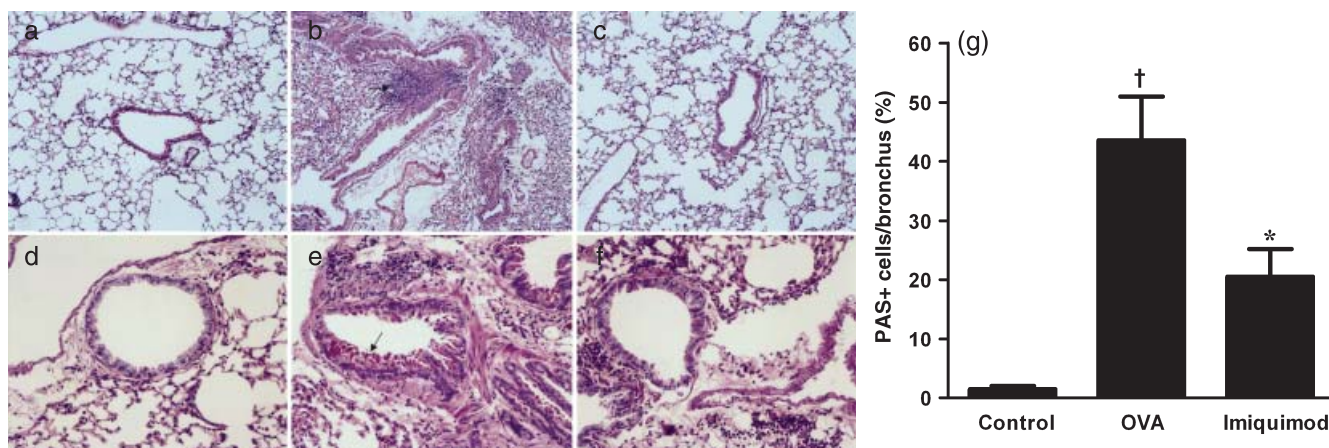


Fig. 2 Treatment with imiquimod attenuates chronic airway inflammation and antigen-induced mucus production in a murine model of chronic asthma. (a–c) Haematoxylin and eosin staining (original magnification $\times 100$) of lung tissue from (a) control, (b) ovalbumin (OVA)-sensitized and -challenged and (c) imiquimod-treated mice. (d–f) Periodic acid-Schiff (PAS) staining (original magnification $\times 200$) of lung tissue from (d) control, (e) OVA and (f) imiquimod-treated mice. The arrow indicates areas of peribronchial and perivascular inflammation, as well as airway goblet cell hyperplasia. (g) Percentage of PAS-positive cells per bronchiole. Data are the mean $\pm$ SEM ($n = 6$ in each group). $^*P < 0.05$ compared with the OVA group; $^{\dagger}P < 0.01$ compared with the control group.

