

ORIGINAL ARTICLE

I κ B kinase β inhibitor IMD-0354 suppresses airway remodelling in a *Dermatophagoides pteronyssinus*-sensitized mouse model of chronic asthma

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Summary

Background Nuclear factor (NF)- κ B is a transcription factor that regulates cytokine and chemokine production in various inflammatory diseases, including bronchial asthma. I κ B kinase (IKK) β is important for NF- κ B activation in inflammatory conditions, and is possibly related to airway remodelling. Thus, inhibition of the IKK β -NF- κ B pathway may be an ideal strategy for the management of airway remodelling.

Objective We examined the effects of a newly synthesized IKK β inhibitor, IMD-0354, in a chronic allergen exposure model of bronchial asthma in mice.

Methods A chronic mouse model was generated by challenge with house dust mite antigen (*Dermatophagoides pteronyssinus*). IMD-0354 was administered intraperitoneally in therapeutic groups. Lung histopathology, hyperresponsiveness and the concentrations of mediators and molecules in supernatants of lung homogenates were determined.

Results NF- κ B activation was inhibited by prolonged periods of IMD-0354 administration. IMD-0354 reduced the numbers of bronchial eosinophils. IMD-0354 also inhibited the pathological features of airway remodelling, including goblet cell hyperplasia, subepithelial fibrosis, collagen deposition and smooth muscle hypertrophy. Inhibition of these structural changes by IMD-0354 was the result of the suppressing the production and activation of remodelling-related mediators, such as TGF- β , via inhibition of IKK β . IMD-0354 inhibited IL-13 and IL-1 β production, and it restored the production of IFN- γ . It also ameliorated airway hyperresponsiveness.

Conclusion IKK β plays crucial roles in airway inflammation and remodelling in a chronic mouse model of asthma. A specific IKK β inhibitor, IMD-0354, may be therapeutically beneficial for treating airway inflammation and remodelling in chronic asthma.

Keywords airway inflammation, airway remodelling, IKK β inhibitor, IMD-0354, NF- κ B, TGF- β
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Introduction

Bronchial asthma is characterized both by allergic inflammation and airway remodelling including mucus cell hyperplasia, subepithelial fibrosis and smooth muscle cell hypertrophy/hyperplasia. The severity of asthma is related to subepithelial fibrosis [1], and asthmatic airway smooth muscle remodelling is related to airway responsiveness [2]. These structural changes arise from the influence of various cytokines and mediators, including TGF- β , platelet-derived growth factor (PDGF)-AB, plasminogen activator inhibitor (PAI)-1 and matrix metalloproteinase (MMP)-9 [1, 2]. Current therapies, including inhalant

corticosteroids, have little or no effect on these structural changes [3]. Although studies in mice have shown that leukotriene receptor antagonists are effective against airway remodelling [4, 5], some asthmatic patients do not respond to these drugs. Therefore, new molecular targets are needed to suppress airway remodelling without additional adverse consequences.

It is known that nuclear factor (NF)- κ B plays important roles in inflammatory diseases. Recently, it was shown that NF- κ B activation contributed to the pathogenesis of bronchial asthma. In its inactive state, NF- κ B binds to I κ B α and is normally found in the cytoplasm. Phosphorylation of I κ B α leads to the release of NF- κ B, which

promotes gene transcription. Accordingly, we have focused attention on I κ B kinase (IKK) β . The multi-subunit IKK complex, including IKK α , IKK β and IKK γ , plays a key role in the phosphorylation of I κ B α . IKK β appears to be more critical than the other subunits for activating the NF- κ B pathway during inflammation, as it has been reported that the inhibition of IKK α and NF- κ B-induced kinase did not suppress NF- κ B activation [6, 7]. Therefore, inhibition of IKK β may be an attractive therapeutic strategy.

We demonstrated previously that IMD-0354, a recently synthesized low-molecular-weight compound, inhibited allergic inflammation in an acute mouse model of asthma [8] and bleomycin-induced lung fibrosis in mice [9]. IMD-0354 selectively inhibits IKK β particularly when it is induced by pro-inflammatory cytokines, such as TNF- α and IL-1 β [8, 10]. Previous reports showed that IMD-0354 was effective in acute and subacute inflammatory diseases, such as myocardial ischaemia/reperfusion injury [10] and insulin resistance [11]. These reports also demonstrated the safety of IMD-0354 *in vitro* and *in vivo* [8–10]. Moreover, IMD-0354 is now in phase I/II studies for patients with atopic dermatitis and COPD. Based on these

previous observations, IMD-0354 may be a suitable agent for clinical applications.

It is not known, however, if this specific IKK β inhibitor can inhibit the development of airway remodelling in bronchial asthma. Broide et al. [12] demonstrated an important role for NF- κ B-regulated genes in airway epithelium during allergen-induced airway remodelling, including peribronchial fibrosis and mucus production. In this study, we examined if IMD-0354 affected airway remodelling, including mucus cell hyperplasia, subepithelial fibrosis and smooth muscle cell hypertrophy, in a chronic antigen exposure model of asthma in mice.

Materials and methods

Preparation of IMD-0354

A synthetic IKK β inhibitor, IMD-0354 (*N*-[3,5-bis-trifluoromethyl-phenyl]-5-chloro-2-hydroxy-benzamide), was kindly provided by the Institute of Medicinal Molecular Design Inc. (Tokyo, Japan) (Fig. 1a). IMD-0354 powder was dissolved in 0.5% carboxymethylcellulose (CMC; Sigma-Aldrich Japan,

