

ORIGINAL ARTICLE

Blockade of cysteinyl leukotriene-1 receptors suppresses airway remodelling in mice overexpressing GATA-3

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Summary

Background We demonstrated previously that GATA-3 overexpression markedly enhanced allergen-induced airway inflammation and airway remodelling, including subepithelial fibrosis, and smooth muscle cell hyperplasia, in transgenic mice.

Objective Because cysteinyl leukotrienes (cysLTs) have been shown to be involved in such structural changes, the effects of a specific cysLT1 receptor antagonist, montelukast, were evaluated in a mouse model of chronic asthma.

Methods GATA-3-overexpressing mice and wild-type Balb/c mice were sensitized and repeatedly challenged by ovalbumin (OVA) or saline. The effects of montelukast on the development of airway remodelling were compared between the two mouse genotypes.

Results CysLTs in the lung were increased after repeated allergen challenges, and significantly enhanced in GATA-3-overexpressing mice. The enhanced cysLT levels were accompanied by the development of eosinophilia, smooth muscle cell hyperplasia, and increased stromal cell-derived factor-1 gene expression with a small increase in pro-collagen gene expression in OVA-challenged GATA-3-overexpressing mice, but not in wild-type mice. Montelukast significantly decreased lung cysLT levels and inhibited the GATA-3-overexpression-related airway remodelling, potentially preventing smooth muscle cell hyperplasia, but partially suppressed the increased pro-collagen gene expression and eosinophilic inflammation. Increases in the levels of IL-4, IL-5, IL-13, and eotaxin in bronchial lavage and TGF- β gene expression in the lungs were induced by OVA in both mouse genotypes. Montelukast treatment also significantly reduced these levels to the levels seen after saline challenges in GATA-3-overexpressing mice.

Conclusion Montelukast efficaciously prevented airway inflammation and remodelling in a GATA-3-overexpression antigen challenge mouse model by decreasing the cysLT-driven Th2 cytokine cycle of amplification of airway pathologies.

Keywords airway inflammation, airway remodelling, cysteinyl leukotrienes, GATA-3, Th2 cytokines

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Introduction

Chronic asthma is characterized by airway inflammation, hyperresponsiveness, and reversible airway obstruction. Persistent inflammation and airflow obstruction cause a gradual decline in lung function due to the structural changes called airway remodelling [1, 2]. The pathological features of airway remodelling include airway wall thickening, subepithelial fibrosis, airway smooth muscle cell

hyperplasia and hypertrophy, and excessive mucus secretion from hyperplastic goblet cells [3–5]. Therapies to arrest or reverse these pathologies would be beneficial in the management of chronic asthma.

Airway remodelling in allergic asthma is particularly driven through Th2 cytokine production. GATA-3 is a transcription factor required for Th2 differentiation of common T precursor cells [6–8]. We have demonstrated previously that GATA-3 overexpression enhanced

antigen-induced airway inflammation and the remodelling that included subepithelial fibrosis and smooth muscle cell hyperplasia and hypertrophy in an animal model of chronic asthma [9]. However, mucous secretion was regulated by T-bet overexpression with a Th1 cytokine bias rather than via GATA-3 overexpression [9].

GATA-3 also transactivates Th2 cytokine promoters for gene transcription. It is known to selectively activate the promoters of IL-4, IL-5, and IL-13 through chromatin remodelling [10–13]. The expression of targeted ILs produces airway phenotypes that demonstrate the development of airway remodelling. Selective IL-13 overexpression in the lung produced eosinophilic and mononuclear inflammation and airway remodelling, which included mucus cell metaplasia, subepithelial fibrosis, airway obstruction, and epithelial cell hypertrophy [14]. Similarly, IL-4 and IL-9 overexpression produced a mixed mononuclear and eosinophilic inflammation [15, 16], and IL-5 overexpression caused tissue eosinophilia [17]. IL-4, IL-5, and IL-9 also caused extensive mucus metaplasia, subepithelial fibrosis, and airway hyperresponsiveness [15–17].

Leukotrienes are a family of bioactive lipids derived from the oxygenation of arachidonic acid. LTC₄, LTD₄, and LTE₄ are collectively known as the cysteinyl leukotrienes (cysLTs). The cysLTs signal through several high-affinity G protein-coupled receptors including the cysLT1 receptor. Activation of the cysLT1 receptor can result in airway inflammation and bronchoconstriction in respiratory diseases [18]. Ovalbumin (OVA)-induced pulmonary inflammation was markedly reduced in mice lacking LTC₄ synthase, the terminal enzyme of cysLT generation [19]. Moreover, clinical and preclinical studies with cysLT1 receptor antagonists have shown that the cysLTs are involved in asthmatic airway remodelling [20–22].

It has been suggested that Th2 cytokines and cysLTs positively and mutually regulate the activity of the other. Th2 cytokines up-regulate the expression of both cysLT1 receptor gene and protein [23]. However, the correlation between Th2 cytokines and cysLT has not been well understood in the development of airway remodelling.

In the present study, the effects of montelukast, a specific cysLT1 receptor antagonist, were examined on airway remodelling enhanced by GATA-3 overexpression in an animal model of antigen-induced chronic asthma.

Methods

Mice and animal model of airway remodelling

GATA-3-overexpressing transgenic (*GATA-3-tg*) mice were generated using a 2.0 kb full-length GATA-3 cDNA inserted into the VA CD2 transgene cassette containing the up-stream gene regulatory region and locus control region of the human CD2 gene, and backcrossed with Balb/c mice for eight generations [10, 24]. To create an animal

model of airway remodelling, *GATA-3-tg* mice and the wild-type Balb/c mice (6–8 weeks old) were intraperitoneally sensitized with 100 µg of OVA with the adjuvant Al(OH)₃ on days 0 and 14 and repeatedly challenged with 10 µg of intranasal OVA or saline for 5 days a week for eight consecutive weeks from day 21 [9]. Montelukast (2 mg/kg/day), kindly provided by Merck & Co. Inc. (Whitehouse Station, NJ, USA), or vehicle was continuously administered using mini-osmotic pumps (Alzet; model 2004; Durect, Cupertino, CA, USA) for 8 weeks starting 24 h before the initial antigen challenge. The protocol of the animal study was approved by the institutional review board.

Lung histopathology and morphometry

The lung sections were stained immunohistochemically using anti- α -smooth muscle actin antibody for contractile elements of smooth muscle (Sigma Chemical Co., St Louis, MO, USA), with Masson's Trichrome stain for extracellular matrix, with Congo red stain for eosinophils, and with periodic acid Schiff (PAS) stain for mucin production and secretion. The percentages of the tissue area positively stained for extracellular matrix, contractile elements or mucin production and secretion in a 20 µm region of the airway epithelium were morphometrically analysed using a method described previously [25].

Bronchoalveolar lavage

The lungs were lavaged by six repeated instillations of 1 mL of saline each through the trachea cannula. The first bronchoalveolar lavage (BAL) fluids were centrifuged, and the supernatant was used for the measurements of cytokines and MUC5AC protein. Cells were counted using a haemocytometer. Differential cell counts were performed under standard light microscopy after Diff-Quik staining.