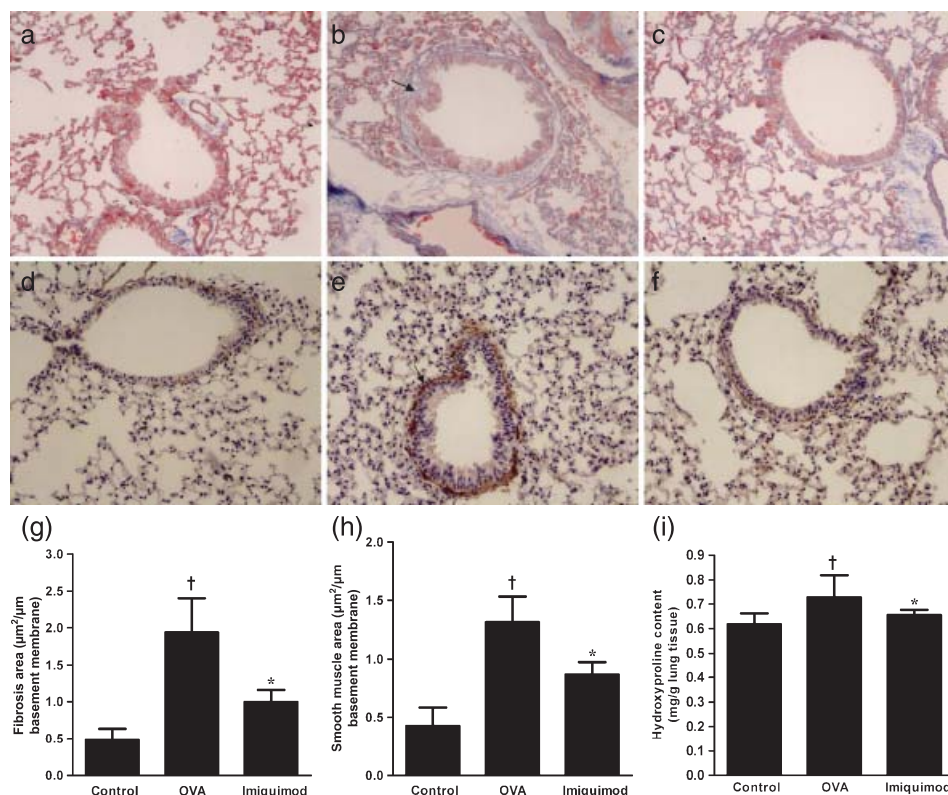


Fig. 3 Treatment with imiquimod inhibits peribronchial fibrosis and areas of α -smooth muscle actin (SMA)-stained smooth muscle in chronic experimental asthma. (a–c) Masson's trichrome-positive peribronchiolar collagen layer (original magnification $\times 200$) of lung tissue from (a) control, (b) ovalbumin (OVA)-sensitized and -challenged and (c) imiquimod-treated mice. (d–f) Peribronchial α -SMA immunostaining (immunohistochemistry, original magnification $\times 200$) of lung tissue from (d) control, (e) OVA and (f) imiquimod-treated mice. The arrow indicates extensive collagen deposition or airway smooth muscle hypertrophy. (g) Mean area of airway fibrosis, (h) immunostaining for peribronchial α -SMA and (i) total lung hydroxyproline content were determined as described in the Methods. Data are the mean $\pm$ SEM ($n = 6$ in each group). $^*P < 0.05$ compared with the OVA group; $^{\dagger}P < 0.01$ compared with the control group.

addition, imiquimod treatment reduced levels of Th2 cytokines (IL-4, IL-5 and IL-13) in BALF and total serum IgE. Moreover, airway eosinophilia was markedly reduced in lungs of asthmatic mice treated with imiquimod. Because eosinophils are critical for the development of airway remodelling,¹⁷ we presume that imiquimod may mediate its antiremodelling action, at least in part, through modulation of eosinophilic airway inflammation.

Mucus hypersecretion and plugging in airways are predominant during the exacerbation of asthma.¹⁸ Mucous gland hyperplasia is dependent on Th2-biased airway inflammation,¹⁹ because it has been shown that adoptive transfer of Th2 cells induces airway mucus expression²⁰ and that the Th2 cytokines IL-4, IL-9 and IL-13 induce goblet cell hyperplasia in the murine lung.²¹ In the present study, administration of imiquimod significantly abolished the number of