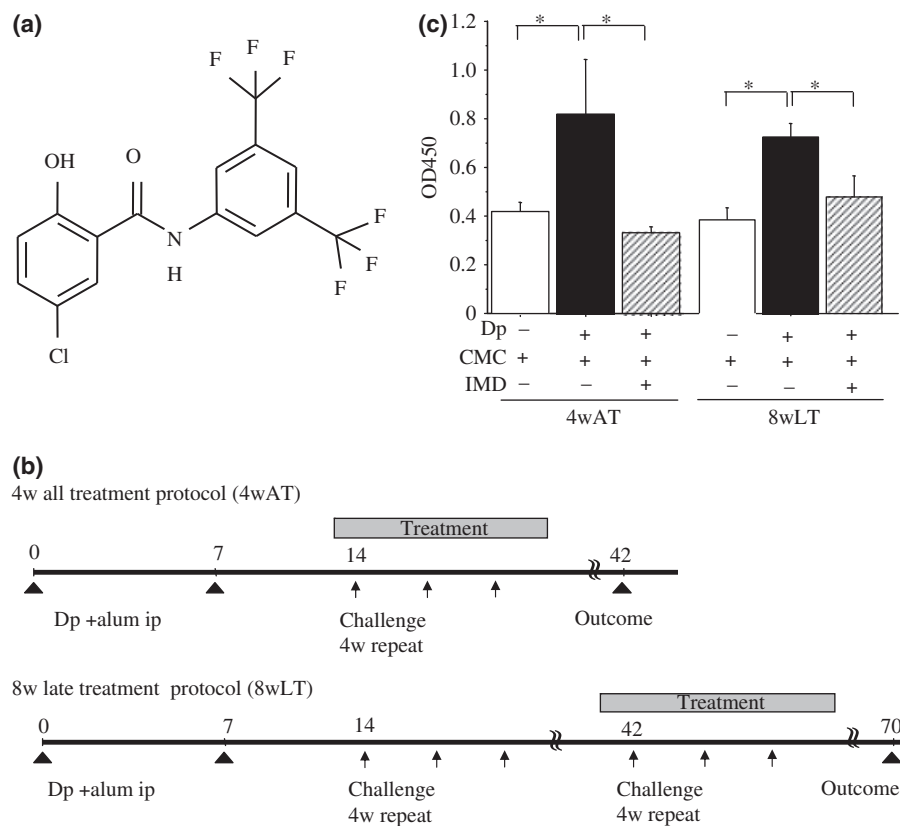


Fig. 1. (a) Structure of IMD-0354. These data were provided by the Institute of Medicinal Molecular Design Inc. (b) Mouse experimental protocols. (c) NF- κ B activation in lung tissue nuclear extracts. Levels of the activated p65 subunit in the nucleus were measured using a Trans AM NF- κ B p65 Transcription Factor Assay kit described in "Material and methods". Results are absorbance at 450 nm (OD 450 nm; mean $\pm$ SE obtained from three different experiments. Each experiment used in five mice per group). * $P < 0.05$. Number of mice (n) used for statistical analysis was as follows: white bars, control/CMC mice (4wAT: $n = 8$, 8wLT: $n = 11$); black bars, Dp/CMC mice (4wAT: $n = 12$, 8wLT: $n = 13$); shaded bars, Dp/IMD (20 mg/kg) mice (4wAT: $n = 10$, 8wLT: $n = 14$). Dp, *Dermaphagoides pteronyssinus*; NF, nuclear factor; CMC, carboxymethylcellulose.

Tokyo, Japan). The optimal dose of IMD-0354 for *in vivo* use was previously shown to be 20 mg/kg [8].

Preparation of house dust mite antigen

House dust mite antigen [*Dermatophagoides pteronyssinus* (Dp)] was purchased from LSL (Tokyo, Japan). This extract included the major allergens, Der p 1 and Der p 2 and was proteolytically active [13]. Endotoxin removal solution (Sigma-Aldrich Japan) was used to reduce the endotoxin concentration. After removal, Dp endotoxin was 0.308 IU/mg.

Mouse experimental protocols

Six-week-old female BALB/c mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Mice were maintained in the animal facility of the University of Tokushima under specific pathogen-free conditions, according to the guidelines of and approval by the ethics committee of our university [14]. Experimental protocols are shown in Fig. 1b. Each experiment was performed using three to five mice per group.

All mice were sensitized on days 0 and 7 by intraperitoneal injections of 10 µg of Dp dissolved in 500 µL saline and mixed with 1 mg of Alum (Sigma-Aldrich Japan). We then used two different treatment protocols. The periods of Dp challenge and IMD-0354 administration were decided upon from preliminary experiments in which subepithelial fibrosis was established after more than 4 weeks of Dp challenge and was reduced after more than 4 weeks of IMD-0354 administration (data not shown). Administering CMC and IMD-0354 alone to control mice did not affect NF-κB activation [8].

One protocol was an all-period treatment model (4wAT model) in which we examined the role of IKKβ during airway remodelling. For this protocol, Dp-sensitized mice with a CMC vehicle treatment (Dp/CMC group) and mice with IMD-0354 treatment (Dp/IMD group) were challenged intranasally with 10 µg (10 µL) of Dp in 70 µL saline every other day, 3 days per week, from days 14 to 39. IMD-0354 (20 mg/kg) [8] and CMC were administered intraperitoneally every day during this period.

The second protocol was a late-period treatment model (8wLT model) in which we examined the therapeutic effects of IMD-0354 on established pathological features of airway remodelling. For this protocol, Dp challenges were performed from days 14 to 67, every other day, 3 days per week, and IMD-0354 (20 mg/kg) treatment was administered every day from days 49 to 67. As a control (control/CMC group), mice were challenged with saline and given CMC during this period.

Mice were killed on day 42 for the 4wAT model, and day 70 for the 8wLT model. Bronchoalveolar lavage was performed [8]. The lungs were harvested for histopathol-

ogy, to obtain supernatants after lung homogenization, and for nuclear extracts [8].

Nuclear factor-κB enzyme-linked immunosorbent assay

To evaluate NF-κB activation, translocation of the p65 subunit into the nucleus was measured using a Trans AM NF-κB p65 Transcription Factor Assay kit (Active Motif Inc., Carlsbad, CA, USA) according to the manufacturer's instructions [15]. Activated NF-κB levels were determined by measuring the absorbance at 450 nm, with results expressed as optical density (450 nm).

Histopathology and immunohistochemistry

Lung tissue was fixed in 10% formalin and embedded in paraffin. Sections (3 µm thick) were stained with haematoxylin and eosin, Luna, periodic acid-Schiff (PAS) or Azan Mallory. A Dako CSA immunohistochemistry kit (Dako, Tokyo, Japan) was used for immunohistochemistry according to the manufacturer's instructions. Anti-mouse smooth muscle α-actin antibody (Thermo Fisher Scientific Inc., Fremont, CA, USA) was used as a primary antibody.

Morphological analyses

An OLYMPUS BX61 microscope (Olympus, Tokyo, Japan) with Scion Image software was used for morphological analysis. The methods for counting the number of eosinophils and measuring the PAS-positive areas were described previously [8]. The thickness of subepithelial fibrosis and the smooth muscle layer was analysed as follows. The area of subepithelial fibrosis (stained blue) and smooth muscle layer (α-SMA positive) around a bronchus was measured. The average thickness was determined by the area of the positive layer divided by the length of the internal circumference of the area. The mean values for thickness were calculated for 8–10 bronchi per left lung lobe.

Collagen assay

The left lungs, harvested on days 42 and 70, were used for collagen assays. Total lung collagen was determined using a Sircol Collagen Assay kit (Biocolor Ltd, Belfast, Northern Ireland) according to the manufacturer's instructions [9, 16]. Collagens contain about 14% hydroxyproline by weight. According to the manufacturer's data, collagen contents obtained with this method are correlated well with the hydroxyproline content.

Measurements of total protein and cytokine concentrations

Protein concentrations were determined by the BCA method (Pierce, Rockford, IL, USA). Cytokines, growth

factors, enzymes and serum Igs were determined using commercial ELISA kits. ELISA kits and their sensitivities were as following: IL-1 β , 4, 5, 13, IFN- γ , TGF- β , PDGF-AB, total MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 (R&D Systems, Minneapolis, MN, USA), with sensitivities of 3, 2, 7, 1.5, 2, 1.7, 2.3, 7 and 1.4 pg/mL, respectively; PAI-1 (Innovative Research Inc., Sarasota, FL, USA) with a sensitivity of 20 pg/mL; active MMP-9 (MMP-9 Biotrak Activity Assay, GE Healthcare, Buckinghamshire, UK) with a sensitivity of 0.5 ng/mL; Serum IgE (ELISA mouse IgE kit, Seikagaku Biobusiness Corp., Tokyo, Japan) with a sensitivity of 0.5 ng/mL; and Serum IgG1 and IgG2a (Cygnus Technologies, Southport, NC, USA) with sensitivities of 0.4 and 0.3 ng/mL, respectively.