Enzyme-linked immunosorbent assay

The contents of cysLTs in the lung homogenates and the contents of IL-4, IL-5, IL-13, and eotaxin in the first BAL supernatant were determined by ELISA according to the manufacturers' instructions (cysLTs: Cayman Chemical, Ann Arbor, MI, USA; IL-4, IL-5, and eotaxin: BioSource International, Camarillo, CA, USA; IL-13: R&D Systems, Minneapolis, MN, USA). MUC5AC in BAL was also measured by ELISA as described previously [26].

Identification of fibrocytes and their expression of cysteinyl leukotriene-1 receptor

Fibrocytes located at the bronchial subepithelium were identified immunohistochemically. Paraffin sections (4 µm thick) were cut and mounted on poly-L-lysine-coated

glass slides. After removing the paraffin, the sections were reacted with rabbit anti-mouse collagen I (1 : 100 dilution; Chemicon, Temecula, CA, USA) and rat anti-mouse CD34 (1 : 100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. To identify cysLT1/LT2 receptors on fibrocytes, the sections were co-incubated with goat anti-cysLT1 receptor or with goat anti-cysLT2 receptor antibodies (1 : 100 dilution; Santa Cruz). After washing, sections were incubated with fluorescein-conjugated anti-rabbit IgG (1 : 100 dilution; Santa Cruz), rhodamine-conjugated anti-rat IgG (1 : 100 dilution; Santa Cruz), and Alexa Fluor 633-conjugated anti-goat IgG (1 : 100 dilution; Invitrogen, Carlsbad, CA, USA) secondary antibodies, respectively. The number of fibrocytes positive for both collagen I and CD34 was counted in the subepithelium of each section and expressed as cells/mm². Non-immune IgGs were used as the negative controls.

Circulating fibrocytes were isolated from mouse peripheral blood mononuclear cells (PBMC) according to the method reported previously [27]. Briefly, PBMC were isolated from blood by centrifugation over Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden). After overnight culture on a fibronectin-coated plate (3×10^6 PBMCs per well), the non-adherent cells were removed by gentle aspiration. Following 10 days of continuous culture, the cells were harvested and fibrocytes were identified by flow cytometry using the above-named antibodies. Cells positive for both collagen I and CD34 were gated and the expression of cysLT1 receptor among these cells was analysed by flow cytometry.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from the lung tissues, and RT-PCR was performed using a One Step RT-PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The sequences of the PCR primers were as follows: 5'-ATTGGTAATGTTGGTGCT-3' and 5'-GTGACCCCTTATGCCTCTGT-3' for pro- $\alpha 1$ (²) pro-collagen, 5'-TTGCTTCAGCTCCACAGAGA-3' and 5'-TGGTTGTAGAGGGCAAGGAC-3' for TGF- $\beta 1$, and 5'-CTTCATCCCCATTCTCCTCA-3' and 5'-GACTCTGCTCTGGT GGAAGG-3' for stromal cell-derived factor-1 (SDF-1/CXCL12). The expression level was quantified using NIH Image software (National Institute of Health, Bethesda, MD, USA). The expression levels were normalized against glyceraldehyde-3-phosphate dehydrogenase mRNA.

Statistical analysis

The data are expressed as mean $\pm$ SE. The difference between values after OVA challenges and saline challenges, and between the mice overexpressing GATA-3 and the wild-type control were evaluated by ANOVA and compared with the Tukey's multiple comparison test for all pairs of conditions. Values of $P < 0.05$ were considered to indicate statistical significance.

Results

Ovalbumin-induced increase in lung cysteinyl leukotriene levels was enhanced in GATA-3-tg mice

The concentration of cysLTs was elevated after repeated OVA challenges in the lungs of both wild-type and GATA-3-tg mice (Fig. 1). The concentration of cysLTs was significantly higher in the lungs of GATA-3-tg mice than in those of wild-type mice after repeated OVA challenge (Fig. 1). Montelukast treatment significantly inhibited the increase in the lung cysLT content after repeated OVA challenge in both mouse genotypes (Fig. 1).

Montelukast completely suppressed the airway smooth muscle cell hyperplasia induced by GATA-3 overexpression

The layer of smooth muscle cells in the airway was examined for cells positive for α -smooth muscle actin. The layer of smooth muscle cells was thin after repeated saline challenges in both mouse genotypes (Figs 2a and d). On the other hand, significantly increased thickness of the smooth muscle cell layer was observed in GATA-3-tg mice (Fig. 2e), but not in the wild-type mice (Fig. 2b). Montelukast treatment potently inhibited the thickening of the smooth muscle cell layer in GATA-3-tg mice (Fig. 2f).

When the percentage of area occupied by α -smooth muscle actin-positive cells in the 20 μ m region of the epithelium was examined with morphometric analysis (Fig. 2g), the area was significantly increased after

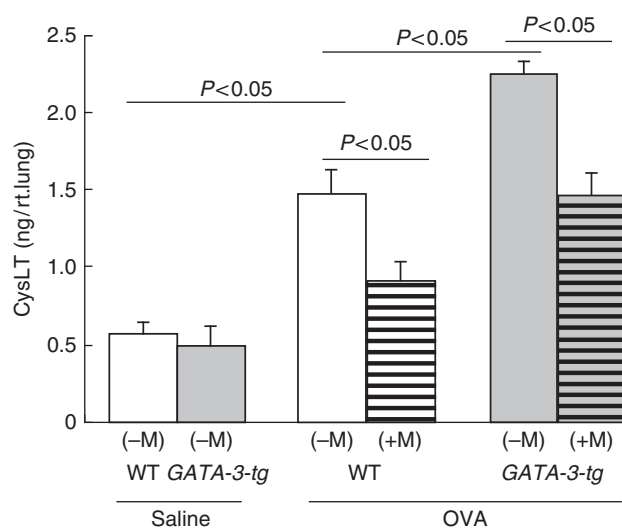


Fig. 1. GATA-3 overexpression increased the level of lung cysteinyl leukotrienes (cysLTs) in a mouse model of chronic asthma. CysLTs in the lung homogenate were measured by ELISA in mice overexpressing GATA-3 (GATA-3-tg) and Balb/c wild-type control mice (WT) after sensitization and repeatedly challenges by saline (saline) or ovalbumin (OVA). Data are expressed as the mean $\pm$ SE of four mice in each group.