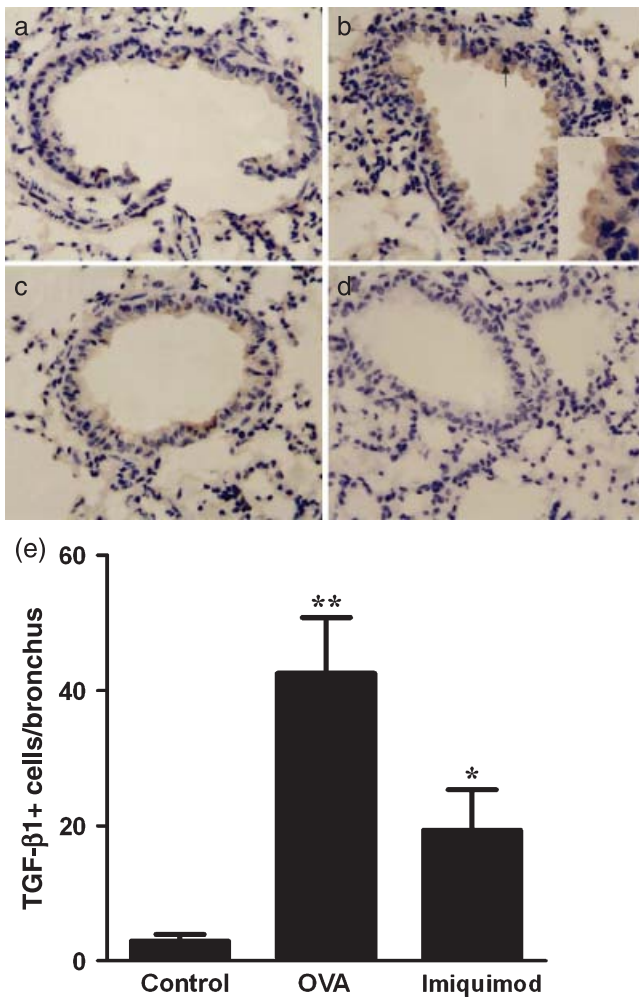


Fig. 4 Imiquimod ameliorates the expression of transforming growth factor (TGF)- β 1 protein in the lungs of asthmatic mice. (a–d) Peribronchial TGF- β 1 immunostaining (immunohistochemistry; original magnification $\times 400$) of lung tissue from (a) control, (b) ovalbumin (OVA)-sensitized and -challenged and (c) imiquimod-treated mice. (d) IgG negative control. The arrow indicates strong cytoplasmic staining in bronchial epithelial cells. (e) The number of peribronchial cells expressing TGF- β 1 was determined as described in the Methods. Data are the mean $\pm$ SEM ($n = 6$ in each group). * $P < 0.05$ compared with the OVA group; ** $P < 0.01$ compared with the control group.

mucus-producing goblet cells within the bronchiolar epithelium. Thus, the mechanism by which imiquimod reduced mucus production likely results from its ability to inhibit Th2 cytokines, including IL-4 and IL-13, in the chronic model of airway remodelling.

In the present study, we demonstrated for the first time that administration of imiquimod significantly suppresses the deposition of subepithelial collagen in chronic experimental asthma, as determined by morphometric and hydroxyproline analyses. Collagen deposition in the airways is a key feature of airway remodelling in association with the assessment of the severity and the decline of pulmonary function in human asthma,²² with IL-4 and IL-13 considered key mediators of tissue fibrosis in the pathophysiology of asthma.^{23,24} The imiquimod-mediated drop in BALF IL-4 and IL-13 levels shown here may be associated with its antifibrosis action. Furthermore, imiquimod treatment resulted in decreased lung expression of the profibrotic

cytokine TGF- β 1, which is suggested to play an important role in the progression of airway remodelling.²⁵

Airway smooth muscle hyperplasia is attributable to the structural changes associated with airway remodelling, such as the exaggerated airway narrowing in response to a variety of stimuli in asthma.^{2,26} In the present study, the amount of α -SMA was increased significantly after chronic exposure to an allergen, consistent with previous reports in animal models of chronic asthma.^{27,28} The allergen-induced increase in airway smooth muscle was significantly decreased following treatment with imiquimod. Because airway smooth muscle plays a critical role in AHR, the ability of imiquimod to reduce the thickness of the airway smooth muscle layer may account for its suppression of AHR.

In conclusion, the data suggest that the TLR7 ligand imiquimod inhibits airway inflammation and airway remodelling in a murine model of chronic asthma by suppressing Th2 cytokine levels in BALF and TGF- β 1 expression in the lung. Overall, imiquimod shows promise for the treatment of asthma by ameliorating chronic allergic responses through manipulation of TLR7-mediated signalling.

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