Western blotting

Lung homogenate extracts were subjected to SDS-PAGE, using a 7.5% PAGE gel (Bio-Rad Laboratories, Hercules, CA, USA) with a Tris-glycine-SDS buffer. Proteins were then electroblotted onto nitrocellulose and incubated with a primary antibody. After this, they were incubated with an HRP-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ, USA). The blot was developed using the enhanced chemiluminescence advanced method (Amersham Biosciences) and exposed to Fuji X-ray film for 5 min. Primary antibodies were for phosphor-SMAD2/3 (Santacruz Biotechnology, Santacruz, CA, USA), SMAD2/3 (Millipore, Billerica, MA, USA), SMAD7 (LIFESPAN Bioscience, Seattle, WA, USA) and β -actin (antibody; Sigma-Aldrich Japan).

Measurement of airway resistance

Lung resistance (R_L) was measured by restrained whole-body plethysmography (Buxco Electronics, Troy, NY, USA). On days 42 or 70, mice were anaesthetized with 10 mg/mL of 2,2,2-tribromoethanol (Sigma-Aldrich Japan) with 2% of 2-methyl-2-butanol (Sigma-Aldrich Japan). While mice were tracheostomized and cannulated with a 19 G tracheostomy tube, they were placed in the chamber for plethysmography and ventilated mechanically. Aerosolized methacholine (Mch) was administered by an inline aerosol delivery system at increasing concentrations (0–50 mg/mL). After each Mch challenge, data were continuously collected for 3 min. R_L values were expressed as a percentage of the baseline R_L . The provocative concentration of Mch that caused a 200% increase in R_L , PC200, was calculated by linear interpolation of the dose-response curves [17].

Statistical analysis

Experimental results are given as means $\pm$ SE. Experimental groups were compared using a one-way ANOVA. If statistical significance was identified by ANOVA, a

Tukey–Kramer *post hoc* test was used to correct for multiple comparisons. For airway resistance measurements, a two-way repeated measures ANOVA was used, followed by a Tukey–Kramer test. Stat-View 5.0 (Abacus Concept Inc., Berkeley, CA, USA) was used for data analysis. *P*-values < 0.05 were considered significant.

Results

Inhibition of nuclear factor- κ B by IMD-0354

As shown in Fig. 1c, for both experimental protocols, NF- κ B activation was increased in Dp/CMC mice compared with control/CMC mice. NF- κ B p65 levels in Dp/IMD mice were significantly lower than those in Dp/CMC mice. These results suggest that prolonged administration of IMD-0354 inhibits NF- κ B activation in the lungs of a mouse model of chronic asthma.

Eosinophil infiltration

We analysed airway inflammation and enumerated eosinophils in the airways of Dp-sensitized mice. Increased inflammatory cell infiltration into the subepithelium area by chronic exposure to Dp was inhibited by IMD-0354 treatment in both protocols (Figs 2a–c and g–i). The effect on eosinophil infiltration was similar (Figs 2d–f and j–l). As shown in Table 1, treatment with IMD-0354 significantly decreased the numbers of eosinophils in the subepithelium for both models. In bronchoalveolar lavage fluid (BALF), the total number of cells in Dp/CMC mice was higher than those in control/CMC mice in the 4wAT model (Fig. 2m). In particular, lymphocytes and eosinophils were increased. The numbers of total cells and eosinophils were significantly reduced in Dp/IMD mice. In the 8wLT model, the numbers of total cells, lymphocytes and eosinophils were increased in the BALF of both Dp/CMC and Dp/IMD mice compared with control/CMC mice (Fig. 2m). Again, IMD-0354 significantly lowered the number of total cells and eosinophils, but not lymphocytes, compared with that in Dp/CMC mice.

Pathological features of airway remodelling

The number of PAS-positive cells in the bronchial epithelium of Dp/CMC mice was also increased compared with control/CMC mice (Figs 3b and k). Both periods of IMD-0354 administration reduced mucus production by goblet cells (Figs 3c and l). Areas of goblet cells were significantly increased in Dp/CMC mice and were decreased in Dp/IMD mice for both protocols (Table 1).

To analyse the effects of IMD-0354 on subepithelial fibrosis, we histologically examined subepithelial fibrosis and collagen deposition. IMD-0354 markedly reduced subepithelial fibrosis in Dp/IMD mice compared with