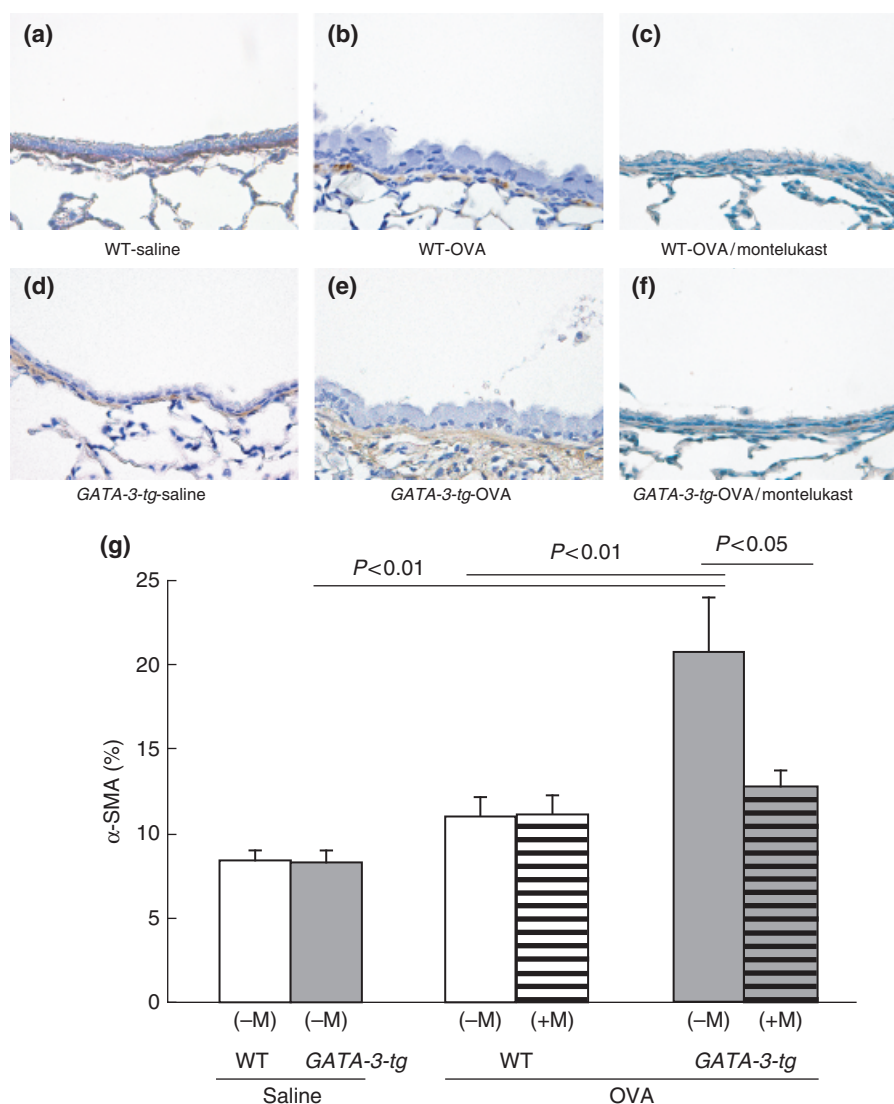


Fig. 2. Montelukast suppressed the airway smooth muscle cell hyperplasia induced by GATA-3 overexpression. (a–f) Smooth muscle cells in the airway wall were immunohistochemically stained using anti- α -smooth muscle actin in the wild-type mice (WT, a–c) and in the mice overexpressing GATA-3 (*GATA-3-tg*, d–f). The animals were sensitized and repeatedly challenged by saline (a, d) or ovalbumin (OVA) without treatment (b, e), and treated with montelukast during OVA challenges (c, f). (g) The percentage of area occupied by smooth muscle cells in the 20 μ m region beneath the epithelium in the airway was evaluated by morphometric measurement in *GATA-3-tg* mice and the WT control, after saline or OVA challenges. A group of mice for each genotype was treated with montelukast (+M) during OVA challenges. Data are expressed as the mean $\pm$ SEM of four mice in each group. SMA, smooth muscle actin.

repeated OVA challenges in *GATA-3-tg* mice, but was not changed in the wild-type control. Montelukast treatment significantly reduced the increased area percentage in *GATA-3-tg* mice to the level of the wild-type mice.

Montelukast suppressed the pro-collagen gene expression induced by GATA-3 overexpression

The subepithelium was examined for development of extracellular matrix with Masson's trichrome staining. Substantial subepithelial deposition of extracellular matrix was strongly observed after repeated OVA challenges in both wild-type

mice and *GATA-3-tg* mice (Figs 3b and e). By comparison, only a small amount of extracellular matrix deposition without obvious fibrosis was observed after saline challenges in both mouse genotypes (Figs 3a and d). Montelukast treatment reduced the OVA-induced subepithelial extracellular matrix deposition to the level induced by repeated saline challenges in both mouse genotypes (Figs 3c and f).

Morphometric analysis revealed that the area percentage of extracellular matrix in the 20 μ m region beneath the epithelium was significantly higher after OVA challenges as compared with saline challenges in both mouse genotypes. However, the increases were similar between