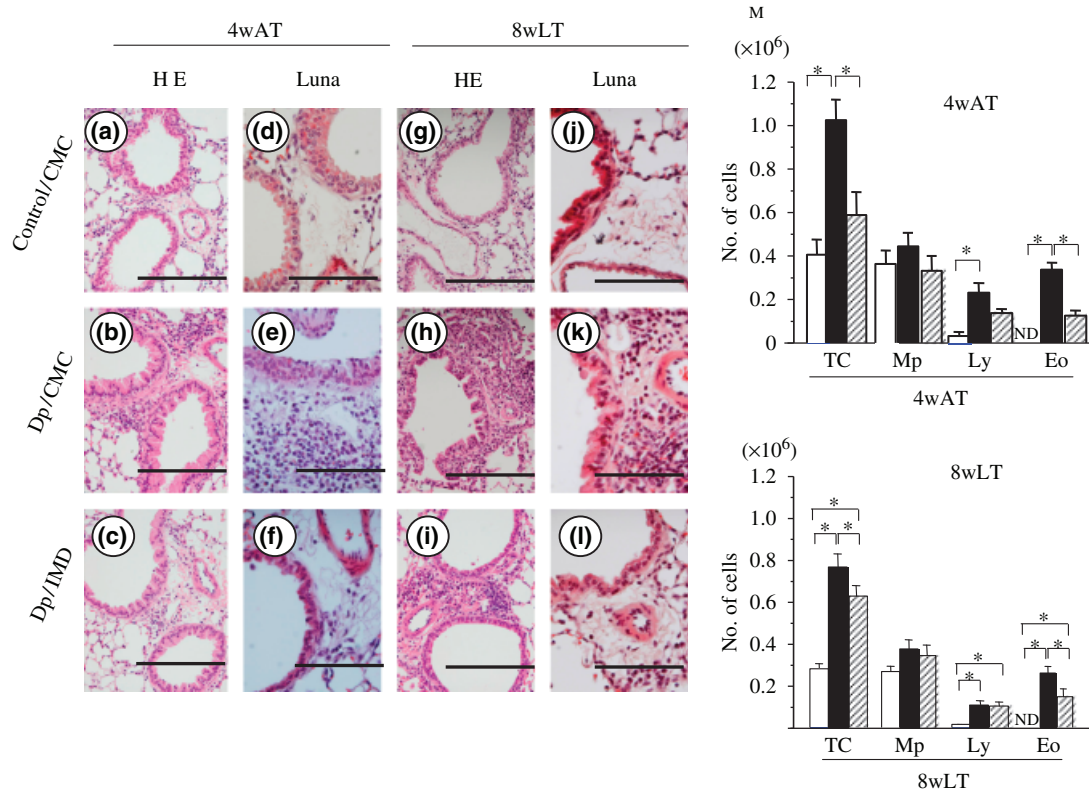


Fig. 2. Histopathology of allergic airway inflammation. (a–c and g–i) Haematoxylin and eosin sections (original magnification $\times 200$, scale bars = $100\ \mu\text{m}$). (d–f and j–l) Luna-modified method sections (original magnification $\times 400$, scale bars = $50\ \mu\text{m}$). Results are representative of three experiments. M, BALF cell analysis; TC, total cells; Mp, macrophage; Ly, lymphocytes; Eo, eosinophils. Values are means $\pm$ SE obtained from three different experiments. Each experiment used four mice per group * $P < 0.05$. Number of mice (n) used for statistical analysis was as follows: white bars, control/CMC mice (4wAT: $n = 10$, 8wLT: $n = 11$); black bars, Dp/CMC mice (4wAT: $n = 11$, 8wLT: $n = 13$); shaded bars, Dp/IMD (20 mg/kg) mice (4wAT: $n = 14$, 8wLT: $n = 14$). ND, not detected; Dp, *Dermatophagoides pteronyssinus*; CMC, carboxymethylcellulose; BALF, bronchoalveolar lavage fluid.

Table 1. Morphological analyses of chronic mouse models of asthma

	Number of eosinophils (no./mm)	% of PAS-positive cells (%)	Thickness of subepithelium ($\mu\text{m}^2/\mu\text{m}$)	Collagen (mg/lung)	Thickness of smooth muscle ($\mu\text{m}^2/\mu\text{m}$)
4wAT					
Control/CMC ($n = 10$)	0.04 ± 0.0	0	3.90 ± 0.46	0.37 ± 0.04	4.80 ± 0.60
Dp/CMC ($n = 11$)	$9.69 \pm 1.14^*$	$35.1 \pm 8.6^*$	$8.45 \pm 0.68^*$	$0.52 \pm 0.03^*$	$7.07 \pm 0.48^*$
Dp/IMD ($n = 14$)	$5.43 \pm 0.61^{*\dagger}$	$16.1 \pm 2.5^\dagger$	$3.73 \pm 0.61^\dagger$	0.43 ± 0.03	6.13 ± 0.41
8wLT					
Control/CMC ($n = 11$)	0	0	3.55 ± 0.43	0.56 ± 0.04	2.98 ± 0.48
Dp/CMC ($n = 13$)	$13.42 \pm 1.76^{**}$	$36.5 \pm 9.3^{**}$	$9.30 \pm 0.96^{**}$	$1.60 \pm 0.12^{**}$	$8.50 \pm 0.60^{**}$
Dp/IMD ($n = 14$)	$6.32 \pm 0.83^{**\dagger}$	$13.3 \pm 4.7^{\dagger\dagger}$	$4.98 \pm 0.47^{\dagger\dagger}$	$1.04 \pm 0.06^{**\dagger\dagger}$	$5.31 \pm 0.40^{**\dagger\dagger}$

The methods for morphological analysis are described in "Materials and methods". Values are means $\pm$ SE obtained from three different experiments.

* $P < 0.05$ compared with control/CMC mice in the 4wAT model.

** $P < 0.05$ compared with control/CMC mice in the 8wLT model.

$^\dagger P < 0.05$ compared with Dp/CMC mice in the 4wAT model.

$^{\dagger\dagger} P < 0.05$ compared with Dp/CMC mice in the 8wLT model.

Dp, *Dermatophagoides pteronyssinus*; CMC, carboxymethylcellulose; PAS, periodic acid-Schiff.

Dp/CMC mice (Figs 3d–f, 3m–o). Dp/IMD mice had significantly smaller areas of subepithelial fibrosis than Dp/CMC mice (Table 1). The increased total collagen in the lungs of Dp/CMC mice tended to decline after an admin-

istration of IMD-0354 in the 4wAT model, and was significantly reduced in the 8wLT model (Table 1).

Dp exposure also resulted in a significantly thickened subepithelial smooth muscle; this thickness decreased

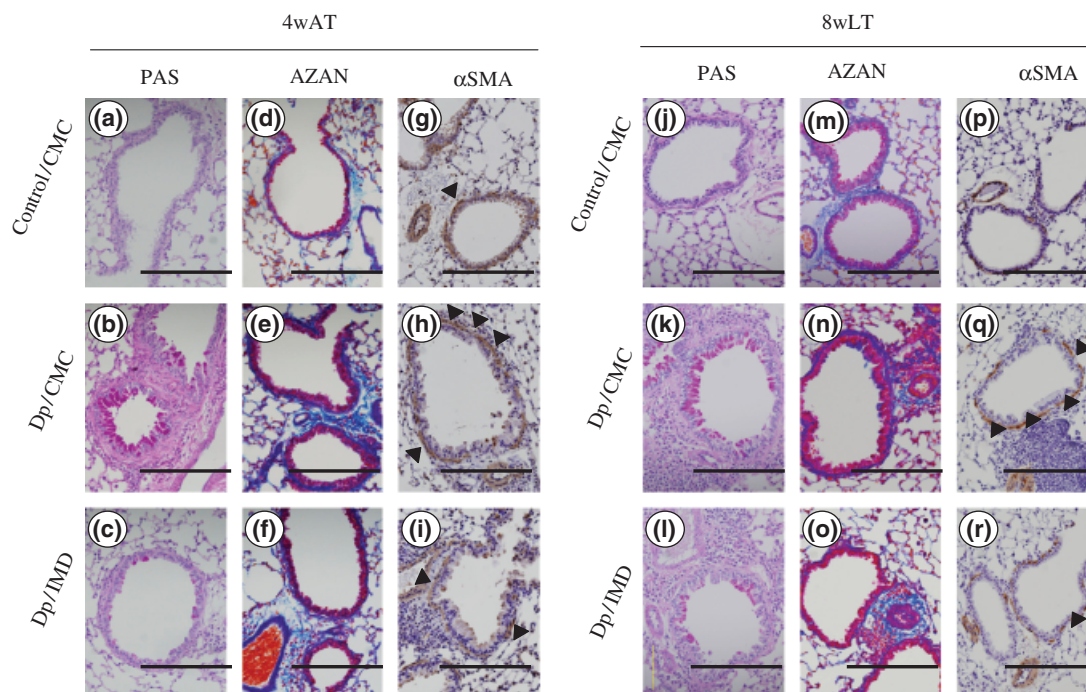


Fig. 3. Histopathology of airway remodelling. Photographs are representative of four experiments. (a–c) and (j–l) Periodic acid-Schiff section. (d–f) and (m–o) Azan–Mallory sections. (g–i) and (p–r) α -SMA immunohistochemistry sections. Arrowhead showed α -SMA-positive subepithelial smooth muscle cells. Original magnification in all figures: $\times 200$. Scale bars in all figures = 100 μ m.

with IMD-0354 treatment in both protocols (Figs 3g–i, 3p–r). By morphological analysis, IMD-0354 diminished the subepithelial smooth muscle thickness in both protocols (Table 1).

Transforming growth factor- β 1 in bronchoalveolar lavage fluid and lung homogenates

Total TGF- β 1 in the BALF was increased in Dp/CMC mice, and IMD-0354 significantly reduced it in both protocols (Fig. 4a). The active form of TGF- β 1 was not detected in BALF. Instead of the BALF, we evaluated the TGF- β 1 in lung homogenate supernatants. There were no differences in total TGF- β 1 levels in whole lungs among the groups of mice [4wAT model (ng/mL): control/CMC = 19.4 ± 3.3 ; Dp/CMC mice = 27.1 ± 5.2 ; Dp/IMD = 28.3 ± 5.2 . 8wLT model (ng/mL): control/CMC = 42.2 ± 5.1 ; Dp/CMC = 28.3 ± 3.6 ; Dp/IMD = 32.3 ± 5.7]. In contrast, the active form of TGF- β 1 tended to increase with Dp chronic exposure, and to decrease after IMD-0354 administration in both protocols, although these results were not statistically significant [4wAT model (pg/mL): control/CMC = 109.1 ± 15.6 ; Dp/CMC mice = 334.5 ± 5.2 ; Dp/IMD = 218.5 ± 54.7 . 8wLT model (pg/mL): control/CMC = 60.3 ± 13.7 ; Dp/CMC = 83.2 ± 18.6 ; Dp/IMD = 46.3 ± 11.1]. As shown Fig. 4a, the proportion of active TGF- β 1 as percentage of total TGF- β 1 was increased with Dp chronic exposure and was decreased after IMD-0354 treatment. This suggested that localized TGF- β 1

production and activation had occurred in the allergic inflammatory airway, and was suppressed by the IMD-0354 treatment.

SMAD2/3 and SMAD7 expression in lung homogenates

SMAD2/3 is a known positive regulator and SMAD7 is a known negative regulator of TGF- β signalling [18]. Therefore, we examined phosphorylated-SMAD2/3, SMAD2/3 and SMAD7 expressions in lung homogenates. There were no differences in SMAD2/3 expression among the three groups in both protocols (Fig. 4b). Phosphorylation of SMAD2/3 was increased by Dp chronic exposure in Dp/CMC mice and was decreased after IMD-0354 treatment (Fig. 4b). The band for SMAD7 in Dp/CMC mice was decreased markedly compared with control mice, and the IMD-0354 restored these in both protocols (Fig. 4c). These results suggested that IMD-0354 inhibited TGF- β activation and signalling by restoring SMAD7 expression.

Mediator production related to airway remodelling

Remodelling-related mediators were measured in lung homogenates, as they were not detected in BALF. PAI-1 and PDGF-AB levels were significantly increased in Dp/CMC mice, and decreased in Dp/IMD mice for the 8wLT model (Fig. 4c). The 4wAT model showed the same trends for PAI-1 and PDGF-AB, although there were no