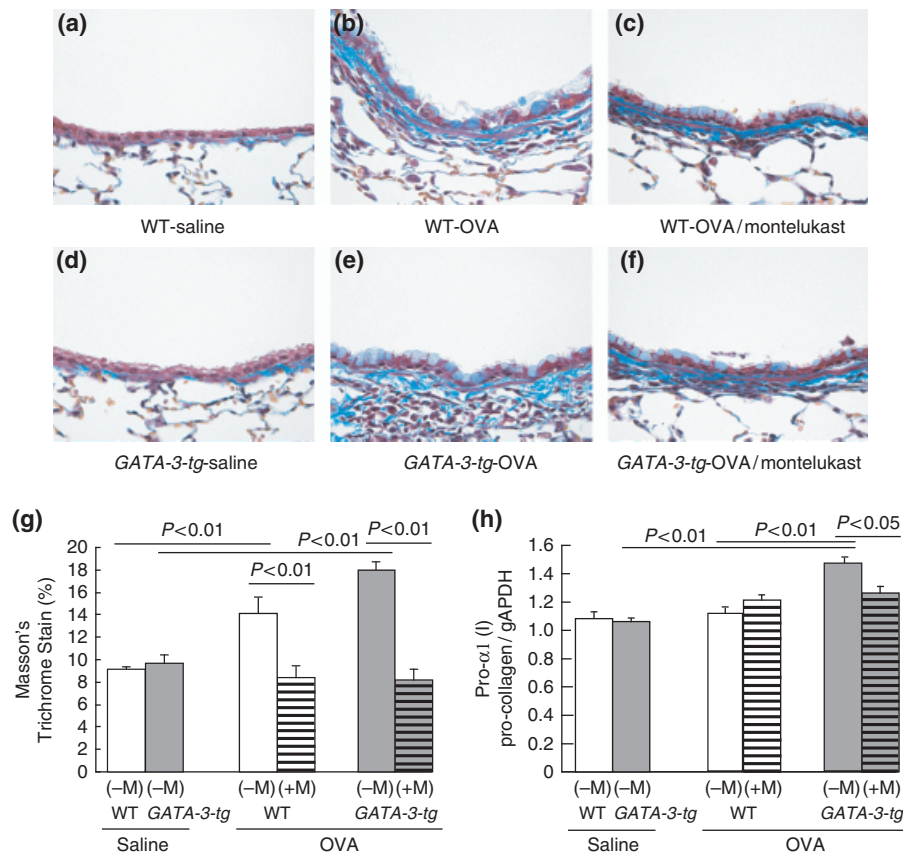


Fig. 3. Montelukast suppressed the increase in extracellular matrix induced by ovalbumin (OVA) challenges and the pro-collagen gene expression induced by GATA-3 overexpression. (a–f) Extracellular matrix deposition in the lungs was stained with Masson's Trichrome in the wild-type mice (WT, a–c) and in the mice overexpressing GATA-3 (*GATA-3-tg*, d–f). Animals were challenged by saline (a, d) or OVA (b, e) or treated with montelukast during the OVA challenges (c, f). (g) The percentage of area occupied by cells with deposition of extracellular matrix in the 20 μ m region beneath the epithelium in the airway was evaluated by morphometric measurement in WT and *GATA-3-tg* mice after challenges of saline or OVA. A group of mice for each genotypes was treated with montelukast (+M) during OVA challenges. (h) Pro- α 1 (I) pro-collagen gene expression was measured in the lung homogenates after OVA or saline challenges in the *GATA-3-tg* mice and the WT control. A group of mice for each genotypes was treated with montelukast (+M) during OVA challenges. Data are expressed as the mean $\pm$ SEM of four mice in each group.

the *GATA-3-tg* mice and the wild-type control, showing no enhancement with GATA-3 overexpression. Montelukast treatment significantly reduced the OVA-induced increases to the level seen in the saline-challenged animals in both mouse genotypes (Fig. 3g).

When the pro- α 1 (I) pro-collagen mRNA expression in the lungs was measured, no significant increase in the gene expression was observed after OVA challenges or after saline challenges in either genotype. However, a significant increase in the gene expression was observed after OVA challenges in *GATA-3-tg* mice compared with that in the wild-type control (Fig. 3h). Montelukast treatment significantly suppressed the GATA-3-associated increase in the pro-collagen gene expression.

Montelukast inhibited ovalbumin-induced mucin hyperproduction

Goblet cell hyperplasia and mucus hyperproduction in the lung were observed in both mouse genotypes after re-

peated OVA challenge (Figs 4b and e), whereas few positive epithelial cells were found after saline challenge (Figs 4a and d). Montelukast partially inhibited the mucus hyperproduction in the *GATA-3-tg* mice, but not in the wild-type control (Figs 4c and f).

As demonstrated by morphometric measurements, the percentage of PAS-positive epithelial cells was significantly higher after repeated OVA challenges compared with that after saline challenges in both *GATA-3-tg* mice and the wild-type control. However, no significant difference was observed between the *GATA-3-tg* mice and the wild-type control mice, indicating that the GATA-3 overexpression did not enhance mucin hyperproduction. Montelukast treatment significantly inhibited the OVA-induced mucus hyperproduction in *GATA-3-tg* mice, but not in the wild-type control (Fig. 4g). Similarly, repeated OVA challenge increased the expression of the mucin analogue MUC5AC protein in bronchial lavage in both mouse genotypes, but there was no significant difference between the *GATA-3-tg* mice and the wild-type control

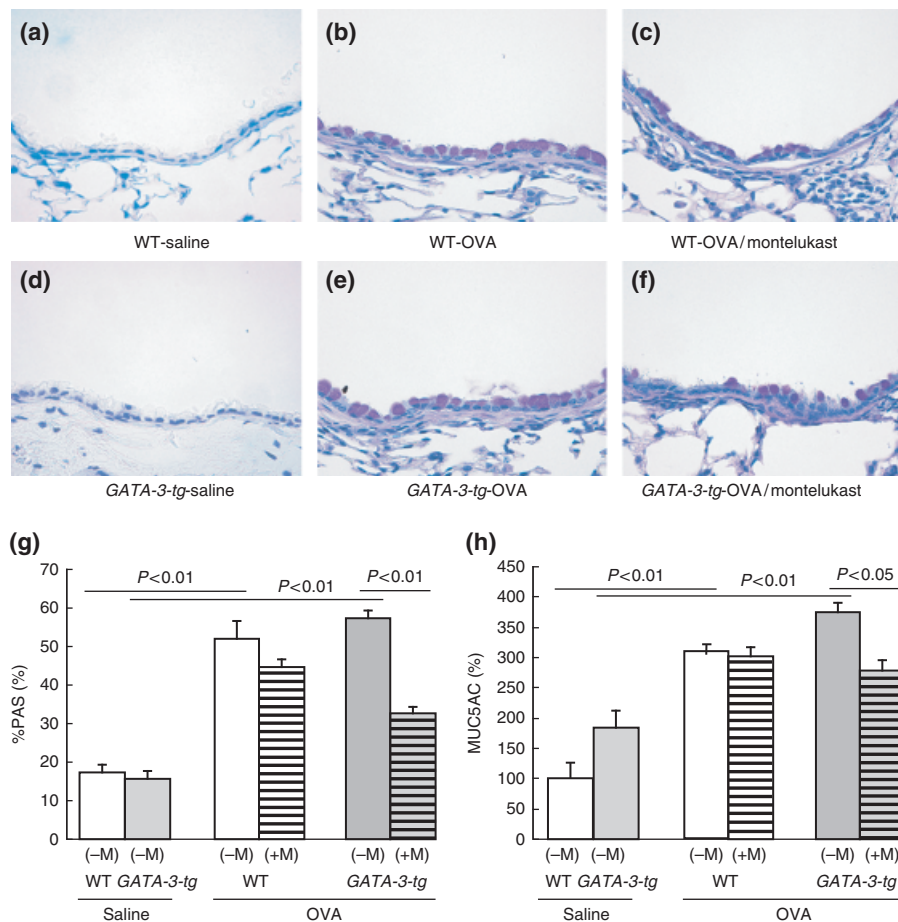


Fig. 4. Montelukast suppressed ovalbumin (OVA)-induced mucus hyperproduction and secretion. (a–f) The lung sections were stained with periodic acid Schiff (PAS). The histology was compared after saline (a, d) or OVA (b, c, e, and f) challenges in wild-type mice (WT, a–c) and in mice overexpressing GATA-3 (*GATA-3-tg*, d–f) and after montelukast treatment during OVA challenges (c, f). (g) The percentage of area occupied by PAS-positive cells in the airway epithelium was measured after saline or OVA challenges with (+M) or without (–M) montelukast in *GATA-3-tg* mice and in the WT control. (h) The level of MUC5AC protein expression was measured in BAL fluid after saline or OVA challenges in *GATA-3-tg* mice and in the WT control with (+M) or without (–M) montelukast administration during OVA challenges. The levels are shown as the percentage changes from the level in the saline-challenged WT mice (100%). Data are expressed as the mean $\pm$ SEM of four mice in each group.

mice. Montelukast treatment partially but significantly inhibited the OVA-induced MUC5AC protein expression in *GATA-3-tg* mice (Fig. 4h).