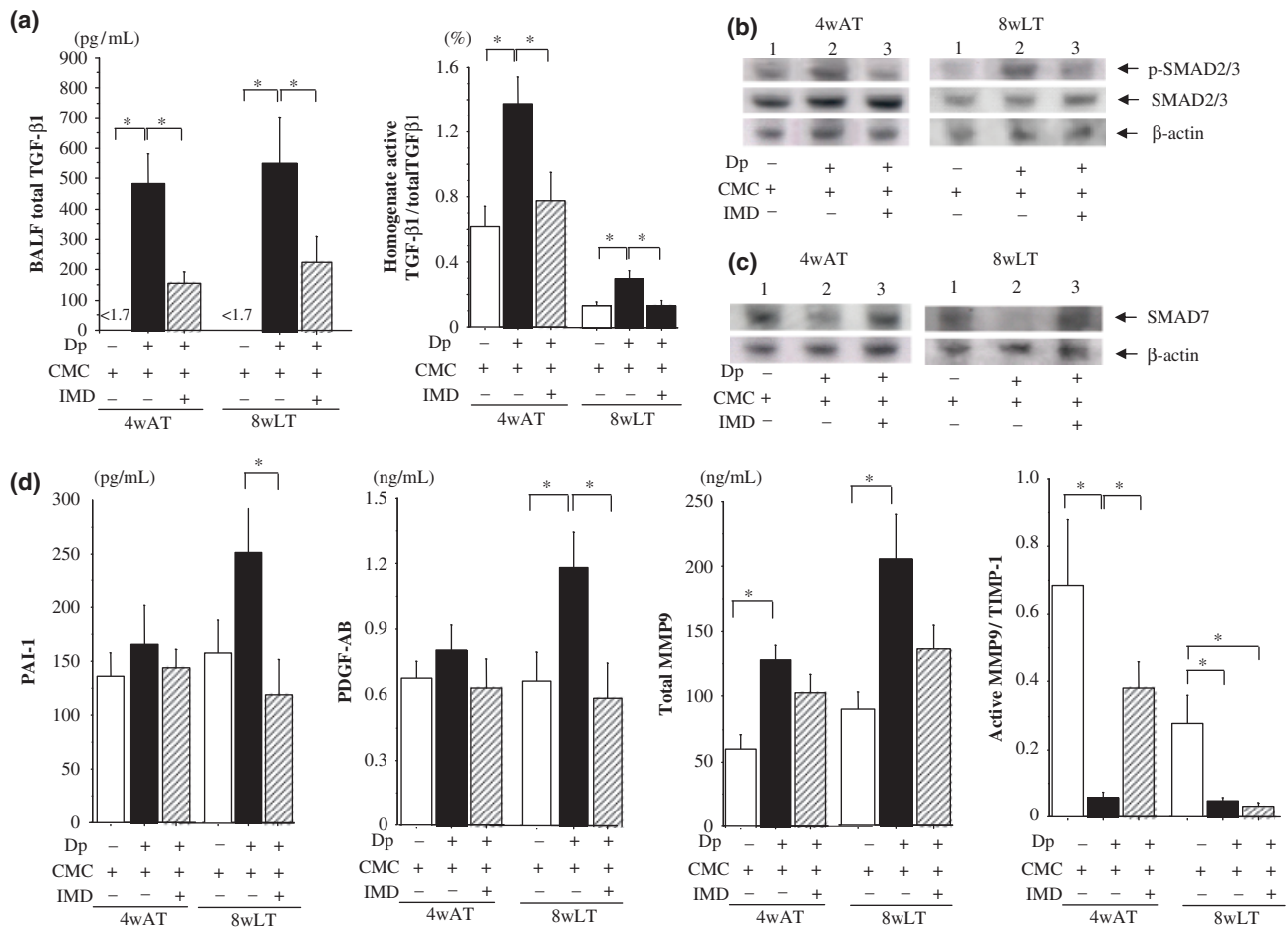


Fig. 4. (a) Total TGF- β 1 concentration in BALF and the ratio of active TGF- β 1 concentration to total TGF- β 1 concentration in lung homogenates. Values are means $\pm$ SE obtained from three different experiments. Each experiment used five mice per group * P < 0.05. The number of mice (n) used for statistical analysis was as follows: white bars, control/CMC mice (4wAT: n = 10, 8wLT: n = 9); black bars, Dp/CMC mice (4wAT: n = 12, 8wLT: n = 13); shaded bars, Dp/IMD (20 mg/kg) mice (4wAT: n = 14, 8wLT: n = 13). (b) Phosphorylated-SMAD2/3 (p-SMAD2/3) and SMAD2/3 expression by Western blotting. Bands are p-SMAD2/3 in the upper panel, SMAD2/3 in middle panel, β -actin in lower panel. Lane 1, control/CMC mice; lane 2, Dp/CMC mice; lane 3, Dp/IMD mice. (c) SMAD7 expression by Western blotting. Bands are SMAD7 in the upper panel and are β -actin in lower panel. Lane 1, control/CMC mice; lane 2, Dp/CMC mice; lane 3, Dp/IMD mice. (d) Airway remodelling-related mediators and enzymes in chronic exposure mice. Values are means $\pm$ SE obtained from three different experiments. Each experiment used five mice per group * P < 0.05. Number of mice (n) used for statistical analysis was as follows: white bars, control/CMC mice (4wAT: n = 10, 8wLT: n = 9); black bars, Dp/CMC mice (4wAT: n = 11, 8wLT: n = 10); shaded bars, Dp/IMD (20 mg/kg) mice (4wAT: n = 14, 8wLT: n = 12). BALF, bronchoalveolar lavage fluid; Dp, *Dermatophagoides pteronyssinus*; CMC, carboxymethylcellulose.

significant differences. We also examined the fibrinolytic enzyme MMP-9. Dp exposure significantly increased MMP-9 production. IMD-0354 tended to decrease the total MMP-9 levels in both models. The ratio of active MMP-9/TIMP-1 was decreased with chronic Dp exposure, and was significantly restored after IMD-0354 administration in the 4wAT model. In the 8wLT model, however, IMD-0354 did not affect this ratio (Fig. 4c).

Cytokine levels in mouse lung homogenates

We also examined cytokine concentrations in the lung homogenates, as some of these were not detected in the BALF. IL-1 β and IL-13 levels in Dp/CMC mice were higher

than for controls in both protocols; IMD-0354 treatment decreased these (Fig. 5). For IFN- γ concentrations in the 8wLT model, IMD-0354 significantly increased its production compared with Dp/CMC mice (Fig. 5). There were no differences among the three groups for IL-4 and -5 in both protocols and IFN- γ in 4w model.

Serum immunoglobulin concentrations

As shown in Table 2, IgE increased by Dp exposure and tended to decreased by IMD0354 administration. In particular, IMD0354 significantly decreased serum IgE in the 8wLT model. IgG1 concentrations increased in Dp/CMC mice compared with control/CMC mice, and decreased after

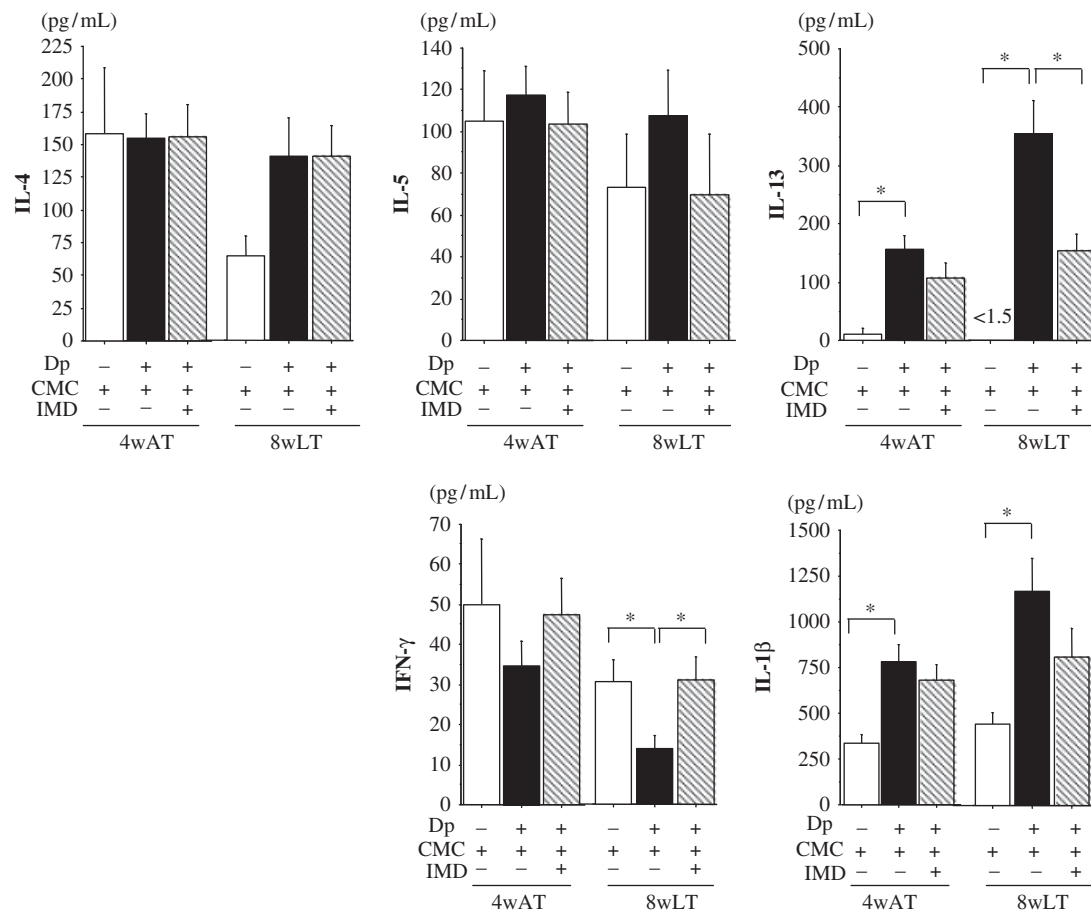


Fig. 5. Cytokines in lung homogenate supernatants. Concentrations of cytokines in mouse lung homogenates were analysed by ELISA. Values are means $\pm$ SE obtained from three different experiments. Each experiment used five mice per group. * $P < 0.05$. The number of mice (n) used for statistical analysis was as follows: white bars, control/CMC mice (4wAT: $n = 10$, 8wLT: $n = 11$); black bars, Dp/CMC mice (4wAT: $n = 11$, 8wLT: $n = 13$); and shaded bars, Dp/IMD (20 mg/kg) mice (4wAT: $n = 14$, 8wLT: $n = 14$). Dp, *Dermatophagoides pteronyssinus*; CMC, carboxymethylcellulose.

Table 2. Serum immunoglobulin concentrations in chronic mouse models of asthma

	IgE (ng/mL)	IgG1 (mg/mL)	IgG2a (μ g/mL)
4wAT			
Control/CMC ($n = 8$)	12.6 $\pm$ 3.4	1.2 $\pm$ 0.2	193.4 $\pm$ 40.3
Dp/CMC ($n = 10$)	156.7 $\pm$ 39.6*	6.3 $\pm$ 1.3*	125.6 $\pm$ 20.6
Dp/IMD ($n = 11$)	116.1 $\pm$ 27.9	4.6 $\pm$ 0.9	174.2 $\pm$ 40.3
8wLT			
Control/CMC ($n = 7$)	10.9 $\pm$ 2.4	0.7 $\pm$ 0.1	166.4 $\pm$ 27.1
Dp/CMC ($n = 9$)	253.0 $\pm$ 44.8**	6.3 $\pm$ 1.4**	149.4 $\pm$ 27.3
Dp/IMD ($n = 9$)	156.9 $\pm$ 24.9**††	4.4 $\pm$ 0.5	197.5 $\pm$ 29.4

Serum immunoglobulin concentrations (total IgE, total IgG1 and total IgG2a) were analysed by ELISA. Values are means $\pm$ SE obtained from three different experiments.