Montelukast partially reduced GATA-3-enhanced ovalbumin-induced eosinophilic airway inflammation

Eosinophilic infiltration was demonstrated after repeated OVA exposure especially in the peribronchial and perivascular areas in both the *GATA-3-tg* mice and the wild-type control. The area of eosinophil infiltration was greater in the *GATA-3-tg* mice than in the wild-type control (Figs 5b and e). Montelukast treatment partially reduced the eosinophil infiltration in the *GATA-3-tg* mice (Figs 5c and f).

When the eosinophil infiltration into the BAL fluids was quantitated, the eosinophil numbers were also higher after repeated OVA challenges than after saline challenges in

both mouse genotypes. However, the increase was significantly greater in the *GATA-3-tg* mice than that in the wild-type control, indicating that eosinophil inflammation was enhanced by GATA-3 overexpression. Montelukast treatment partially reduced the enhanced eosinophil numbers in the *GATA-3-tg* mice, but had no effect on OVA-induced increases in eosinophils in the wild-type mice (Fig. 5g).

Montelukast attenuated the increases in lung Th2 cytokines induced by ovalbumin challenges

The concentrations of the Th2 cytokine IL-4, IL-13, and CC chemokine eotaxin in the BAL fluids were increased after repeated OVA challenges, compared with the saline-challenged controls in both mouse genotypes. However, no enhancement was observed in *GATA-3-tg* mice, compared

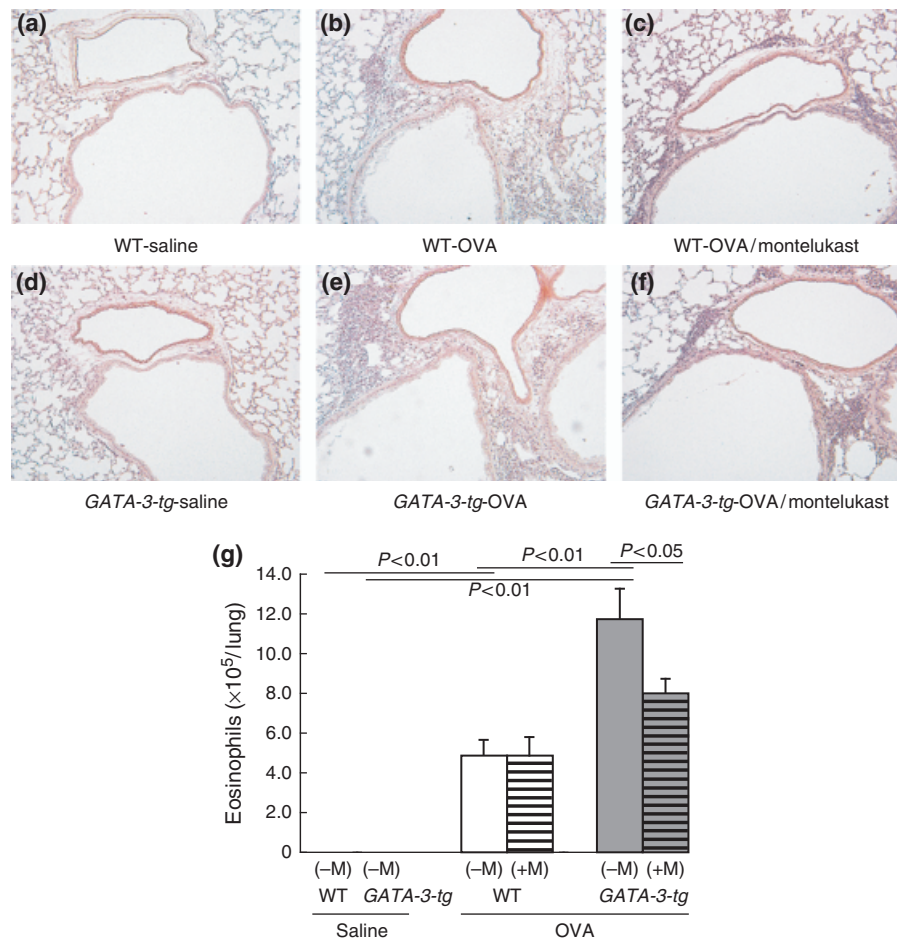


Fig. 5. Montelukast partially attenuated eosinophilia induced by the GATA-3 overexpression. (a–f) The lung sections were stained with Congo red for eosinophils. The histology was compared after saline (a, d) or ovalbumin (OVA) challenge (b, c, e, f) in GATA-3-overexpressing mice (*GATA-3-tg*, d–f) and the wild-type control mice (WT, a–c), and after montelukast administration during OVA challenges (c, f). (g) Eosinophil counts in the BAL fluids were taken after saline or OVA challenge in *GATA-3-tg* mice and the WT control mice with (+M) or without (–M) montelukast administration during OVA challenges. Data are expressed as the mean $\pm$ SEM of four mice in each group.

with wild-type mice after repeated OVA challenges (Figs 6a, c, and d). Although OVA challenges did not induce an increase in IL-5 in the wild-type mice, OVA challenges increased IL-5 in *GATA-3-tg* mice (Fig. 6b). Montelukast treatment significantly reduced all the cytokines to the levels seen following saline challenges in *GATA-3-tg* mice (Figs 6a–d).

Montelukast suppressed fibrocyte localization in the airways

Fibrocytes are unique blood-borne fibroblast-like cells, and their migration plays an important role in the pathogenesis of airway remodelling. To clarify the effects of montelukast on fibrocyte function, the cysLT1 receptor expression on circulating fibrocytes isolated from blood was examined. FACS analysis revealed that more than 60% of circulating fibrocytes obtained from saline-treated animals were positive for cysLT1 receptors (Fig. 7 and

Table 1). The proportion of cysLT1 receptor-positive fibrocytes increased $>80\%$ after repeated challenges with OVA (Table 1). However, the proportion of cysLT1 receptor-positive fibrocytes was not different between wild-type mice and *GATA-3-tg* mice (Table 1). Montelukast treatment did not affect the expression of cysLT1 receptors on fibrocyte in either mouse genotype (Table 1).

Localization of the fibrocytes in the airways was then examined by determining the number of fibrocytes positive for both CD34 and type I collagen in the airways of *GATA-3-tg* mice and the wild-type mice. Immunohistochemical analysis revealed that a significant number of fibrocytes were present in the airway subepithelial regions after repeated challenges with OVA in both mouse genotypes (Figs 8b and c, OVA). However, the degree of fibrocyte localization was much higher in the *GATA-3-tg* mice than in the wild-type mice (Fig. 8d). Montelukast treatment significantly reduced the number of fibrocytes in the airways of *GATA-3-tg* mice (Fig. 8d). No

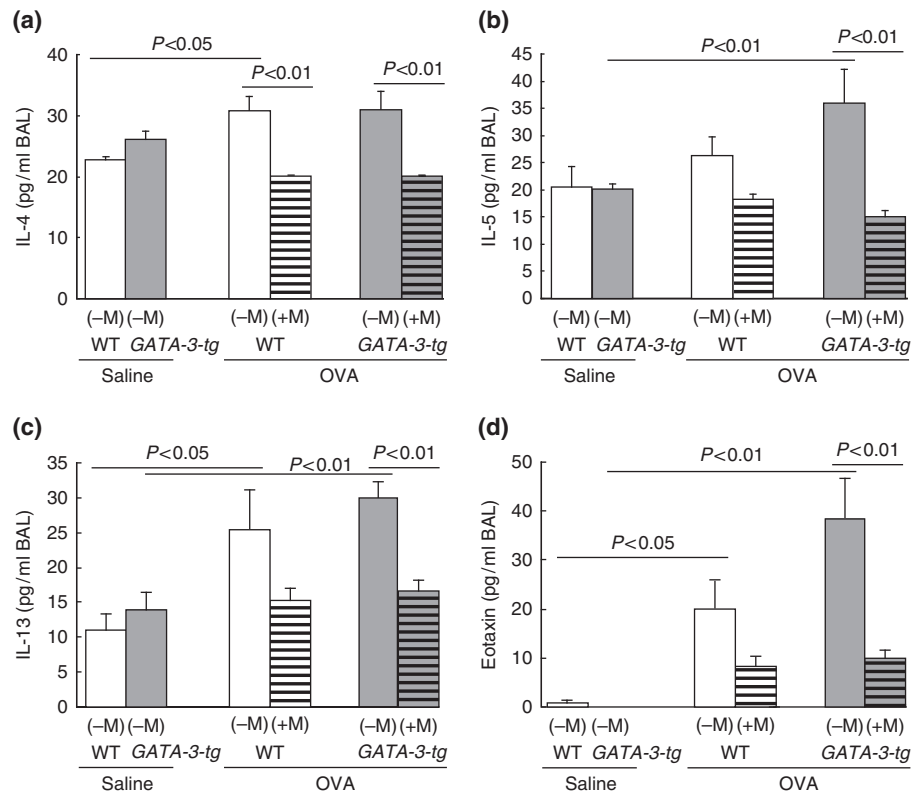


Fig. 6. Montelukast suppressed the increases in cytokines and eotaxin induced by ovalbumin (OVA) challenges. (a–d) The levels of IL-4 (a), IL-5 (b), IL-13 (c), and eotaxin (d) in bronchoalveolar lavage (BAL) fluid were measured using ELISA. The levels were compared between GATA-3-overexpressing (GATA-3-tg) mice and the wild-type (WT) control after challenges with saline or OVA with (+M) or without (–M) montelukast administration. Data are expressed as the mean $\pm$ SEM of four mice in each group.

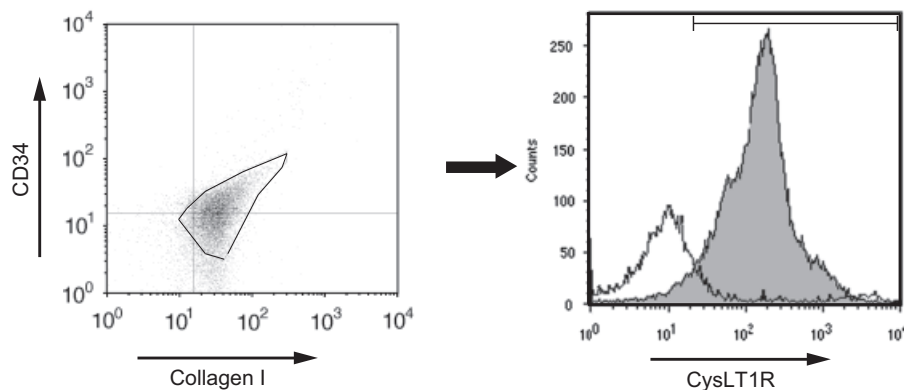


Fig. 7. Cysteinyl leukotriene-1 (cysLT1) receptor was expressed in circulating fibrocytes. Fibrocytes obtained from blood mononuclear cells were identified flow-cytometrically using anti-collagen I and anti-CD4 antibodies. The expression of cysLT1 receptor in the double-positive cells was then analysed by using its specific antibody. A representative blot obtained from GATA-3-overexpressing mouse is shown.

immunoreactive cells were detected in the airways after the saline challenges in either mouse genotype. Consistent with the results of circulating fibrocytes, fibrocytes located in the airway subepithelium expressed cysLT1 receptor (Fig. 8b). Subepithelial fibrocytes also expressed the cysLT2 receptor (Fig. 8c). Control sections stained with an isotype control showed no immunoreactivity (Fig. 8a).

Montelukast suppressed the ovalbumin-induced increase in pulmonary transforming growth factor- β gene expression and the GATA-3-associated increase in stromal cell-derived factor-1/CXCL12 gene expression

Because TGF- β is a cytokine that is thought to play multiple roles in airway wall remodelling [28, 29], we

Table 1. The percentage of cysteinyl leukotriene1 (cysLT1) receptor-positive cells in fibrocytes

	Saline	OVA	OVA/montelukast
Wild-type	64.4±9.8	82.5±0.7	81.5±0.8
GATA-3-tg	68.4±11.8	81.8±1.1	80.3±1.0

Circulating fibrocytes were isolated from peripheral blood mononuclear cells in wild-type mice and GATA-3-overexpressing (GATA-3-tg) mice after challenges of saline or OVA. A group of mice of for each genotypes was treated with montelukast during OVA challenges.

Data are expressed as the mean±SEM of four mice in each group. OVA, ovalbumin.