* $P < 0.05$ compared with control/CMC mice in the 4wAT model.

** $P < 0.05$ compared with Dp/CMC mice in the 8wLT model.

†† $P < 0.05$ compared with Dp/CMC mice in the 8wLT model.

Dp, *Dermatophagoides pteronyssinus*; CMC, carboxymethylcellulose.

IMD-0354 treatment although this was not statistically significant in both models (Table 2). There were no differences among three groups for IgG2a in both protocols (Table 2).

Changes of airway hyperresponsiveness (AHR)

To examine the effects of IMD-0354 on AHR in the chronic model, we assessed R_L by restrained plethysmography. In the 4wAT model, there were significant difference among the curves for all groups ($F(2, 20) = 7.79$, $P < 0.001$). ANOVA also showed an interaction between treatment and Mch dose ($F(8, 80) = 4.13$, $P < 0.001$). The R_L values for Dp/CMC mice in the 4wAT model were increased with increasing Mch concentrations; IMD-0354 significantly reduced lung resistance (Fig. 6a). Likewise, in the 8wLT model, there were significant differences among the curves for all groups ($F(2, 23) = 11.13$, $P < 0.001$). The repeated measured two-way ANOVA showed that the curve for all groups were different ($F(8, 92) = 5.28$, $P < 0.001$). The R_L values for Dp/CMC mice in the 8wLT model were increased at a dose of 12.5 mg/mL and at all higher Mch doses; IMD-0354 significantly reduced lung resistance at 25 and 50 mg/mL of Mch (Fig. 6a). PC200 values were also significantly decreased in Dp/CMC mice compared with control/CMC mice, and were restored in Dp/IMD mice in both protocols, but without statistical significance (Fig. 6a).

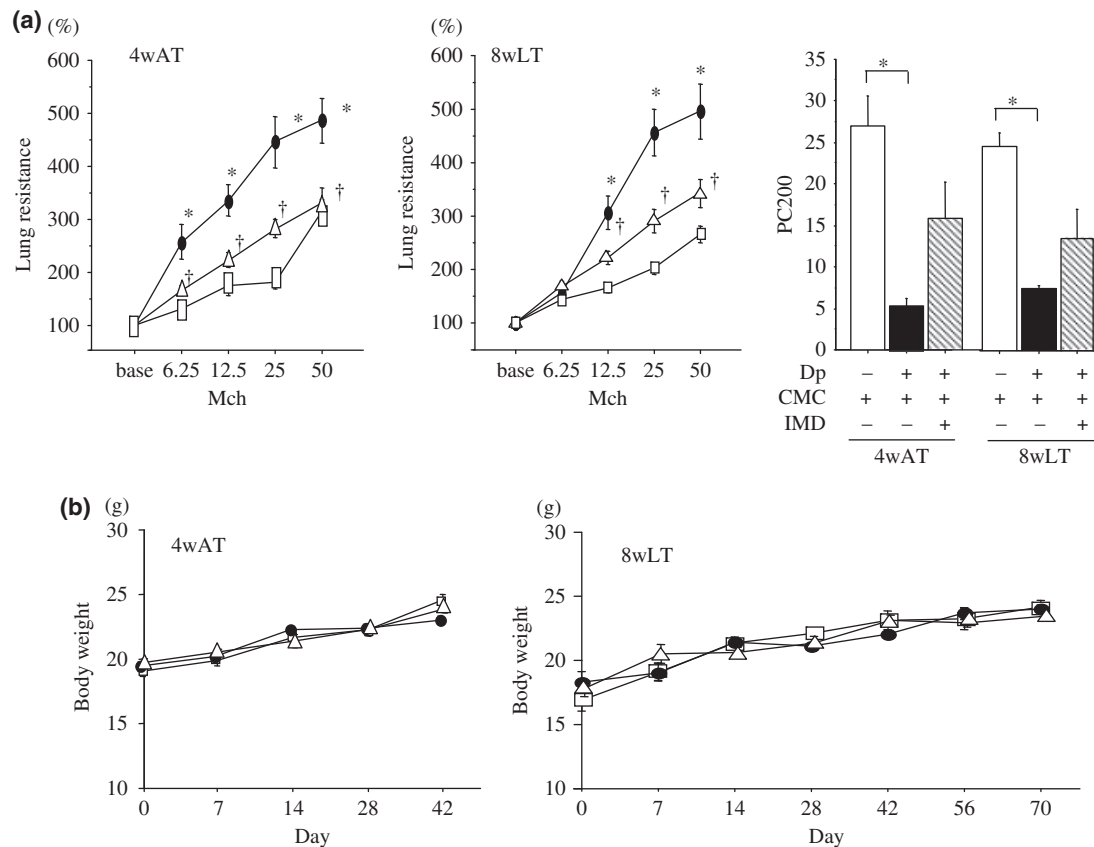


Fig. 6. (a) Assessment of airway resistance and PC200 after Dp challenge. Values are means $\pm$ SE obtained from three different experiments. Each experiment used four mice per group. * $P < 0.05$ compared with control/CMC mice. † $P < 0.05$ compared with Dp/CMC mice. The number of mice (n) used for statistical analysis was as follows: open squares and white bars, control/CMC mice (4wAT: $n = 7$, 8wLT: $n = 8$); closed circles and black bars, Dp/CMC mice (4wAT: $n = 8$, 8wLT: $n = 8$); open triangles and shaded bars, Dp/IMD (20 mg/kg) mice (4wAT: $n = 8$, 8wLT: $n = 10$). (b) Body weight measures in chronic exposure mice. Each experiment used four mice per group. Number of mice (n) used for statistical analysis was as follows: open squares, control/CMC mice (4wAT: $n = 8$, 8wLT: $n = 8$); closed circles, Dp/CMC mice (4wAT: $n = 12$, 8wLT: $n = 10$); open triangles, Dp/IMD (20 mg/kg) mice (4wAT: $n = 10$, 8wLT: $n = 11$). Dp, *Dermatophagoides pteronyssinus*; CMC, carboxymethylcellulose.

Body mass changes after prolonged IMD-0354 administration

For both treatment protocols, we monitored changes of body mass as an index of an adverse event. In the 4wAT model, the body mass was measured on days 0, 7, 14, 28 and 42. In the 8wLT model, body mass was also examined on days 56 and 70. There were no significant differences in body mass among the three groups for both protocols during the experimental periods (Fig. 6b).

Discussion

We have shown that a novel IKK β inhibitor, IMD-0354, effectively ameliorated airway allergic inflammation, AHR, cytokine production, and in particular, airway remodelling changes by reducing the production of remodelling-related mediators and enzymes in a chronic antigen (Dp) exposure mouse model of asthma.