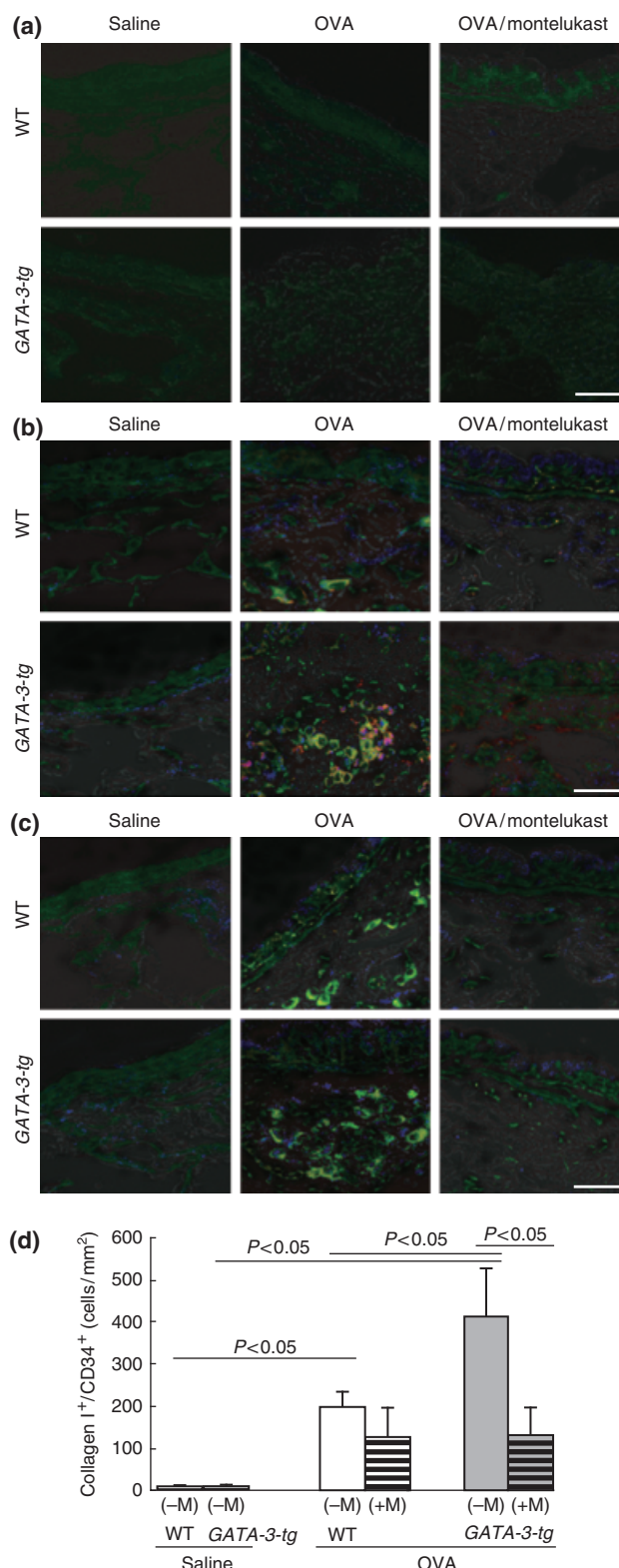
examined the TGF- β gene expression in the lungs in this animal model. OVA challenge induced an increase in TGF- β gene expression in both wild-type mice and *GATA-3-tg* mice. However, the expression after repeated OVA challenges was significantly higher in *GATA-3-tg* mice than that in the wild-type mice (Fig. 9a). In *GATA-3-tg* mice, montelukast treatment significantly reduced the OVA-induced increase to the level of saline challenges (Fig. 9a).

SDF-1/CXCL12 is the most efficacious chemoattractant for T lymphocytes and fibrocytes. Because plasma SDF-1 is high in asthmatic children [30], it may have an important role in the development of airway remodelling. When SDF-1 gene expression was examined in the lung in the present study, the level of the gene expression was similarly increased after repeated OVA challenges and after saline challenges in the wild-type control. However, the expression after repeated OVA challenges was significantly higher in *GATA-3-tg* mice than in the wild-type control (Fig. 9b). Montelukast reduced the GATA-3-overexpression-induced increase in SDF-1 gene expression in *GATA-3-tg* mice to the level of the wild-type control.

Fig. 8. Montelukast suppressed the GATA-3-overexpression-induced fibrocyte localization in the airway. (a–c) Fibrocyte localization and fibrocyte expression of cysteinyl leukotriene (cysLT)1 receptor (b) or cysLT2 receptor (c) in the airway subepithelium were examined using immunofluorescent histochemistry. The expression of collagen I was visualized by FITC (green). The expression of CD34 was visualized by rhodamine (red). And the expression of cysLT1 or cysLT2 receptor was visualized by Alexa Fluor 633 (blue). Cells positive for both CD34 and collagen I were identified as fibrocytes (yellow). All images were digitally merged to demonstrate colocalization. Localization of fibrocytes in the airway subepithelium and their expression of cysLT1 receptor or cysLT2 receptor were compared after saline or ovalbumin (OVA) challenges in *GATA-3-tg* mice and in the wild-type (WT) control. A group of mice for each genotype was treated with montelukast during OVA challenges (right panels). (a) Control sections stained with isotype control IgGs. Bar = 100 μ m. (d) The number of fibrocytes in the airways of *GATA-3-tg* mice and WT control after challenges with saline or OVA with (+M) or without (–M) montelukast administration. Data are expressed as the mean±SEM of four mice in each group.

Discussion

In the present study, mice overexpressing GATA-3 showed greater airway inflammation and greater remodelling after OVA challenges compared with the wild-type control



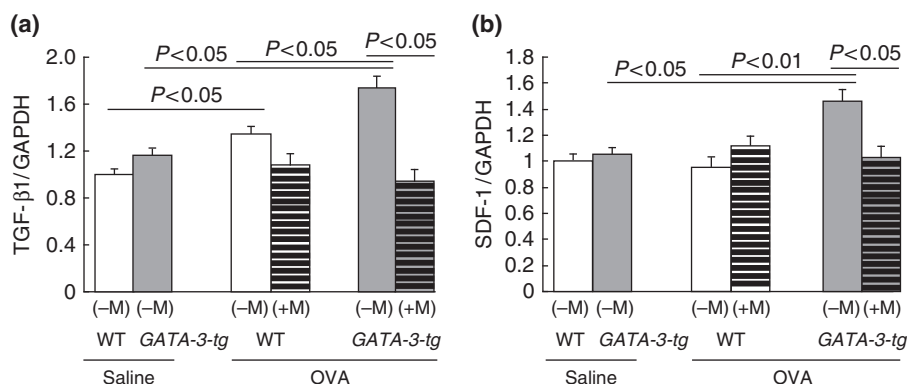


Fig. 9. Montelukast suppressed the GATA-3-induced increase in TGF- β 1, and in stromal cell-derived factor (SDF)-1 gene expression. (a, b) The levels of TGF- β 1 (a) and SDF-1 (b) gene expression in the lungs were examined after ovalbumin (OVA) or saline challenges in the GATA-3-overexpressing (*GATA-3-tg*) mice and the wild-type (WT) control and after montelukast administration (+M) with OVA challenges. Data are expressed as the mean $\pm$ SEM of six mice in each group.

mice. The *GATA-3-tg* mice exhibited eosinophilia, smooth muscle cell hyperplasia and hypertrophy, increased procollagen gene expression, and increased localization of fibrocytes in the airway. The increased remodelling was accompanied by increased concentrations of pulmonary cysLTs and increased pulmonary TGF- β and SDF-1 gene expression. Although the Th2 cytokines IL-4, IL-5, IL-13, and eotaxin were induced by OVA challenges, minimal enhancement was observed by the GATA-3 overexpression. This is consistent with our previous studies, in which overexpression of GATA-3 suppressed Th1 differentiation by inhibiting T-bet rather than promoting Th2 differentiation and Th2 cytokine transcription [9, 31]. Montelukast, a selective cysLT1 receptor antagonist, administered during OVA challenges suppressed GATA-3-related airway inflammation and remodelling as well as OVA-induced GATA-3 independent increases in Th2 cytokines. Another cysLT1 receptor antagonist, pranlukast, has been shown to suppress mite-antigen-induced production of Th2 cytokines in human PBMC [32].

Previous studies demonstrated that the continuous administration of montelukast using osmotic pumps nicely suppresses airway remodelling induced by repeated allergen challenge [21, 22]. The amount of montelukast used in this study (2 mg/kg/day) was twice as much as used in the previous study. We therefore consider that the concentration of montelukast should be sufficient to suppress airway remodelling at least in wild-type mice. Surprisingly, however, montelukast did not suppress some phenotypes of airway remodelling, such as airway smooth muscle hyperplasia and mucus production in wild-type mice but significantly attenuated the phenotypes in *GATA-3-tg* mice. Montelukast also did not suppress the allergen-dependent increase in BAL eosinophil number in wild-type mice. It has been demonstrated that TGF- β can enhance the proliferation of airway smooth muscle cells by up-regulating the cysLT1 receptor [33]. IL-5 and IL-13 induce goblet cell hyperplasia independently of IL-4 [17,

34]. Differentiation, maturation, and activation of eosinophils are regulated by IL-5 [35]. In the present study, the levels of these cytokines were significantly elevated in *GATA-3-tg* mice and montelukast treatment suppressed the expression of these cytokines specifically in *GATA-3-tg* mice. Therefore, a possible explanation as to why montelukast did not efficaciously suppress those remodelling phenotypes is the difference in cytokine expression between wild-type mice and *GATA-3-tg* mice in response to repeated allergen challenges. In addition, the differences in the allergen challenge protocols may have led to different results with respect to the effects of montelukast in wild-type mice between the present and previous studies [21].

CysLTs are released from the lungs following chronic allergen exposure in rats and mice. They are also known to participate in airway remodelling via processes unrelated to inflammation. The cysLTs are pro-inflammatory and also cause airway remodelling through both inflammatory processes and direct effects on mesenchymal cells. They directly promote airway smooth muscle hyperplasia and hypertrophy [33] as well as increase the capacity of lung fibroblasts to synthesize collagen [36]. Because cysLTs were more elevated in the *GATA-3-tg* mice than in the wild-type control mice in the present study, GATA-3 airway pathologies may be mediated, at least in part, by cysLTs.

Th2 cytokines play a role in the regulation of cysLT-mediated lung inflammatory processes. Th2 cytokines regulate the cysLT production via the coordinated induction of LTC4 synthase expression and the nuclear import of 5-lipoxygenase [37]. In addition to cysLT biosynthesis, Th2 cytokines up-regulate the cysLT1 receptor in monocytes, macrophages [27], and lung fibroblasts [38]. Coordinated regulation between cysLTs and cytokines was demonstrated in Th2 cell-dependent pulmonary inflammation [39].

TGF- β has been shown to increase cysLT generation by up-regulating 5-lipoxygenase and LTC4 synthase

activities [40, 41]. Furthermore, TGF- β was reported to play a significant role in the pathogenesis of airway remodelling. Blocking TGF- β activity inhibits remodelling phenotypes including epithelial shedding, mucus hypersecretion, angiogenesis, airway smooth muscle cell hypertrophy, and hyperplasia in an asthmatic mouse model [42]. Our previous study demonstrated that TGF- β expression in the lung was enhanced in *GATA-3-tg* mice after OVA [9] and bleomycin challenges [43]. In the present study, correspondingly, TGF- β gene expression was increased in *GATA-3-tg* mice after repeated allergen challenges. Montelukast treatment reduced the increase in TGF- β gene expression in these mice. Interestingly, the synergism of cysLTs and TGF- β was demonstrated in a study by Espinosa et al. [33], who showed that LTD₄ alone was unable to support smooth muscle cell proliferation without pretreatment of TGF- β 1 or IL-13, both of which increased the cysLT 1 receptor expression. In a study of bleomycin-induced pulmonary fibrosis, montelukast also inhibited the inflammatory process and the development of fibrosis with a decrease in TGF- β expression [44]. Thus, the inhibitory effects of montelukast on the development of airway remodelling may be partially mediated by the regulation of TGF- β .

The present study suggested that fibrocyte recruitment to the lung was enhanced by the GATA-3 overexpression, as shown by localization of both CD34- and collagen I-positive fibrocytes in the airway subepithelium. The localization was increased after repeated allergen exposure. Interestingly, the degree of fibrocyte localization was higher in *GATA-3-tg* mice than in wild-type mice. Fibrocytes are bone-marrow-derived cells that circulate in the peripheral blood, and that uniquely express the haemopoietic stem cell antigen CD34 and collagen I [45]. Fibrocytes can produce connective tissue proteins such as collagens I and III, as well as matrix metalloproteinases [46], causing fibrosis. However, more recent studies have demonstrated that fibrocytes may also play a role in the development of airway smooth muscle hyperplasia. Fibrocyte-like cells accumulate in the bronchial mucosa and differentiate into myofibroblasts in asthmatic airways [47]. Thus, fibrocytes may have multiple functions in the pathogenesis of airway remodelling in asthmatics. Our present finding is consistent with a previous study demonstrating that the cysLT1 receptor was expressed on the majority of fibrocytes [48]. In the present study, montelukast treatment decreased the number of fibrocytes located in the airway of OVA-exposed mice. Montelukast might inhibit local proliferation of fibrocytes, because cysLTs can regulate their proliferation [48].

We observed that the level of SDF-1/CXCL12 gene expression in the lungs was significantly elevated after repeated allergen challenges in *GATA-3-tg* mice, but not in the wild-type mice. A previous study suggested that SDF-1/CXCL12 may play a role in airway remodelling via

the angiogenesis of the bronchial mucosa in asthmatic patients [49]. SDF-1/CXCL12 is the only chemokine known to bind to CXCR4, a chemokine receptor expressed in fibrocytes. It has been demonstrated that circulating fibrocytes migrate and translocate to the lungs in response to SDF-1/CXCL12 in a bleomycin-induced pulmonary fibrosis model [50]. In the present study, we also demonstrated that the expression of SDF-1/CXCL12 was significantly reduced in the lungs of *GATA-3-tg* mice with montelukast treatment. This finding supports previous data showing that montelukast therapy significantly reduced plasma SDF-1/CXCL12 concentrations in asthmatic children [30]. Although the precise mechanisms of the cysLT-mediated production of SDF-1/CXCL12 are not yet fully understood, the activation of nuclear factor-kappa B (NF- κ B) might be involved in the process, because NF- κ B activation is required for the production of SDF-1/CXCL12 [51], and montelukast was found to successfully suppress the activation of NF- κ B [52]. Thus, it is likely that the enhanced recruitment of fibrocytes into the asthmatic airways in *GATA-3-tg* mice was at least in part due to the cysLT-mediated up-regulation of SDF-1/CXCL12.

In summary, our findings revealed that GATA-3 overexpression significantly enhanced airway inflammation and the development of airway remodelling, and markedly increased the concentration of cysLTs in the lungs of chronically allergen-challenged mice. Montelukast, a cysLT1 receptor antagonist, suppressed the GATA-3-related increase in inflammation, the phenotypes of airway remodelling, and the recruitment of fibrocytes into the airways. Concurrent with these changes, it also suppressed the allergen-induced increases in lung Th2 cytokines, SDF-1/CXCL12, and TGF- β . Although the fact that montelukast treatment was not instituted after the initiation of allergic asthma does not model the usual clinical situation in patients, and is a limitation of the study, montelukast might offer additional benefits by preventing airway remodelling, particularly in Th2-biased asthmatics.

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