IMD-0354 is a recently synthesized agent that inhibits IKK β activity only under inflammatory conditions [8, 10]. Because it is a low molecular compound [8, 9], IMD-0354 can be easily incorporated into a variety of cells. IMD-0354 inhibited 98.5% of NF- κ B activity, but did not inhibit other kinases, proteases or proteasome-related immune responses [8]. The plasma concentration after administrating 30 mg/kg IMD-0354 was reached a maximum at 4 hr, and decreased gradually for 12 hr.

Tanaka A et al. [19] showed that IMD-0354 inhibited the proliferation of T cells and mast cells [19]. In addition, the cytokines produced by these cells and IgE production were also inhibited by IMD-0354 *in vitro*. Inhibitors of IKK β , TPCA-1 [20] and COMPOUND A [21], prevented allergic pulmonary inflammation and Th2 responses without adverse events. We have also demonstrated that IMD-0354 inhibited allergic inflammation not only in an acute model [8] but also in our chronic model. NF- κ B appears to be most important for antigen-induced IL-5 and IL-13

gene expressions [22], suggesting a mechanistic effect for IKK β inhibitors, including IMD-0354, to inhibit Th2-driven allergic inflammation.

In this study, we also demonstrated the effects of IMD-0354 for inhibiting airway remodelling. This is the first report that an IKK β inhibitor suppresses airway remodelling in a chronic Dp-exposure asthmatic model and extends the potential of IMD-0354 as an anti-airway remodelling agent. IMD-0354 ameliorated the localized production and activation of TGF- β in the airway, suggesting one possible mechanism for improving airway remodelling. TGF- β is a key molecule, as it stimulates fibroblasts, and promotes their secretion of extracellular matrix (ECM) proteins. It also increases the proliferation of airway smooth muscle cells [23, 24], and decreases the production of enzymes that degrade ECM proteins [25]. In the asthmatic patient, TGF- β production arises from inflammatory cells, including eosinophils, and is correlated with subepithelial fibrosis [26]. As shown here, IMD-0354 decreased eosinophils' infiltration into the subepithelium and its effects were confirmed by the analysis of BALF data. These findings may reflect one mechanism for reducing the production of TGF- β 1 by IMD-0354.

In addition, it has been suggested that bronchial epithelial cells are sources of TGF- β [27], and that NF- κ B induced by IL-1 β directly contributes to TGF- β production in bronchial cells [28]. NF- κ B activation also promotes TGF- β signalling by inhibiting SMAD7 promoter activity, a negative regulator of TGF- β signalling [18]. IFN- γ inhibits TGF- β production in airway epithelial cells via stimulating SMAD7 protein expression [29]. Our findings showed that IMD-0354 inhibited IL-1 β production and restored IFN- γ and SMAD7 expression. Thus, it is likely that the effects of IMD-0354 on subepithelial fibrosis are associated with synergistic mechanisms that inhibit TGF- β production or signalling in inflammatory and structural cells via inhibiting the activation of the IKK-NF- κ B pathway.

PAI-1, MMP-9, TIMP-1 and PDGF also contribute to airway remodelling. PAI-1 inhibits a plasminogen activator, leading to a limited fibrinolytic response by inhibiting plasmin and MMP-9 [30]. Although MMP-9 degradation of ECM proteins leads to improvements of subepithelial fibrosis, active MMP-9 can promote allergic inflammation [1]. Previous reports on MMP-9 knockout mice showed that lymphocyte emigration into the airway submucosa was decreased and that peribronchial fibrosis was decreased slightly [31]. MMP-9 also cleaves the latent form to generate an active form of TGF- β [1]. PDGF is a potent inducer of collagen production [32]. It is also related to airway smooth muscle migration and proliferation [33]. In this study, augmentations of these mediators and enzymes were ameliorated by IMD-0354. Because these mediators are produced by inflammatory cells, including eosinophils and mast cells, and are linked to NF- κ B activation [31, 34], IMD-0354 could inhibit the production of these media-

tors. These findings suggest that an anti-remodelling effect of IMD-0354 may result from the suppressing both TGF- β and its synergistic effects on mediators and enzymes related to fibrinolysis.

We used a 'late treatment' model to examine any improvements for established pathological features of airway remodelling. Our findings showed that IMD-0354 was effective in suppressing allergic inflammation, AHR and established remodelling by late treatment. These mechanisms may have been involved the suppression of new allergic inflammation and airway remodelling after IMD-0354 treatment was started. The magnitudes of inhibitory effects on some parameters were less than for the all treatment model (e.g. lymphocytes infiltration in the BALF and active TGF- β production). It is likely that other pathways independent of the IKK β -NF- κ B pathway were established before administrating IMD-0354.

The question is if these airway remodelling features are reversible by IMD-0354, because these features are generally regarded as irreversible in asthma [3]. This remains unknown. Late treatment with IMD-0354 improved the remodelling features to the same degree as the 4wAT Dp exposure model. In the 8wLT model, the MMP-9/TIMP-1 ratio, which is decreased in asthmatic patients and restored after using current therapies [35, 36], was not restored by IMD-0354. One possibility is that this suppression may contribute to the inhibition of new production of Th2 cytokines, growth factors and remodelling-related mediators after beginning IMD-0354 treatment. TIMP-1, an inhibitor of MMP-9, binds active MMP-9 and inactivates its degradation of ECM proteins; TIMP-1 expression is not regulated by NF- κ B [37]. It is likely that TIMP-1 expressed before starting IMD-0354 treatment may have affected the MMP-9/TIMP-1 ratio in the 8wAT model. However, in the 4wAT model, IMD-0354 restored the MMP-9/TIMP-1 balance. Based on this observation, it is possible that IMD-0354 has the potential to reverse pathological airway remodelling changes by restoring the balance of the fibrinolytic/anti-fibrinolytic response. Additional studies are necessary to clarify the details.

Interestingly, AHR was improved by IMD-0354 treatment in this chronic model of asthma. Some reports have shown that improvements in allergic inflammation led to improvements in AHR [21, 38]. In contrast, Poynter *et al.* [39] showed that NF- κ B inhibition in airway epithelium modulated allergic inflammation, but not hyperresponsiveness, as NF- κ B inhibition did not modulate fibrin deposition. A CysLT1 receptor blocker reversed airway remodelling, but did not affect AHR [4]. Thus, the relationships among allergic inflammation, AHR and airway remodelling are controversial. Although inhibition of allergic inflammation and the progression of airway remodelling by IMD-0354 may be a possibility for improving hyperresponsiveness, these mechanisms remain to be elucidated.

In conclusion, IKK β may play an important role during airway remodelling, and IMD-0354 has the potential to improve not only allergic airway inflammation but also airway remodelling. Previous reports have demonstrated the safety of IMD-0354 administration [8, 9, 11]. Similar results were shown in the present study for the long-term use of IMD-0354. These findings suggest that IMD-0354 may be a candidate drug for the treatment of asthma, including airway remodelling. It remains uncertain what influence IMD-0354 has on host defense. Activation of IKK β -NF- κ B pathway contributes to host defense [40, 41]. However, other reports also showed that IKK α , ck2 and p38 pathways were related the host defense [42]. The effects of IMD-0354 on other critical roles of IKK β -NF- κ B, including host defense, will need to be examined before this agent can be establish as an anti-inflammatory and remodelling drug for refractory asthma.

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